BIOCHEMICALLY ENHANCED METHANE PRODUCTION FROM COAL

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

For many years, biogas was connected mostly with the organic matter decomposition in shallow sediments (e.g., wetlands, landfill gas, etc.). Recently, it has been realized that biogenic methane production is ongoing in many hydrocarbon reservoirs.

This research examined microbial methane and carbon dioxide generation from coal. As original contributions methane production from various coal materials was examined in classical and electro-biochemical bench-scale reactors using unique, developed facultative microbial consortia that generate methane under anaerobic conditions. Facultative methanogenic populations are important as all known methanogens are strict anaerobes and their application outside laboratory would be problematic. Additional testing examined the influence of environmental conditions, such as pH, salinity, and nutrient amendments on methane and carbon dioxide generation.

In 44-day ex-situ bench-scale batch bioreactor tests, up to 300,000 and 250,000 ppm methane was generated from bituminous coal and bituminous coal waste respectively, a significant improvement over 20-40 ppm methane generated from control samples. Chemical degradation of complex hydrocarbons using environmentally benign reagents, prior to microbial biodegradation and methanogenesis, resulted in dissolution of up to 5% bituminous coal and bituminous coal waste and up to 25% lignite in samples tested.

Research results confirm that coal waste may be a significant underutilized resource that could be converted to useful fuel. Rapid acidification of lignite samples resulted in low pH (below 4.0), regardless of chemical pretreatment applied, and did not generate significant methane amounts. These results confirmed the importance of monitoring and adjusting in situ and ex situ environmental conditions during methane production.

A patented Electro-Biochemical Reactor technology was used to supply electrons and electron acceptor environments, but appeared to influence methane generation in a negative manner. Provision of electron acceptor environment might have given an advantage to methanotrophs present in the consortium. Availability of electron acceptors is a limiting step in methanotrophy under anaerobic conditions.

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1. LITERATURE REVIEW

1.1. Global Carbon Cycle and Methanogenesis

On Earth, carbon constantly changes form through a set of complex aerobic and anaerobic conversions known as the carbon cycle (Figure 1). The atmosphere holds about 600-720 Gt of carbon, mostly as carbon dioxide and methane, 15% of which is fixed by plants annually through the process of photosynthesis (Figure 1a) [Falkowski et al., 2000; Killops and Killops, 2005; Opara, 2007]. When exposed to air, dead biomass decomposes back to CO_2 (Figure 1b). In an anaerobic environment, such as subsurface sediments, wetlands or the rumen of animals, carbon is converted into methane. The organic matter is first fermented into fatty acids (such as acetate or formate), CO_2, and H_2 (Figure 1c) [Bryant, 1979; McInerney et al., 1979; Mah, 1982]. Then methanogenic microorganisms from the *Archaea* domain metabolize the fermentation products into methane (Figure 1d). Only a few percent of the biogenic methane is buried in suitable formations to form natural gas deposits [Thauer, 1998]. Most of it leaks into aerobic zones where it is oxidized photochemically and converted by menthanotrophic bacteria into CO_2 (Figure 1e) [Cicerone, 1998; Zehnder and Brock, 1979].

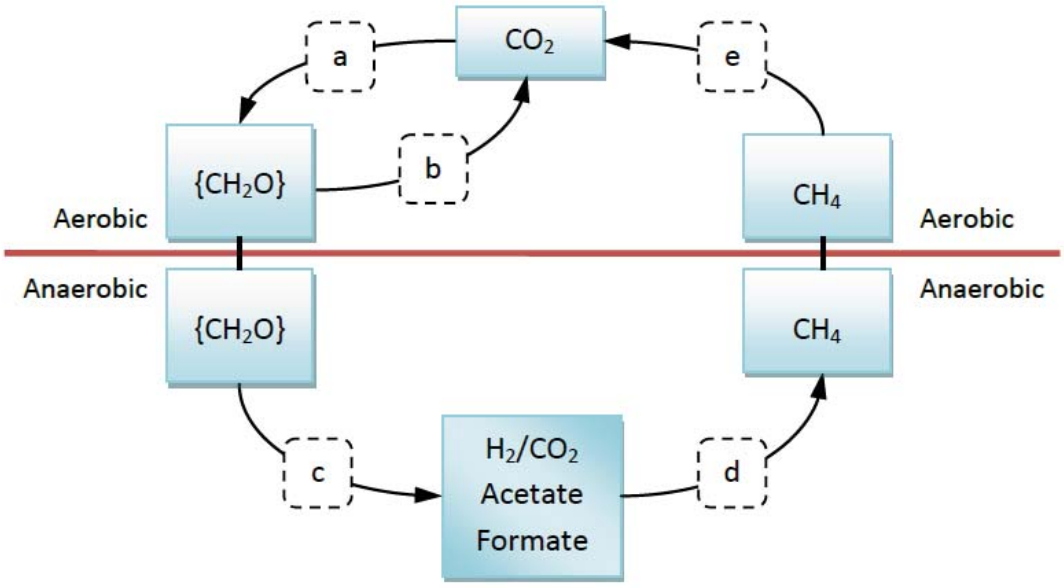

Figure 1. Global carbon cycle (adapted from Ferry 2010a, Ferry 2010b).

1.2. Methane as a Green Fuel

Methane is the main component of natural gas, fuel that – among other things - is used for residential and commercial heating, electricity generation, transportation, and as an industrial feedstock. Over 86% of natural gas used in the United States comes from within the country and 90% of imports come from North America [EIA, 2010b]. Because of its variety of uses, natural gas could replace coal as a main electricity source or crude oil as a main transportation fuel. Increased use of this domestically abundant fuel could reduce the American dependence on foreign oil. Additionally, methane offers many environmental benefits over other fossil fuels. In comparison to coal, burning natural gas releases half the amount of CO_2, 80% less CO and NOx, and virtually no SOx, particulates or mercury. Comparison of air pollutant emissions resulting from combustion of natural gas, crude oil, and coal is given in Table 1. A transition period from fossil fuels towards renewable and

Table 1. Air pollutant emissions by source in lbs/billion BTU energy consumed [EIA, 1999].

Pollutant	Natural Gas	Crude Oil	Coal
CO_2	117,000	164,000	208,000
CO	40	33	208
NOx	92	448	457
SOx	1	1,122	2,591
Particulates	7	84	2,744
Mercury	0.000	0.007	0.016

sustainable fuels is needed. With expansion of currently identified resources, natural gas can be used in conventional steam-turbine power plants or to power vehicles as an exceptional transition fuel.

The proven reserves of conventional natural gas in the United States amount to about 244 TCF (trillion cubic feet) [EIA, 2008], while the annual consumption reaches 23 TCF [EIA, 2010a]. Assuming no imports, conventional reserves will last for roughly 10 years. Since the American economy depends nearly as much on natural gas as it does on crude oil, the search for unconventional resources of natural gas within the United States has a high priority. In recent decades, capturing and burning landfill gas or methane produced in wastewater treatment plants became a significant portion of energy source for these facilities. Nevertheless, many undeveloped unconventional natural gas sources exist. Estimates of global identified sources of methane are given in Table 2. Some of these methane sources, such as landfill gas or municipal wastewater treatment, have been utilized. Intensive research is being performed around the world to tap into some other methane sources, including methane hydrates or gas produced by termites and

Table 2. Estimated annual global sources of methane in Tg/year [Kvenvolden and Rogers, 2005; Opara, 2007].

Source	Range	Accepted average
Natural	110-210	170
Wetlands	55-150	115
Termites and other insects	10-50	20
Oceans	5-50	15
Freshwater	10-35	5
Methane hydrates	0-10	10
Anthropogenic	300-450	375
Fossil fuel related	70-120	110
Rice paddies	20-120	110
Enteric fermentation	65-120	115
Animal waste	20-30	25
Domestic sewage treatment	15-80	25
Landfills	20-70	40
Biomass burning	20-80	40

other insects. However, large volumes of methane are being generated from area sources, i.e., wetlands and rice paddies or multiple point sources, i.e., enteric fermentation, which would be difficult to capture.

1.3. Taxonomy of Methanogens

The taxonomy and ecology of methanogenic microorganisms have been studies thoroughly, due to their importance in the global carbon cycle. They are relatively slow growing anaerobes that are neither prokaryotes nor eukaryotes. They belong to

a newest domain of life, proposed in 1990 by Woese and coworkers, *Archaea* (Figure 2) [Woese et al., 1990]. The creation of the domain of *Archaea* was based on the relationships derived from the 16S rRNA sequencing, which showed that they are not closely related to either of existing domains [Gupta, 1998]. It consists of two kingdoms: *Crenarchaeota* (thermophiles) and *Euryarchaeota* (extreme halophiles, methanotrophs, and methanogens) [Bintrim et al., 1997; Brown and Doolittle, 1997].

Furthermore, methanogens can be divided into five orders: Methanobacteriales, Methanosarcinales, Methanopyrales, Methanomicrobiales, and Methanococcales [Bapteste et al., 2005]. Within these five orders, there are over 50 described species of methanogens, which do not form a monophyletic group, although they all belong to *Archaea*. Methanogens are anaerobic microorganisms and cannot function under aerobic conditions. They are very sensitive to the presence of oxygen, even at trace levels. Usually, they cannot survive oxygen stresses for a prolonged time. Specific taxonomy of methanogenic microorganisms is given in Table 3.

1.4. Methanogenic Pathways

There are two main methanogenic pathways: conversion of CO_2 and H_2, formate or alcohols, and conversion of methylated compounds or acetate to methane [Worm et al., 2010]. In the first methanogenic pathway, the substrate (hydrogen, formate) is the electron donor (Equations 1 and 2, Figure 3), while CO_2 is the carbon source and the electron acceptor (Equation 3, Figure 3). As seen from Table 3, most of the methanogens can use hydrogen as the electron donor. This pathway, however, accounts for only a third of methane generated from freshwaters and bioreactors, such as domestic wastewater treatment facilities or landfills. Substrates in the

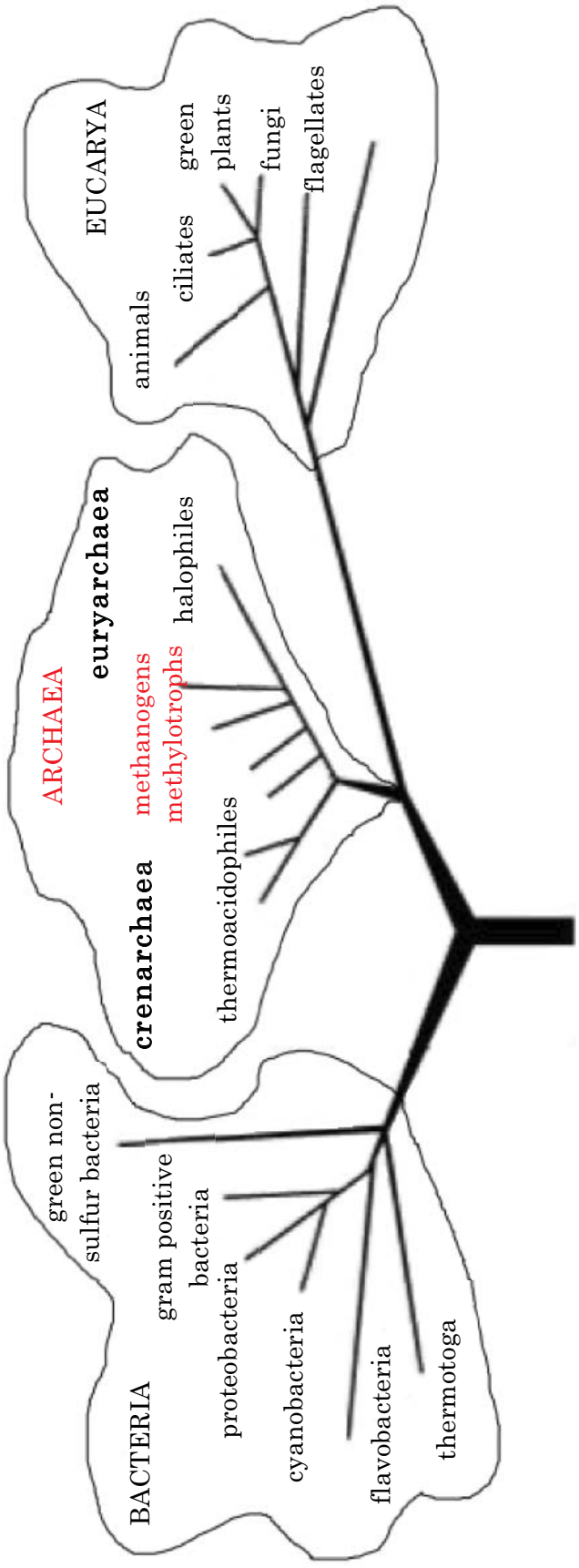

Figure 2. Consensus drawing of the phylogenetic tree showing three domains of life. Groups of organisms and their location is based on rRNA sequence comparison [Woese et al., 1990; Lecointre and Le Guyader; 2006].

Table 3. Taxonomy of methanogens [Garcia, 1990; Zinder, 1993].

Organism (Order, *Family*, ***Genus***)	Morphology	Substrates	Examples of species	Optimum growth conditions		
				Temp. [°C]	pH	NaCl [M]
Methanobacteriales						
Methanobacteriaceae						
Methanobacterium	Rod	H_2/CO_2, formate*, alcohols*	*alcaliphilum, wolfei*	35-70	5.6-9.1	<1.7
Methanosphaera	Coccus	H_2/methanol	*cuniculi, stadtmanae*	35-40	6.5-6.9	NA
Methanobrevibacter	Short rod	H_2/CO_2, formate	*arboriphilus, smithii,*	30-39	7.0-8.0	NA
Methanothermaceae						
Methanothermus	Rod	H_2/CO_2	*fervidus, sociabilis*	80-88	6.5	NA
Methanosarcinales						
Methanosarcinacae						
Methanosarcina	Irregular coccus	H_2/CO_2*, methanol, methylamines, acetate	*alcaliphilum, mazei, thermophila*	30-50	6.5-7.5	<0.7
Methanolobus	Irregular coccus	methanol, methylamines	*siciliae, tindarius*	37	6.5	0.5
Methanococcoides	Irregular coccus	methanol, methylamines	*euhalobius , methylutens*	28-37	6.8-7.3	<1.0
Methanohalophilus	Irregular coccus	methanol, methylamines	*mahii, zhilinae*	35-45	7.4-9.2	<2.5
Methanolohalobium	Irregular coccus	methanol, methylamines	*evestigatus*	NA	NA	NA
Methanoseataceae						
Methanosaeta	Filament	acetate	*thermoacetophila*	35-60	6.5-7.5	NA

Table 3. (continued)

Organism (Order, *Family,* ***Genus***)	Morphology	Substrates	Examples of species	Optimum growth conditions		
				Temp. [°C]	pH	NaCl [M]
Methanopyrales						
Methanopyraceae						
Methanopyrus	Rod	H_2/CO_2	*thermophila*	98	6.5	0.25
Methanomicrobiales						
Methanoinicrobiaceae						
Methanomicrobium	Short rod	H_2/CO_2, formate*, alcohols*	*mobile*	40	6.1-6.9	NA
Methanoculleus	Irregular coccus	H_2/CO_2, formate, alcohols*	*marisnegri*	20-25	6.8-7.3	0.1
Methanogenium	Irregular coccus	H_2/CO_2, formate, alcohols*	*anulus, friitonii*	20-60	6.2-7.3	<0.3
Methanocorpusculaceae						
Methanocorpusculum	Irregular coccus	H_2/CO_2, formate, alcohols*	*aggregans, parvum*	30-37	6.4-7.5	<0.8
Methanospirillaceae						
Methanospirillum	Long spirals	H_2/CO_2, formate, alcohols	*hungatei*	30-37	NA	NA
Methanococcales						
Methanococcaceae						
Methanococcus	Coccus	H_2/CO_2, formate*	*maripludis,*	26-85	5.0-8.0	<1.7

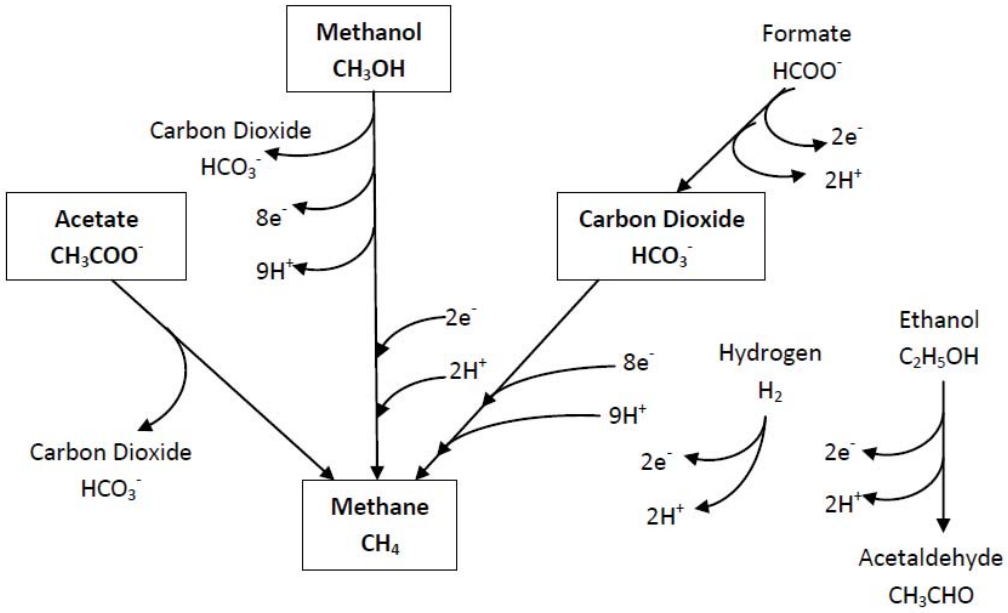

Figure 3. Simplified view of three methanogenesis pathways (acetoclastic, methylotrophic, and hydrogenotrophic) with electron and hydrogen ion flows indicated.

second group (acetate, methylated compounds) act as both, the electron donor and the carbon source [Vandecasteele, 2008]. Even though there are only two genera identified as using the acetoclastic pathway, acetate decarboxylation is responsible for about two-thirds of methane production in freshwaters and bioreactors [Zinder, 1993].

Conversion of CO_2 is the only methanogenic pathway having a net negative electron flow (Figure 3). Moreover, only a handful of electron donors, including hydrogen, formate, and alcohols, have been identified as suitable for this pathway.

$$HCO_3^- + 8e^- + 9H^+ \rightarrow CH_4 + 3H_2O \quad (1)$$

$$H_2 \rightarrow 2e^- + 2H^+ \quad (2)$$

$$HCOO^- + H_2O \rightarrow HCO_3^- + 2e^- + 2H^+ \quad (3)$$

The lack of electrons and availability of the electron donors could be the reason why there is not more methane produced through this pathway. Methylated compounds, on the other hand, can be simultaneously oxidized to CO_2, releasing six electrons, and reduced to methane through the reaction with coenzyme B, accepting two electrons. Lack of electron acceptors could be the limiting factor in this case. Finally, during the acetoclastic pathway two electrons are donated through the conversion of the carboxylic group into CO_2, while a series of reactions between the methyl group with coenzymes B, M, and tetrahydrosarcinapterin accepts two electrons, resulting in net zero free electrons [Ferry, 2011].

Methanogenic reactions are shown in Table 4, indicating the importance of electron donors and acceptors in the process. From the thermodynamic standpoint, the most favorable conditions for methanogenesis are negative Eh and low pH values (Figure 4). At neutral pH, methanogens require ORP (oxidation-reduction potential) of -400mV or lower [Khanal, 2008], (Figure 4). By controlling pH and ORP of the environment, an optimal generation of methane might be obtained.

1.5. Biogenic Natural Gas from Complex Hydrocarbons

Biogenic gas, produced from anoxic decomposition of organic matter by microorganisms, is considered an unconventional natural gas resource. For many years, biogenic gas (or biogas) was connected mostly with the decomposition of organic matter in shallow anoxic sediments (e.g., wetlands, marsh gas, methane associated with or produced from municipal wastewater treatment facilities and landfills, or from rice paddies). Recently, it has been realized that biogenic methane production is ongoing in many hydrocarbon reservoirs. Coalbed methane (CBM), for

Table 4. Most important methanogenic reactions in ordered from the most to least thermodynamically favored, as defined by free energy change [Zinder, 1993; Thauer, 1998].

Electron donor	Carbon source	Reaction	ΔG [kJ/mol CH_4]
Formate	CO_2	$4HCO_2^- + H_2 + H_2O \rightarrow CH_4 + 3HCO_3^-$	-145
Hydrogen	CO_2	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-135
Alcohol	CO_2	$2CH_3CH_2OH + HCO_3^- \rightarrow 2CH_3COO^- + H^+ + CH_4 + H_2O$	-116
Methanol	Methanol	$4CH_3OH \rightarrow 3CH_4 + HCO_3^- + H_2O + H^+$	-105
Methylamine	Methylamine	$4(CH_3)_3NH^+ + 9H_2O \rightarrow 9CH_4 + 3HCO_3^- + 4NH_4^- + 3H^+$	-76
Acetate	Acetate	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31

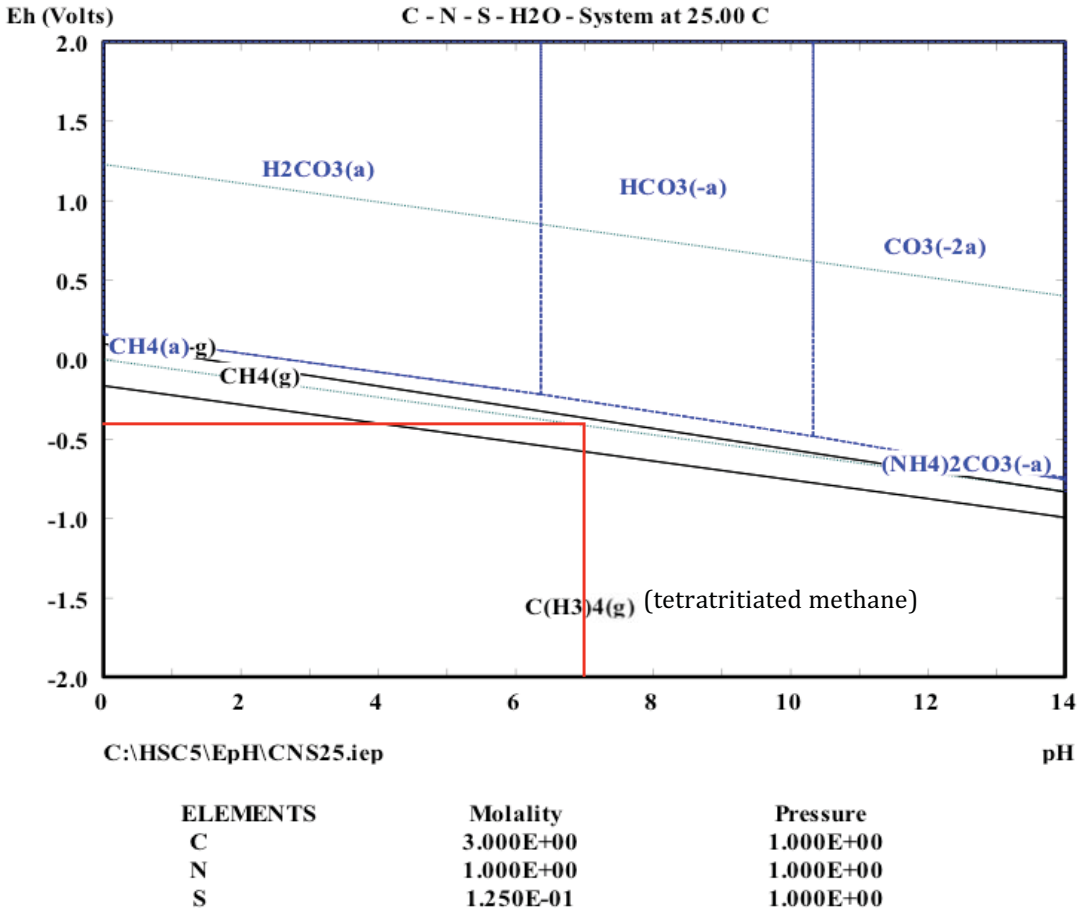

Figure 4. Pourbaix diagram of C-N-S-H-O system at 25°C. Red lines indicate boundary under neutral pH. Note that $C(H_3)_4(g)$ species is a tetratritiated methane.

instance, has been believed for many years to have thermogenic as well as biogenic origins through decomposition of organic matter occurring during early stages of coalification [Thomas 2002]. Recent studies show, however, that coalbed methane may also be of a more recent biogenic origin, produced through microbial degradation and utilization of complex carbon compounds [Ulrich and Bower 2008].

The mechanism of anoxic coal biodegradation is not well understood [Strąpoć et al., 2011]. It is believed, however, that after initial fragmentation and activation of

coal compounds, fermentation and oxidation of intermediate compounds leads to methane precursors (Figure 5).

Similarly, large biogenic shale gas plays, such as the Antrim Shale of the Michigan Basin or the Colorado shale in Alberta, have been recently described [Curtis 2002; Jarvie et al. 2007]. Biogenic natural gas wells, however, tend to have low production rates and are poorly researched, which discourages many operators [Shurr and Ridgley, 2002]. From a different perspective, mining of coal and other hydrocarbon sources (e.g., tar sands and oil shales) results in mountainous waste heaps of mineral waste and lower grade coal materials, a potentially useful, but under-utilized, fuel source. In the United States alone, accumulated culm and gob (waste products of anthracite and bituminous coal mining, respectively) are estimated to be about two billion tons [Akers and Harrison 2000]. Annually, about 55 million tons of waste coal are generated [Tillman and Harding 2004] and pile up on mine sites as unprofitable mountains or valley fills that can potentially contribute to generation of metal contaminated waters, or are gravitationally

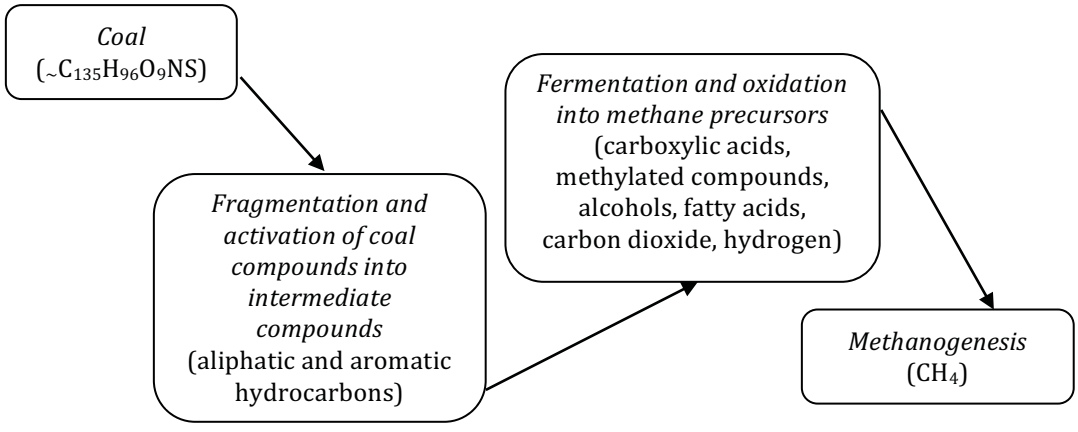

Figure 5. Simplified diagram of coal biodegradation (adapted from Strąpoć et al., 2011).

unstable and a risk for slope failure. Acid mine drainage and leaching of various metals into neighboring watersheds is a problem. Moreover, waste coal heaps often catch fire and release toxic gases into the atmosphere [Stracher and Taylor, 2004]. Successful conversion of even a fraction of this waste material into useful fuel could prove advantageous to the mining industry and the environment. Coal heaps could be designed in a similar fashion to modern landfills allowing for active methanogenesis and collection and utilization of collected gases.

Enhancing the recovery of low production gas wells and stimulating the methanogenesis in depleting gas wells for methane production have been the research topic of scientists in the last few years. Most of the published research has been focused on characterizing the microbial populations present in hydrocarbon reservoirs. Limited attention has been given to the microbiology of crude oil fields. Using molecular techniques, Grabowski and colleagues (2005), Higashioka and colleagues (2010), and Pham and colleagues (2009) described the microbial diversity of various petroleum reservoirs. Gieg and colleagues (2011) characterized the microbial population responsible for methanogenesis and crude oil biodegradation in an Alaskan oil field. Youssef with colleagues (2009) and Strąpoć with colleagues (2009) described various roles of microorganisms in oil fields, including crude oil degradation and active methanogenensis.

Coalbed methane has been by far the focus point of most of the research in this area; nevertheless, only a small amount of work has been published. Scott and Kaiser discussed in 1995 the potential for microbially enhanced recovery of CBM. In recent years, numerous studies have been published describing microbial populations present in coal seams and utilizing coal as the sole substrate. The diversity of the cultured organisms was large. Proteobacteria have been the bacteria

most commonly associated with coal seams, even though their role has not been determined [Li et al., 2008; Penner et al., 2008, Midgley et al., 2010]. Bacteria from the phyla Firmicutes, capable of demethylating aromatic compounds, and Bacteroidetes, responsible for anaerobic degradation of cellulose, proteins, polysaccharides, and polyaromatic hydrocarbons, have been found in coal beds in large quantities as well [Shimizu et al., 2007; Strąpoć et al., 2008a]. Archaea were found to be present in coal seams but their relative abundance and type depended strongly on the location. Methanogens belonging to the archaeal phylum Euryarchaeota were common in coal beds but representatives from the phylum Crenarchaeota (extremophiles) were often present as well [Green et al., 2008; Strąpoć et al., 2008a]. By characterizing microbial populations present, many of the aforementioned studies suggested that coal biodegradation and methanogenesis are active within some coal beds. Some studies analyzed biogeochemistry of coalbed plays, connecting present microbial populations and chemistry of formation waters with the biogenic origin of natural gas [McIntosh et al., 2008; Rice et al., 2008; Strąpoć et al., 2008b; Warwick et al., 2008; Flores et al., 2008; McIntosh et al., 2010; Aikuan et al., 2010; Schlegel et al., 2011]. Regardless of all the indirect evidence of biogenic gas production in coal seams, the mechanism of anaerobic coal biodegradation is not well understood. Under laboratory conditions, many intermediate products were discovered (e.g., acetate, alkanes, long-chain fatty acids, and low molecular weight aromatics) [Orem et al., 2010]. Moreover, Formolo and colleagues (2008) analyzed the biodegradation indices of coals associated with biogenic coalbed methane in the Powder River Basin and San Juan Basin and observed a removal of n-alkanes and isoprenoids from the coal matrix.

In recent years, the research focus shifted towards stimulating the biogenic methane production from coal by addition of nutrients and/or microbial consortia. Common nutrient amendments included phosphate, yeast extract, ammonia, trace metals, and vitamins [Jones et al., 2010; Strąpoć et al., 2011]. Added microbial consortia contained methanogens collected from coal seams and other natural environments (e.g., wetlands) [Jones et al., 2008]. Successful results reported gas generation potential between $10^1 - 8x10^3$ SCF per ton per year [Strąpoć et al., 2011].

Even though many authors discuss addition of nutrients to stimulate methanogens present in coal, no published paper discusses the potential of stepwise chemical degradation of coal followed by biodegradation resulting in methane production. Moreover, the literature focuses mostly on coalbed methane, ignoring other potential applications; utilization of waste hydrocarbon material is not considered. No published studies discuss an ex-situ methane generation from coal or waste hydrocarbon materials and no large scale bioreactor designs have been presented for these applications. The technology for large-scale generation of biofuels exists; both gaseous fuels (e.g., biogas) and liquid fuels (e.g., biodiesel) have been successfully obtained from simple hydrocarbons. Furthermore, the availability of electron donors and acceptors as well as the oxidation-reduction potential, though being important factors in methanogenesis, are not discussed in the published literature. The studies focus instead on the bioavailability of carbon sources, availability of hydrogen ions, presence of methanogenesis inhibitory compounds, etc.

2. RESEARCH OBJECTIVES

The research objectives are to:

1. Examine various minimally characterized microbial consortia from different environments for their potential use in methane generation from coal and other solid carbonaceous materials.

2. Evaluate methane and carbon dioxide generation potential from coal and other solid carbonaceous materials pretreated with various chemical reagents and microbial consortia.

3. Evaluate bench-scale generation of methane and carbon dioxide from coal and/or other solid carbonaceous materials and examination of the influence of environmental conditions, such as pH, salinity, and nutrient amendment.

4. Examine directly supplying electrons and electron acceptors to stimulate methanogenesis through the use of electro-biochemical reactors.

3. ORIGINAL CONTRIBUTION OF THE RESEARCH

As original contributions, the research will:

- Examine the potential of degradation of complex hydrocarbon materials by chemical reagents prior to microbial biodegradation and methanogenesis;

- Test various hydrocarbon materials, including coal, lignite, and waste coal, since the published literature focuses mostly on enhancement of coalbed methane recovery, while other carbonaceous materials, such as waste solid hydrocarbons, are under-utilized;

- Examine ex-situ use of complex hydrocarbons in bench scale batch bioreactors, since the published literature in the field of complex hydrocarbons, such as coal, focuses mostly on in-situ methane production;

- Perform initial characterizations for unique microbial consortia and their potential for methane generation;

- Examine the influence of environmental conditions, such as pH, salinity, nutrient amendments, on methane and carbon dioxide generation in bench scale bioreactors;

- Test an electrobiochemical reactor to examine the influence of electrons and electron acceptor environments on methanognesis.

4. MATERIALS AND EXPERIMENTAL PROCEDURES

4.1. Collection and Preparation of Materials

4.1.1. Coal Samples

Coal and coal waste samples used in this study were provided from the Deer Creek Mine in Utah. The samples came from the same mining operation and types of mined materials to permit a more direct comparison of the results obtained. The coal sample had a total moisture content of 4.28% and 6.15% ash content as received. With over 76% carbon (dry) and a caloric value of about 14,000 BTU/lb it is classified as a bituminous coal. The coal waste product, as received, contained over 50% ash, 28% carbon (dry), and had a caloric value of about 4,400 BTU/lb. More specific elemental composition, as well as ash analysis for these coal and coal waste samples, is given in Tables 5 and 6.

A commercially available North Dakota lignite sample was purchased as a bulk pack from Ward's Natural Science (#47-2133). Chemical composition of characteristic North Dakota coals is presented in Table 7, as given by Tang and colleagues (1996), and Gale and colleagues (1996).

4.1.2. Coal Grinding

All coal samples were pulverized to -200 mesh particle size in a ball grinder to provide maximum surface area. Sample preparation station was swept and washed

Table 5. Analysis of bituminous coal and coal waste samples.

Parameter	Coal	Waste Coal
Total Moisture (as received)	4.28%	6.89%
Ash (dry)	6.43%	57.61%
Volatile Matter (dry)	48.13%	29.30%
Fixed Carbon (dry)	45.44%	13.09%
Carbon (dry)	76.60%	28.24%
Gross Calorific Value [BTU/lb] (dry)	13,949	4,370
Sulfur (dry)	0.38%	0.30%
Organic Sulfur (dry)	0.37%	0.16%
Oxygen (dry)	9.15%	11.40%
Hydrogen (dry)	6.02%	1.99%
Nitrogen (dry)	1.42%	0.46%

Table 6. Ash analysis of bituminous coal and coal waste samples.

Component	Coal	Waste Coal
SiO_2	52.72%	62.05%
Al_2O_3	13.16%	8.72%
Fe_2O_3	5.27%	2.30%
CaO	12.10%	16.88%
MgO	1.50%	6.34%
K_2O	0.18%	1.55%
Na_2O	4.19%	0.35%
SO_3	8.89%	1.11%
P_2O_5	0.75%	0.18%
TiO_2	0.90%	0.42%

Table 7. Analysis of North Dakota lignite samples [Tang et al. 1996 and Gale et al. 1996].

Parameter	Tang et al., 1996	Gale et al., 1996
Total Moisture (as received)	3.90%	23.3%
Ash (dry)	5.58%	6.0%
Volatile Matter (dry)	44.83%	NA
Fixed Carbon (dry)	45.69%	NA
Carbon (dry)	61.20%	67.2%
Sulfur (dry)	0.25%	1.06%
Oxygen (dry)	27.98%	26.5%
Hydrogen (dry)	3.97%	4.3%
Nitrogen (dry)	1.01%	1.0%

with water and detergent. Grinding vessels, grinding balls, and all the dishes and tools were thoroughly washed. Bituminous coal consisted mostly of large and hard chunks; therefore, it was first manually crushed in a ceramic mortar to a size less than 8 mesh. Waste bituminous coal contained a large quantity of moisture; thus, drying was necessary prior to grinding. Moreover, the waste bituminous coal was upgraded prior to grinding, i.e., large inorganic rocks were removed from the material. Lignite material was soft and did not require manual grinding.

In order to ensure minimal contamination, a 1 kg aliquot of crushed coal was ground for 30 minutes and discarded before the regular grinding started. Coal samples were ground for 3-4 hours and the resulting dust was screened through an 8-mesh sieve into a collection bucket. After 30 kg of coal dust were collected, the workstation and all the tools were thoroughly washed.

4.1.3. Collection of Microbial Populations

Microbial populations were cultured from coal samples and natural environments believed to be suitable for methanogens. Summary of collected samples is given in Table 8. Two 55-gallon drums containing bituminous coal and coal waste rock were obtained (for coal characterization see Section 4.1.1, page 19). Two sediment cores from the Great Salt Lake's wetlands and one from the Jordan River were sampled (Figures 6A and 6B). These three samples had a higher salt content [Jones et al., 2009]. Gas collected over the Jordan River sample site produced a self-sustaining flame, when ignited (Figure 6C).

A 500 mL anaerobic digester sludge sample was collected from the Central Valley Wastewater Treatment Plant. Additionally, samples from eight locations

Figure 6. Photographs of core samples from (A) the Great Salt Lake wetlands and (B) the Jordan River, and (C) self-sustained flame produced from the gas collected from the Jordan River.

Table 8. Environmental samples collected for isolation of methanogenic microorganisms.

Sample	# of samples	Source	Notes
bituminous coal, bituminous waste coal	2, 2	Deer Creek coal mine, Utah	Microbes were collected from coarse and from pulverized samples
lake sediment	1	The Great Salt Lake - south	Higher salinity
wetland sediment	1	The Great Salt Lake - Farmington	Higher salinity
river sediment	1	The Jordan River – the Legacy Nature Preserve	Higher salinity
digester sludge	1	Central Valley Wastewater Treatment Plant	Anaerobic sludge
oil seep	8	The Great Salt Lake – Rozel Point	Higher salinity; hydrocarbon associated
gas well	6	Drunkard Wash, Price, UT	Coal-bed methane wells

around the Rozel Point oil seeps in the Great Salt Lake and six locations from coal-bed methane wells were collected by Michael Peoples (Department of Metallurgical Engineering, University of Utah).

4.1.4. Microbial Cultivation and Morphology Characterization

Prior to sampling, both, the media preparation station and the microbial sample station were cleaned and disinfected with 10% Clorox solution. The samples were collected from the sediments' core interior to avoid contamination with foreign microorganisms potentially present on the core outside surfaces. Grab samples were collected along the core length to ensure that a variety of microbial communities are captured. The remaining cores were wrapped with aluminum foil, sealed in plastic sample bags, and stored at about 5°C to preserve the natural moisture content of the sediments.

Sterile 50 mL centrifuge vials were filled with collected environmental samples. Seven different sterile media were added to the prepared samples (see Appendix A). Sample vials were stored at about 24°C to allow for microbial growth.

4.2. Microbial Characterization

4.2.1. Morphology Characterization

Six tenfold microbial sample dilutions were prepared with normal saline solution (0.85% NaCl) and plated on TSA plates (30g/L trypticase soy broth and 18mg/L agar) under sterile conditions. After three days, the morphology of the grown colonies was characterized accordingly to their form, size, surface, color, elevation, and margin.

Moreover, the most representative plate from each series (i.e., one having 30-300 colonies) was counted.

For a more detailed procedure description, see Standard Operating Procedure *SOP: Cultivation of Microorganisms for Gas Generation Tests,* Appendix B.

4.2.2. Environmental Influences

The best CO_2 and CH_4 generating microbial populations from coal sample screening tests were selected (see Section 5.3.2.) and combined into five consortia (Table 9). The consortia were selected to contain both methane and carbon dioxide generating microbes that were not sensitive to oxygen exposure during culture. Following selection, the consortia were allowed to establish individual microbial concentrations within the consortia based on the culture medium.

The influence of pH, temperature, and salinity on the created microbial consortia was examined. Growth curves were plotted using an indirect spectrophotometer optical density (OD) measurement. Thermo Spectronic Genesis 8 spectrophotometer was used. Since the Trypticase Soy Broth medium is yellow, the wavelength was set at 600 nm to minimize the effect of color on sample measurement. Direct counts were performed on selected samples in order to correlate the sample absorbance with colony counts.

Even though both methods are not extremely precise, they provide a relevant indication of growth (OD measures the turbidity of the sample, which may differ between various microbes; while colony count takes into account only the aerobic, aerotolerant and culturable organisms).

Table 9. Five created consortia and their microbial sources.

Consortium	Microbial Sources
in1	2x coal, 2x coal waste, river sediments
in2	2x oil seep, natural gas well
in3	2x oil seep, lake sediment, 4x coal, 3x coal waste, natural gas well, river sediment
in4	3x coal waste, 3x natural gas well, 2x oil seep
in5	2x oil seep, coal, natural gas well

Moreover, growth kinetics parameters can be assessed based on the created growth curves. Growth rate constant, μ [hr^{-1}], denotes the number of generations that occur per unit time and can be calculated as:

$$\mu = \frac{lnN_t - lnN_0}{t - t_0} \quad (4)$$

where N_t and N_0 are the amount of cells per milliliter at the time t and t_0 (the initial time), respectively. The time required for the population to double, or the doubling time, g [hr], can be calculated from Equation 5.

$$g = \frac{ln2}{\mu} \quad (5)$$

4.2.3. Oxygen Requirement

Thioglycollate broth medium was used in determination of microbial oxygen requirement. This medium contains:

- dextrose, yeast extract, digest of casein (a mixture of nutrients),

- L-cystein and sodium thioglycollate (compounds removing oxygen from the medium),
- agar (slowing down the return of oxygen to the sample and thus creating an oxygen gradient),
- and resazurin (an oxygen indicator).

The growth pattern in the tubes indicates the type of oxygen requirement for a given microbe or consortium (Figure 7). For a detailed description of the procedure, please see *SOP: Oxygen Requirement Test with Thioglycollate,* Appendix B.

4.2.4. Community Level Metabolic Profiling

Each microbial species and microbial consortium or population has a specific and usually unique set of carbon compounds they can utilize as an energy substrate. Describing the pattern of carbon utilization by a given microbial species or

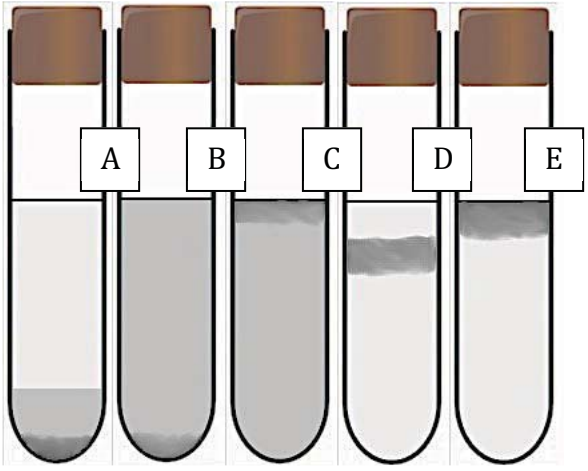

Figure 7. Growth patterns in thioglycollate broth indicating: A – obligate anaerobes, B – aerotolerant anaerobes, C – facultative anaerobes, D – microaerophiles, E – obligate aerobes.

consortium is called Community Level Physiological Profiling (CLPP). The CLPP system was developed by BIOLOG in the late 1980s for a rapid identification of clinically important bacteria. Three most common CLPP systems include GN (Gram negative bacteria), GP (Gram positive bacteria), and ECO (environmental) microtiter plates. GN and GP plates contain 95 carbon sources, while ECO plates contain 31 carbon sources. An ECO plate consists of 96 wells, each well contains one of 31 carbon sources, which are present in triplicates for the reproducibility purpose. The remaining three wells are filled with sterile DI water as a control. Additionally, each plate contains tetrazolium dye, which is transparent initially, but under respiration-dependent reduction, it turns purple. This colorimetric reaction can be monitored with time, indicating which of the carbon sources can be utilized by the consortium. Figure 8 shows the distribution of carbon sources on the ECO plate.

	1	2	3	4
A	Water	ß-methyl-D-glucoside	D-galactonic acid γ-lactone	L-arginine
B	Pyruvic acid methyl ester	D-xylose	D-galacturonic acid	L-asparagine
C	Tween 40	i-erythritol	2-hydroxy benzoic acid	1-phenylalanine
D	Tween 80	D-mannitol	4-hydroxy benzoic acid	L-serine
E	α-cyclodextrin	N-acetyl-D-glucosamine	γ-hydroxy butyric acid	L-threonine
F	Glycogen	D-glucosamic acid	Itaconic acid	Glycyl-L-glutamic acid
G	D-cellobiose	Glucose-1-phosphate	α-ketobutyric acid	Phenylethyl-amine
H	α-D-lactose	D,1- α-glycerol phosphate	D-malic acid	Putrescine

Figure 8. Distribution of carbon compounds on the BIOLOG ECO plate. Each well is repeated in triplicates on the actual plate. Colored matrix corresponds to the type of compound; pink – carboxylic acids, blue – complex carbon sources, yellow – carbohydrates, green – phosphate-carbon, orange – amino acids, gray – amines. Adapted from Chazarenc et al., 2010.

The BIOLOG plates were used in this research as a tool for metabolic profiling of microbial consortia. Dilution series was performed on the five microbial consortia until the desired concentration of approximately 10^3 CFU/mL were obtained. Using a multichannel pipette, 100 µL of the consortia were transferred into each well of the BIOLOG plate. The plates were covered by a moist towel and incubated at 30°C. The elevated temperature was chosen based on the results of environmental influences on the microbial growth experiments (see Section 5.1.2.).

Color development in the wells was noted after 24, 48, 72, and 96 hours. Community Metabolic Diversity (CMD) factor was calculated by summing the number of positive responses observed (violet wells), while a related Functional Diversity (FD) factor was calculated using Equation 6. Similarity between the consortia was calculated accordingly to Equation 7, while the Variation within the results was calculated based on Equation 8.

$$FD = 100 * \frac{\#\ of\ possitive\ wells}{total\ \#\ of\ wells} \tag{6}$$

$$Similarity_{i-k} = 100 * \frac{a+b}{a+b+c+d} \tag{7}$$

where a is a number of carbon sources used (indicated by the color development in the well) by both consortium i and consortium k, b is a number of carbon sources used by consortium k but not by consortium i, c is a number of carbon sources used by consortium i but not by consortium k, and d is a number of carbon sources not used by either consortium.

$$Variation = 100 * \frac{F}{31} \quad (8)$$

where F is the number of false results (i.e., number of carbon sources, in which the three replicates were not all positive or all negative). For a detailed description of the procedure, please see *SOP: Community Level Physiological Profiling,* Appendix B.

4.3. Pretreatments of Carbonaceous Materials

4.3.1. Dissolution Tests

A small quantity of coal material (less than 1 g) was placed in a clean, sterilized mortar under sterilized hood. Using a sterilized pestle, rock was crushed until completely pulverized. More material was added and crushing continued. Pulverized material was placed in sterile containers and its weight was calculated.

Chemical reagent was prepared and filtered into a sterile flask. A list of tested reagents' composition is provided in Appendix C). Desired amounts of ground samples were weighed under the sterilized hood and placed in 50mL centrifuge tubes. A 20mL aliquot of the prepared chemical reagent was added into the sample. Tubes were stored at room temperature for 14, 30, 90, and 120 days, and shaken thoroughly once a week. After the desired reaction time, samples were filtered.

The filtration method was based upon the standard method 2540D *Total Suspended Solids Dried at 103-105°C*. It was used to determine the amount of solids remaining after coal material chemical dissolution. Using a vacuum filtration apparatus, glass microfiber filters (Whatman GF/C, 1.2 µm) were washed with DI water and dried at 105°C for an hour (or until a stable weight was achieved). Dried

filters were weighed, placed in the filtration apparatus and a small aliquot of DI water was applied onto the filter to create a seal. Sample was mixed thoroughly and poured onto the filter and the suction was applied. The sample container and its cap were washed with DI water and detergent, if necessary, until all the solids were transferred onto the filter. Suction was applied until all the water was evacuated. Filter papers with solids were dried at 105°C for 24 hours (or until a stable weight was achieved), cooled in a desiccator, and weighed. The amount of solids was calculated as a difference between the final and initial weight of the filter.

For more detailed procedure descriptions, see *SOP: Coal Crushing – Small Scale, SOP: Chemical Dissolution of Coal – Sample Preparation,* and *SOP: Chemical Dissolution of Coal - Filtration* (Appendix B).

4.3.2. Raman Spectroscopy

In order to assess the biochemical degradation of coal materials, pulverized bituminous coal and coal waste were immersed in five liquid media and DI water (for media composition, see Appendix A). The Raman spectroscopy analysis was performed on the liquid phase of these samples after 48 hours and six months. The resulting spectra were compared to the spectra obtained from the five liquid media and DI water.

A 3 mL aliquot of each sample was filtered through a 0.2 μm syringe filter into a glass vial and dried at 45°C. Samples were analyzed with a Raman Systems R-3000 QE portable spectrometer.

4.4. Gas Generation Tests in Serum Bottles

4.4.1. Microbes in Liquid Media

Carbon dioxide, methane, and heavier gaseous hydrocarbons (C_2-C_6) generated from environmental samples, immersed in various media, were measured using gas chromatography (for environmental sample preparation see Section 4.1.4.).

Gas chromatography analysis begins with turning on the computer and flaming the GC unit. The column and gastight syringe were cleaned with fresh air. The tip of the gastight syringe was placed in the sample vial through an opening made in the septum. The gases were allowed to fill the syringe up to the 500μL mark, the plunger was pushed down to the 200μL mark, while the syringe valve was closed, and excess gases were evacuated into DI water to prevent contamination with the atmospheric gases. After preparing the GC unit, the sample was injected into the injection port. Using calibration data, integrated peak areas were recalculated into gas concentrations.

For a more detailed procedure description, see *SOP: Gas Chromatography* (Appendix B).

4.4.2. Microbes in Coal Samples with and without Nutrient Amendment

Gas generation results combined with microbial colony counts were used to design the experimental matrix. Four microbial population categories as well as their consortia were selected (methane producers, carbon dioxide producers, producers of carbon dioxide and methane, and producers of other gases). The most representative TSA plate was chosen from each series of plated environmental

samples (see Section 4.2.1.) and 2-5 mL of a medium used in a given environmental sample was added to it. Harvested microbes and liquid were collected from the plate and placed in a sterile 15 mL graduated centrifuge tube containing 10 mL of the appropriate medium. Tubes were vortexed and stored in room temperature. After 2-3 days microbes were washed with saline solution and used to inoculate coal samples.

Glass serum bottles (20mL, Wheaton #223742) were used as bioreactors. Four-gram aliquots of pulverized hydrocarbon material (coal, waste coal, and lignite) were placed in serum bottles; five milliliters of liquid solution and one and a half milliliter of microbial consortia were added; each vial had a headspace of approximately 14 milliliters. Microbial samples were centrifuged and washed with saline solution three times, in order to remove remaining carbon sources that could have been introduced through the culture media. A Teflon silicone septum (Wheaton #224173) was placed on top of a bottle and was sealed with an aluminum seal (Wheaton #224178) using a crimper.

Three levels of nutrient amendments were selected; 0%, 10%, and 50%. Thus, the liquid solution added to hydrocarbon samples contained either only normal saline solution (corresponding to 0% nutrient amendment) or normal saline solution with 10% or 50% of additional nutrients. The composition of nutrient amendments was identical to liquid media, in which the consortia were cultured.

Over 650 samples, containing solid hydrocarbons, microbial consortia from various environments, and nutrient solution amendments, were created. The samples were left at room temperature for 30 days and were not disturbed beyond

the normal handling conditions. Produced gases were analyzed with gas chromatography.

For more detailed procedure descriptions, see *SOP: Gas Chromatography* and *SOP: Gas Generation Tests* (Appendix B).

4.4.3. Microbial Consortia in Pretreated Coal Samples

Coal samples were pretreated with three selected chemical pretreatments, analyzed for gas generation with GC after 14 days, inoculated with four selected microbial consortia, and analyzed for gas generation again after an additional 14 and 30 days, for a total of 44 days (Figure 9).

Glass serum bottles (20mL, Wheaton #223742) were used as bioreactors. Four-gram aliquots of pulverized hydrocarbon material (coal, waste coal, and lignite) were placed in serum bottles; eight milliliters of liquid solution and two milliliters of microbial consortia concentrated by a factor of three were added. Four microbial consortia (consortium 1, 3, 4, and 5) were selected. Consortia were washed and centrifuged three times prior to usage. A Teflon silicone septum (Wheaton #224173) was placed on top of a bottle and was sealed with an aluminum seal (Wheaton #224178) using a crimper.

The composition of chemical pretreatments is given in Table 10. Based on coal dissolution tests, lactic acid was selected as a carbon based pretreatment and a potential direct methane precursor, while Nickel/Alumina/Silica was selected as a non-carbon based catalytic pretreatment. Hydrogen peroxide was used both as a pretreatment, since it achieved high coal dissolution rates, and as a control, since all other pretreatments contained it. Sodium dodecyl sulfate (SDS) was selected as a

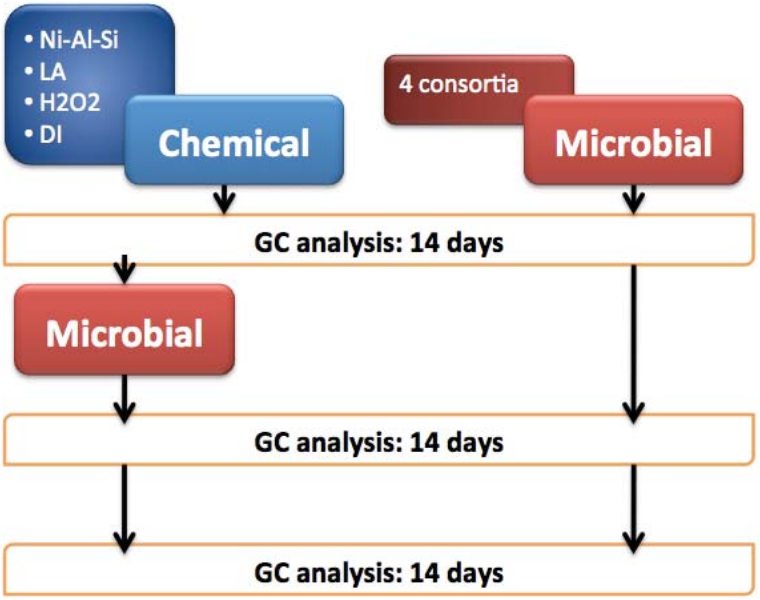

Figure 9. Simplified test matrix for the combined chemical and microbial pretreatment tests.

Table 10. Chemical pretreatment composition.

Abbreviation	Ni	LA	HP	DI
Hydrogen peroxide	3%	3%	3%	-
Sodium dodecyl sulfate (SDS)	0.001M	0.001M	0.001M	-
Active reagents	30mg/L $NiCl_2$ 20mg/L Al_2O_3 100mg/L SiO_2	0.1M lactic acid	-	0.85% NaCl

surfactant instead of Tween 20. Both of these are relatively non-toxic for microorganisms, however, SDS (an anionic surfactant) performed better in coal dissolution tests than Tween 20 (a non-ionic surfactant).

Additional tests, using the same testing matrix and conditions, were performed on four solid hydrocarbon samples (anthracite, subanthracite, bituminous coal, and coaly shale) as well as corn samples. This was done for a comparison of applicability of this technology to various carbonaceous materials.

Moreover, a separate set of tests was performed using enzyme extracts as a pretreatment step. Microbial consortia were grown to the top of the exponential log phase and washed twice with normal saline solution. These concentrated cells were immersed in cold NP40 lysis buffer (chemical cell lysis) and placed in the bead-beater chamber filled with cold glass beads for three minutes (mechanical cell lysis). That allowed for rupture of microbial cell walls and extraction of enzymes. Some of the enzyme preparations, as well as some microbial inocula, were immobilized in alginate beads. One of the advantages of immobilization of cell homogenate is that multiple enzymes can be introduced to the reaction, eliminating the need for separate immobilization of multiple enzymes. Immobilized enzymes or microbes are more convenient to use, usually provide higher stability, and offer protection from the environment. Immobilization material allows for diffusion between the microbial cells and the environment. It also provides nutrients for the microbial growth and will eventually be degraded by the immobilized microbes, releasing them into the environment. Biodegradation of the immobilization material can be designed to be a slower or a faster process. Moreover, chemical degradation of the immobilization material can be performed after the microbes are delivered into the coal seam (e.g.,

exposure to citrate dissolves alginate material). Microbial immobilization would be a preferred technique during field injections into natural gas wells. For more detailed procedure descriptions, see *SOP: Extraction and Immobilization of Enzymes* and *SOP: Immobilization of Microbial Cells* (Appendix B).

The samples were incubated at 30°C and were not disturbed beyond the normal handling conditions. Slightly elevated temperature was selected as the most suitable one for the created consortia (see Section 5.1.2.). Produced gases were analyzed with gas chromatography.

4.5. Bench Scale Bioreactor Tests

Based on the results of combined chemical pretreatment and microbial gas generation tests, lactic acid pretreatment and inoculum 3 were chosen for the bench tests. Plastic, 500mL gas-tight cylindrical containers, obtained from the Energy and Geoscience Institute, were used as batch bioreactors (Figure 10). One set of bioreactors (electro-biochemical reactors; EBR) using titanium electrodes examined the direct electron provision influence on methane production. Each reactor contained 100 gram aliquots of pulverized hydrocarbon material (coal, waste coal, and lignite); 200 milliliters of liquid solution (nutrient media or normal saline solution), and 50 milliliters of four times concentrated microbial consortia. Additionally, about 150 grams of gravel was added to each reactor for equal distribution of solid, liquid, and gas phases (⅓ of each reactor volume) and to provide routes for gases to escape from the solid/liquid phase. Microbial samples were centrifuged and washed with saline solution three times, in order to remove remaining carbon sources that could have been introduced through the culture

Figure 10. Bench scale bioreactors and electro-biochemical reactors.

media. Control samples included: coal immersed in normal saline solution (to examine gas desorption from coal matrix and gas generation by native coal microbial population); microbial inoculum incubated in saline solution and gravel (to examine gases generated from microbes consuming dead cells); and microbial inoculum incubated in lactic acid pretreatment and gravel (to examine gases generated by decomposition of the chemical pretreatment).

Aside from normal handling conditions, the samples were left undisturbed at about 23°C. One set of especially prepared electro-biochemical reactors were connected to 3.0V potential supplied by a power supply (TekPower HY1803D). Produced gases were analyzed with gas chromatography after 14, 28, and 44 days.

5. RESULTS AND DISCUSSION

5.1. Microbial Characterization

5.1.1. Morphology Characterization

Collected coal and environmental samples were immersed in five different media and in DI water (see Appendix A). All the samples were plated on TSA plates in the dilution range of 10^{-1} to 10^{-6}. After three days from plating, colony morphologies and plate counts were performed on all samples. Results are presented in Appendix D.

5.1.2. Environmental Influences

5.1.2.1. pH Influence on Microbial Growth

Selected results from only one of five consortia are presented below, but all consortia were tested; the results are presented in Appendix E. Figure 11 shows consortium 3 growth curves under various pH conditions. Moreover, normalized microbial distribution graphs were prepared for all the measurements (Figure 12 shows and example of consortium 3 growth under various pH values, the remaining graphs are given in Appendix E). Interestingly, all tested consortia adjusted the pH towards their optimum level either by producing acids or by reducing sulfates (Figure 13). By combining these results, it can be concluded that the favorable pH for every consortium was in the range of 7.0-9.5. Moreover, it was found that none of the consortia grew well in pH below 6 or above 11. Most of the known methanogenic

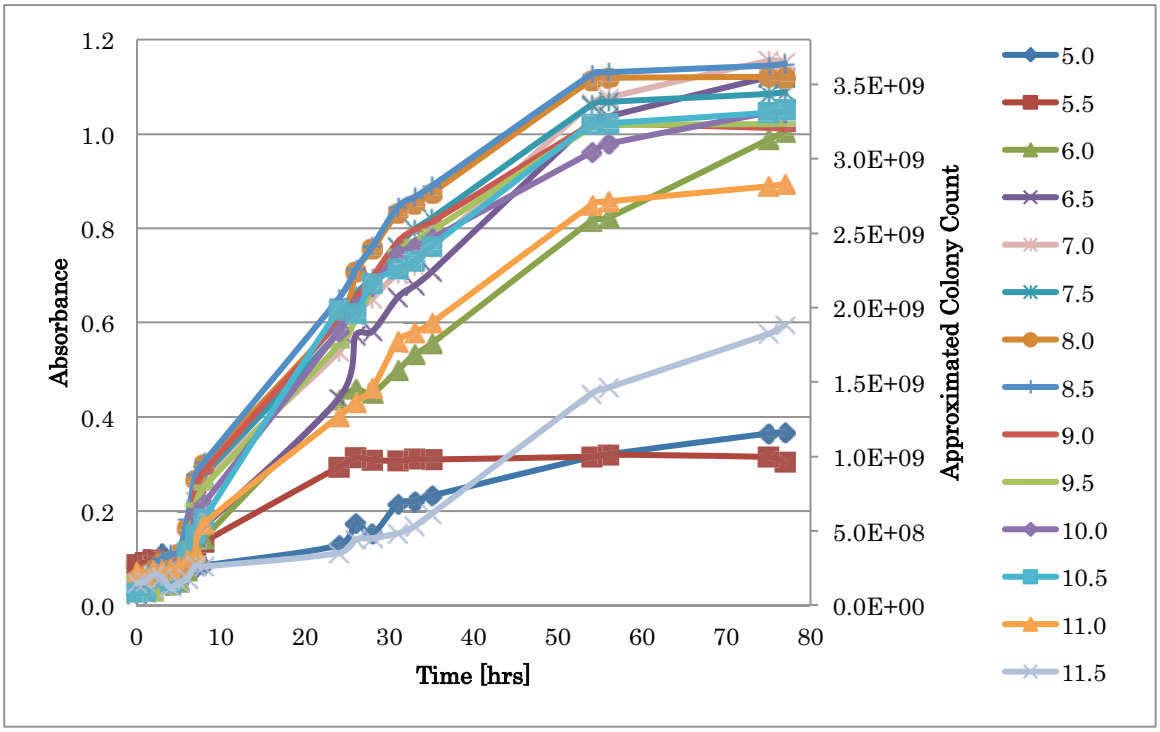

Figure 11. Consortium 3 growth under starting pH conditions ranging between 5.0 and 11.5 at about 23°C.

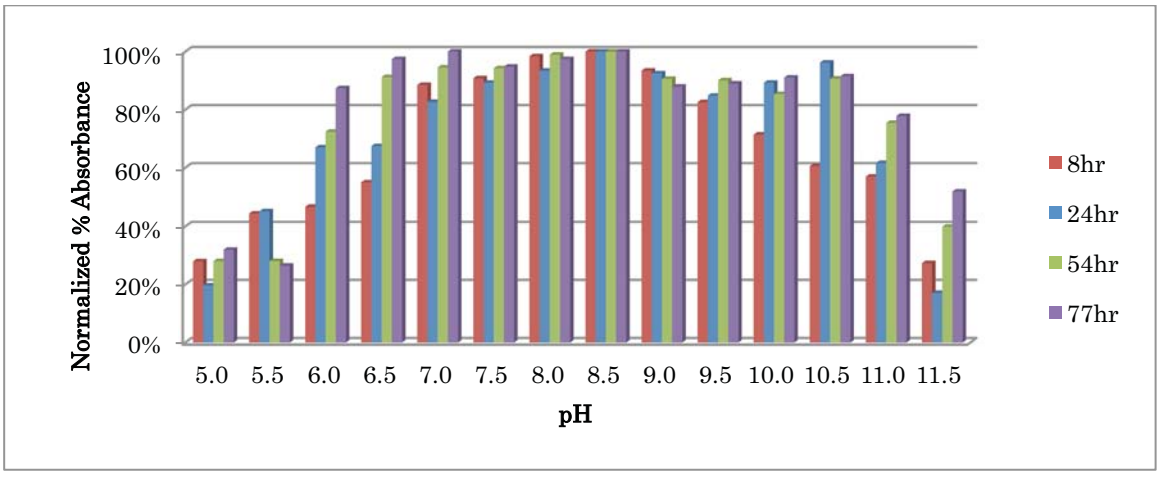

Figure 12. Normalized consortium 3 microbial distribution under starting pH conditions ranging between 5.0 and 11.5.

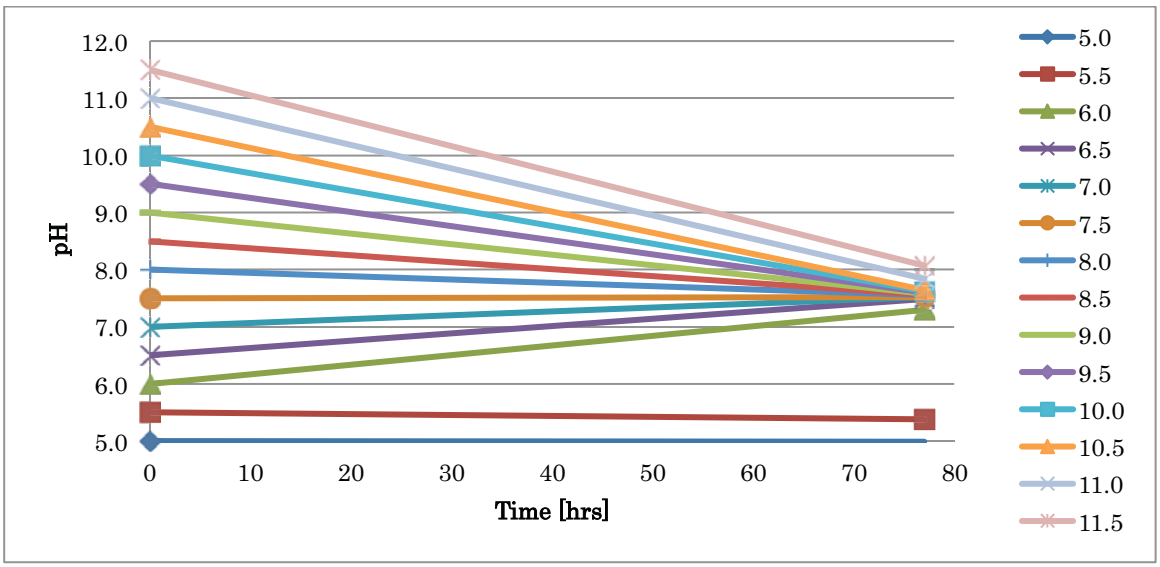

Figure 13. Change of pH in consortium 3 after 77 hours at about 23°C.

microorganisms favor conditions pH close to neutral (Table 3). Moreover, this was expected, since the microorganisms used were collected from neutral pH environments and cultured at neutral pH, making them predisposed to such conditions.

5.1.2.2. Temperature Influence on Microbial Growth

Temperature dependence tests showed that all of the consortia performed best at 30°C, with little decrease in growth at 20°C (Figure 14). Many methanogenic microorganisms are thermophilic, preferring temperatures higher than room temperature (Table 3). The fact that these consortia were collected from natural environments with temperatures of 10-25°C and then grown and stored at room temperature explains why they adapted to or were naturally selected for 20-30°C temperature optimum.

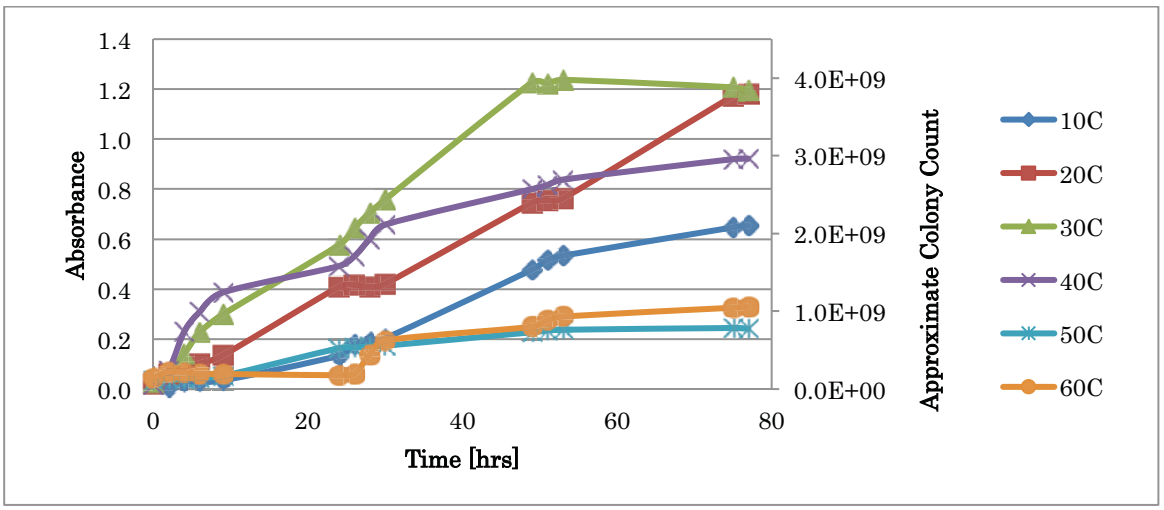

Figure 14. Consortium 3 growth under temperature ranging between 10-60°C.

5.1.2.3. Salinity Influence on Microbial Growth

All consortia preferred a slight addition of salts, with a significant advantage at 2g/L NaCl (Figure 15). This was expected as well, since many environments selected for microbial collection were characterized by elevated salinity.

5.1.2.4. Growth Kinetics Parameters

Finally, growth kinetics parameters were calculated for every consortium and every environmental condition, over the exponential growth phase. As an example, Figure 16 shows the growth kinetics parameters for consortium 3 under various temperature conditions. All the remaining kinetics data is available in Appendix E. Growth kinetics data somewhat differs from the information obtained from the growth curves. Only the exponential growth stage is taken into account during these calculations, regardless of the extent to which it was maintained. For example, consortium 3 experienced the fasted initial growth at 40°C (Figure 14), which is also

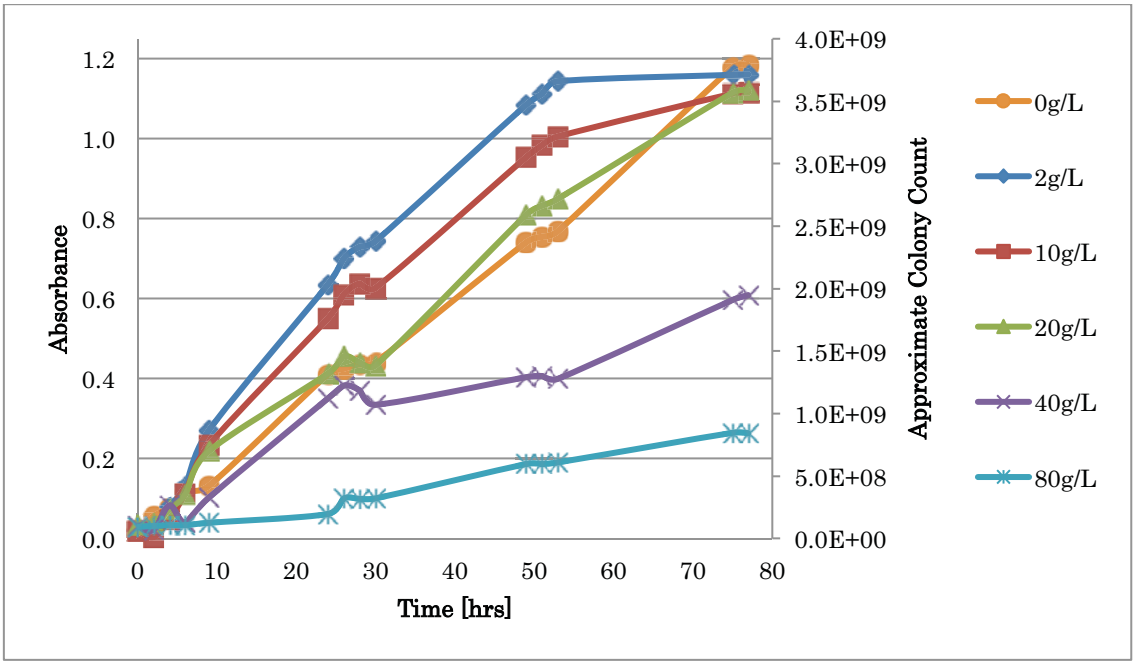

Figure 15. Growth of consortium 3 under salinity (added as NaCl) ranging between 0-80 g/L at about 23°C.

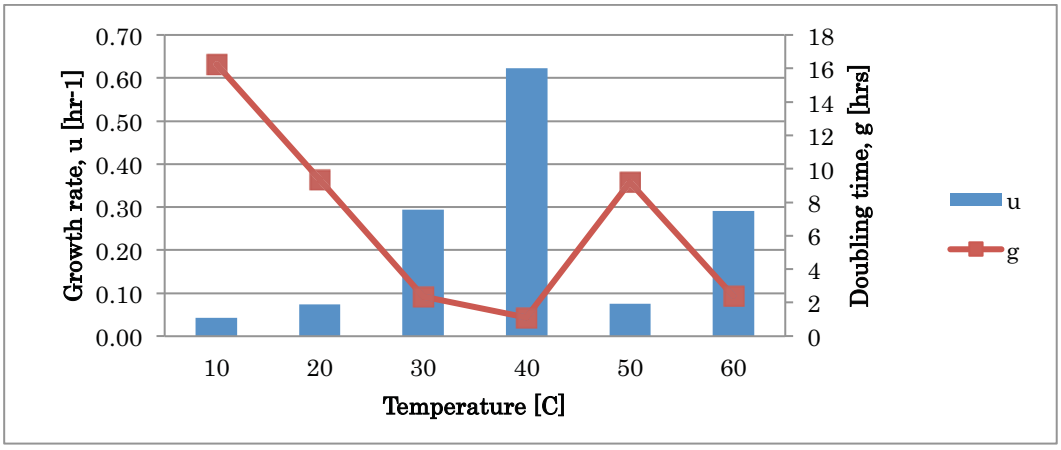

Figure 16. Growth rate constant, μ, and doubling time, g, for consortium 3 under various temperature conditions.

evident in the growth kinetics parameters (Figure 16). Nevertheless, the growth began to slow down at this temperature after 9 hours and the final microbial concentration after 77 hours was much lower here than in the samples incubated at 20°C and 30°C.

5.1.2.5. Implications

By selecting the aforementioned environments for microbial collection, a certain assumption was made about the optimal growth conditions of the desired methanogenic consortia. All samples were collected at 10-25°C and around neutral pH. At least a half of the samples came from higher salinity environments. By doing so, it was realized that the obtained populations would be best adapted to these conditions and would not include, for example, thermophilic microorganisms. Furthermore, the microbes were collected and stored at room temperature and around neutral pH. Therefore, the laboratory testing would need to be performed under the same or similar conditions.

To achieve the best methane and/or CO_2 production in different environments than the ones examined, microbes and consortia need to be developed based upon prevailing environmental conditions and chemistry; this holds for use in coal-bed seams or in bioreactors. For instance, when enhancing methane recovery from a deep coal seam that reaches temperatures above 40°C, methanogenic populations from similar environments will have to be collected. Moreover, consortia created in this research can adapt, to a certain extent, to different conditions than those found optimal herein, through gradual exposure to such conditions. As an example, consortium 3 did not show optimal growth at the pH of 6.0 but did grow favorably at

pH 7.0-8.0. Transferring consortium 3 to growth medium and gradually lowering the pH and letting it adapt prior to the next transfer would possibly allow the microbial population to adapt to new lower pH conditions. However, placing consortium 3 in an environment with pH of 6.0 without attempting to adapt it first would result in microbes experiencing a shock, not growing as fast, and therefore, not metabolizing the substrates into methane or CO_2 at the best possible rates.

5.1.3. Oxygen Requirement

A photograph of the thioglycollate tubes after two-day incubation period at 30°C in the dark is given in Figure 17. The analysis of results in provided in Table 11.

The analysis of the results indicates that consortia 1, 3, 4, and 5 are comprised of obligate and aerotolerant anaerobes, while consortium 2 contains facultative anaerobes, microaerophiles, and obligate aerobes. Consortia 1, 3, 4, and 5 were created from microbes cultured from a variety of environments and in a variety of media. Consortium 2, was created only from microbes cultured in two environments: lake sediments and a coal-bed methane well; and was cultured in DI water without any additional carbon source.

5.1.3.1. Implications

The fact that unique facultative or aerotolerant anaerobic consortia capable of generating large amounts of either methane or CO_2 can be screened and developed from naturally-occurring sources is a significant contribution of this research.

Figure 17. Thioglycollate tubes inoculated with five consortia.

Table 11. Growth pattern of five consortia (numbered 1 through 5) in thioglycollate tubes.

	1	2	3	4	5
Bottom	☑		☑	☑	☑
Throughout	☑		☑	☑	☑
Throughout with higher concentration at the top		☑			
Just below the surface		☑			
Top		☑			

Utilization of characterized and controlled strict anaerobic consortia in laboratory settings is difficult; in the field it is even more so, and the perceived need to conduct methane production enhancement research and testing using only strict anaerobes or in strict anaerobic environments has at least somewhat impeded development of this technology. Aerotolerant anaerobes can be utilized in the coal seams, where they will be exposed to oxygen during the injection process, as well as in the above

ground applications, such as coal waste heaps or bioreactors, where the oxygen exposure can occur more frequently. Moreover, the use of aerotolerant microbes is possible when the use of oxidizing chemical agents (e.g., hydrogen peroxide) to degrade coal may be deemed beneficial.

5.1.4. Community Level Metabolic Profiling

An example of BIOLOG plates after 0, 24, and 72 hours of incubation is given in Figure 18. Violet well indicates that the given carbon source has been utilized by a given consortium. Summarized results of metabolic profiling of the five consortia are presented in Figure 19.

Consortium 3 was the most versatile, utilizing 29 carbon sources. Consortia 1 and 5 were also diverse, using 28 and 24 carbon sources, respectively. Consortium 4 utilized only slightly more than a half of available carbon compound. Finally, consortium 2 was able to metabolize only seven carbon sources. These observations are quantifiable using Community Metabolic Diversity and Functional Diversity calculations (Figures 20 and 21). While both of these factors represent the total number of substrates effectively metabolized by the microbial community; CMD is an absolute value, while FD is represented as a percentage. They both measure the metabolic diversity but do not identify the metabolized and non-metabolized carbon sources.

None of the consortia were able to metabolize 2-hydroxy benzoic acid, also known as salicylic acid. Salicylic acid functions as a plant hormone and is chemically similar to acetylsalicylic acid – the active component of aspirin. That is surprising as the consortia components were selected for biodegradation of more complex carbon

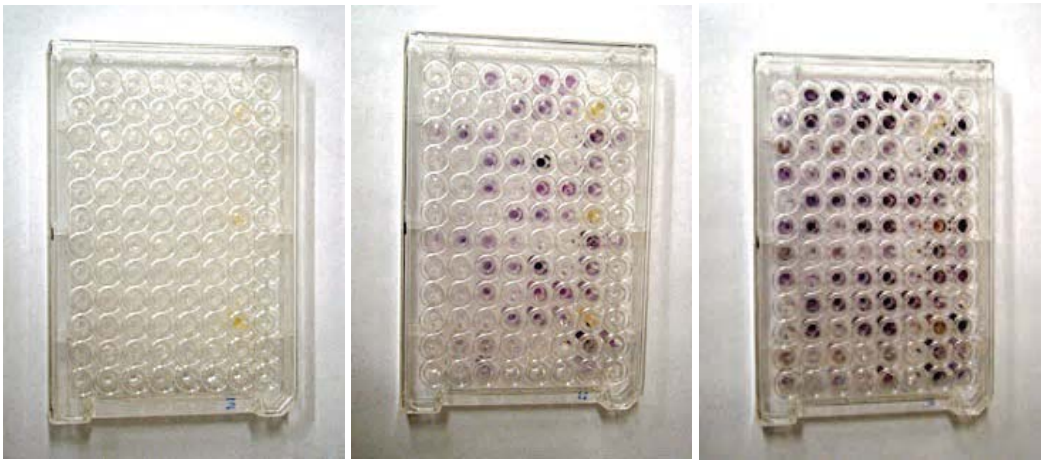

Figure 18. An example of inoculated BIOLOG plates after 0, 24, and 72 hours.

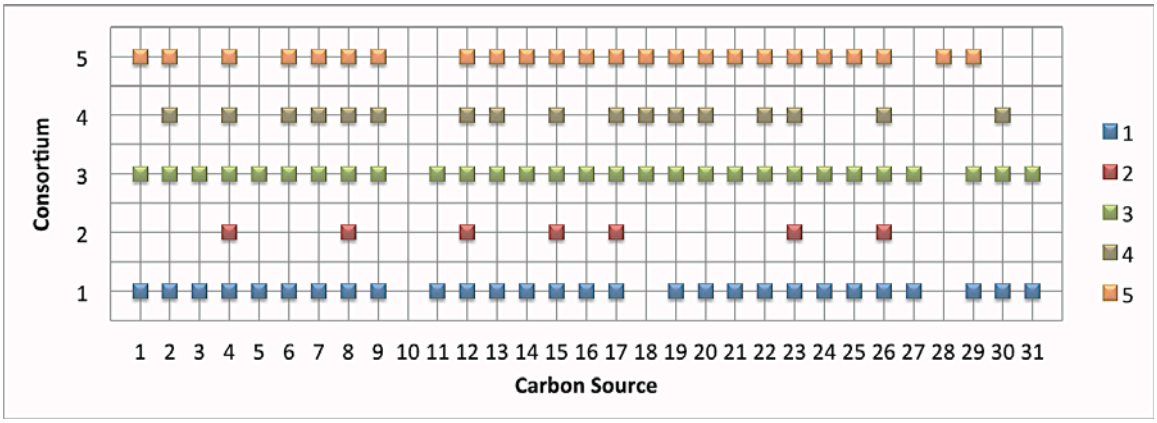

Figure 19. Utilization of 31 carbon sources by five consortia after 96 hours incubation time at 30°C. Carbon sources: **1** – ß-Methyl-D-Glucoside, **2** – D-Galactonic Acid, **3** – L-Arginine, **4** – Pyruvic Acid Methyl Ester, **5** – D-Xylose, **6** – D-Galacturonic Acid, **7** – L-Asparagine, **8** – Tween 40, **9** – i-Erythritol, **10** – 2-Hydroxy Benzoic Acid, **11** – L-Phenylalanine, **12** – Tween 80, **13** – D-Mannitol, **14** – 4-Hydroxy Benzoic Acid, **15** – L-Serine, **16** – α-Cyclodextrin, **17** – N-Acetyl-D-Glucosamine, **18** – γ-Hydroxybutyric Acid, **19** – L-Threonine, **20** – Glycogen, **21** – D-Glucosaminic Acid, **22** – Itaconic Acid, **23** – Glycyl-L-Glutamic Acid, **24** – D-Cellobiose, **25** – Glucose-1-Phosphate, **26** – α-Ketobutyric Acid, **27** – Phenylethyl-amine, **28** – α-D-Lactose, **29** – D,L-α-Glycerol Phosphate, **30** – D-Malic Acid, **31** – Putrescine.

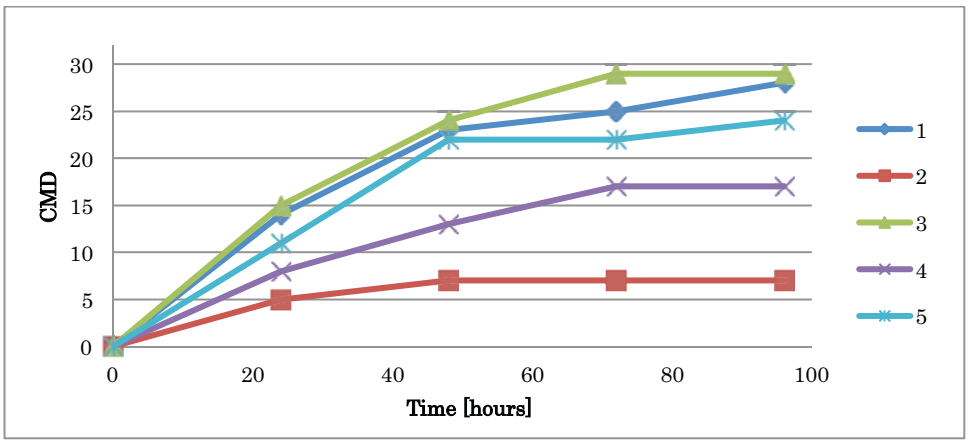

Figure 20. Community Metabolic Diversity of five consortia numbered 1 through 5.

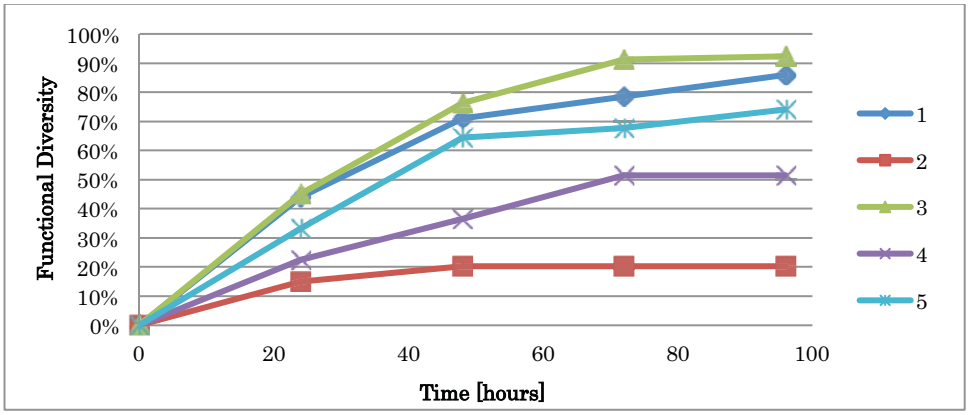

Figure 21. Functional Diversity of five consortia numbered 1 through 5.

compounds than plant matter. Moreover, α-D-Lactose, milk sugar, was only decomposed by consortium 5, indicating that only this consortium contains lactic acid bacteria. On the other hand, all consortia were able to utilize the following: pyruvic acid methyl ester, Tween 40, Tween 80, L-serine, N-acetyl-D-glucosamine, glycyl-L-glutamic acid, and α-ketobutyric acid.

Data variability is an indicator of reproducibility; in these experiments it measures variation within samples tested in triplicate (Figure 22). During the incubation period, consortium 5 had the highest variation, exceeding 25%. This may

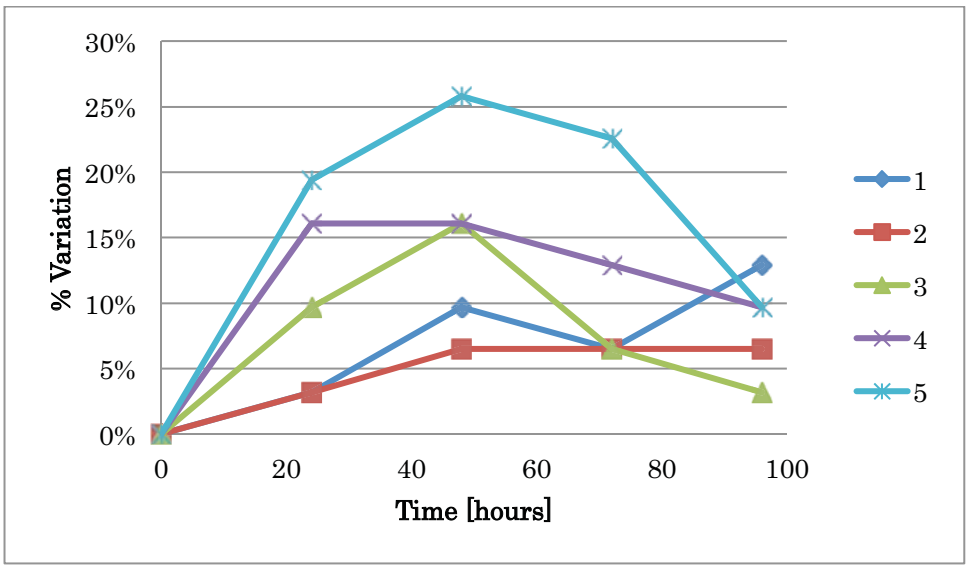

Figure 22. Variation of results with sample of five consortia numbered 1 through 5.

indicate that consortium 5 contains slow growing organisms, which metabolize carbon sources slowly, even though at the end of the 96-hour incubation consortium 5 showed similar metabolic diversity to consortium 3. At the end of the incubation period, all consortia showed variation below 15%, representing good reproducibility of results.

Percent (%) similarity indicates how functionally similar given two consortia are (Figure 23). Consortia 1 and 3 were nearly 97% similar at the end of the incubation period. Populations present in consortia 1 and 3 were collected from similar environments (Table 9), cultured in the same media, and might have contained the same microbial species at a similar population density. Consortia 2 and 3, and consortia 1 and 2 had a functional similarity of only about 30%. Consortium 2 contained three populations cultured from samples incubated with DI water only, while all other consortia were created from a larger number of populations, incubated with a variety of carbon based growth media (TSB, acetate, etc.).

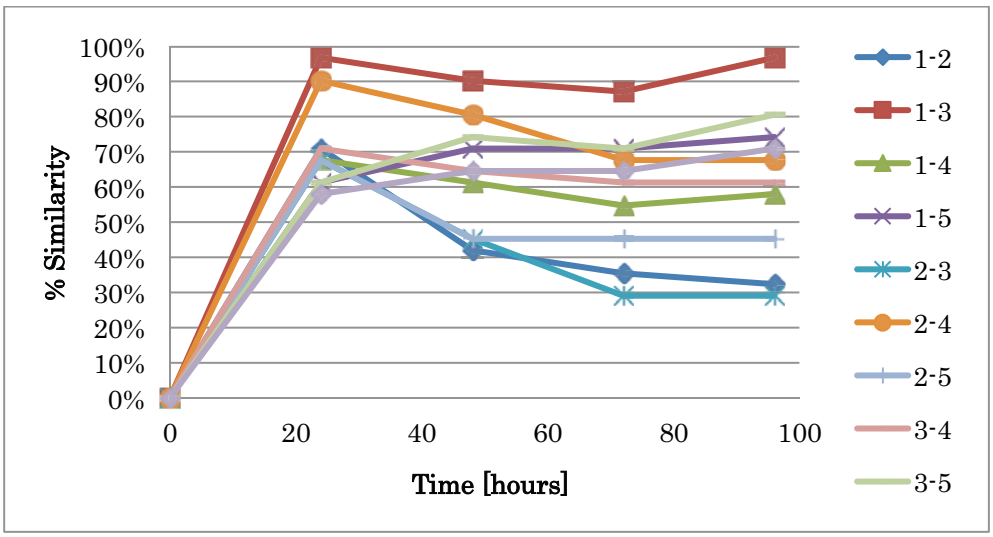

Figure 23. % Similarity between consortia numbered 1 through 5.

Therefore, it was expected that consortium 2 would show the lowest similarity to all other created consortia; however, it was not certain that it would also exhibit the lowest metabolic diversity. Nevertheless, since consortium 2 showed the lowest metabolic diversity and the lowest enhancement of methane production, it was not used in further tests.

5.2. Pretreatments of Carbonaceous Materials

5.2.1. Dissolution Tests

Literature review of possible chemical reagents capable of coal degradation was performed by Dr. Amar Sathyapalan under supervision of Dr. Michael Free (Department of Metallurgical Engineering, University of Utah). Dr. Sathyapalan identified numerous metal catalysts, organic and inorganic acids, and other compounds that could break down aliphatic bonds in the coal structure and performed initial screening tests using aggressive and environmentally benign

reagents. Up to 40% of bituminous coal, 50% of bituminous coal waste, and 100% of lignite were dissolved using aggressive chemical pretreatments in the preliminary study performed by Dr. Sathyapalan. Aggressive reagents included, for example, 5M sulfuric acid, 30% hydrogen peroxide, and 17M acetic acid, dipyridyl. Aggressive chemical pretreatments could be performed in ex-situ setup and would require neutralization steps prior to establishment of microbial populations.

Results presented here were performed using reagents selected from Dr. Sathyapalan's tests and improved methods described in Appendix B. Figure 24 shows results of long-term digestion tests with environmentally benign reagents. These tests resulted in statistically insignificant dissolution of bituminous coal waste (Figure 24) and coal (data not shown) but achieved up to 25% dissolution of lignite. Additionally, several other reagents were tested for their ability to dissolve coal and are shown in Figure 24, including citric acid, ascorbic acid, Fenton's reagent (at normal pH and adjusted to about 4), and ferric citrate. None of these reagents were found to increase digestion of lignite, bituminous coal or bituminous coal waste above that achieved by previously tested reagents.

The influence of surfactant type used in coal degradation was assessed (Figure 25). It was found that an anionic surfactant (SDS) increased the coal dissolution in comparison to a non-ionic surfactant (Tween 20), indicating that ionic forces might be important during coal breakdown.

Selected reagents were tested for their ability to dissolve coal over 14, 30, and 90 days (Figure 26). Results indicate rapid dissolution kinetics and that reaction times longer than 14 days do not provide significant dissolution increases.

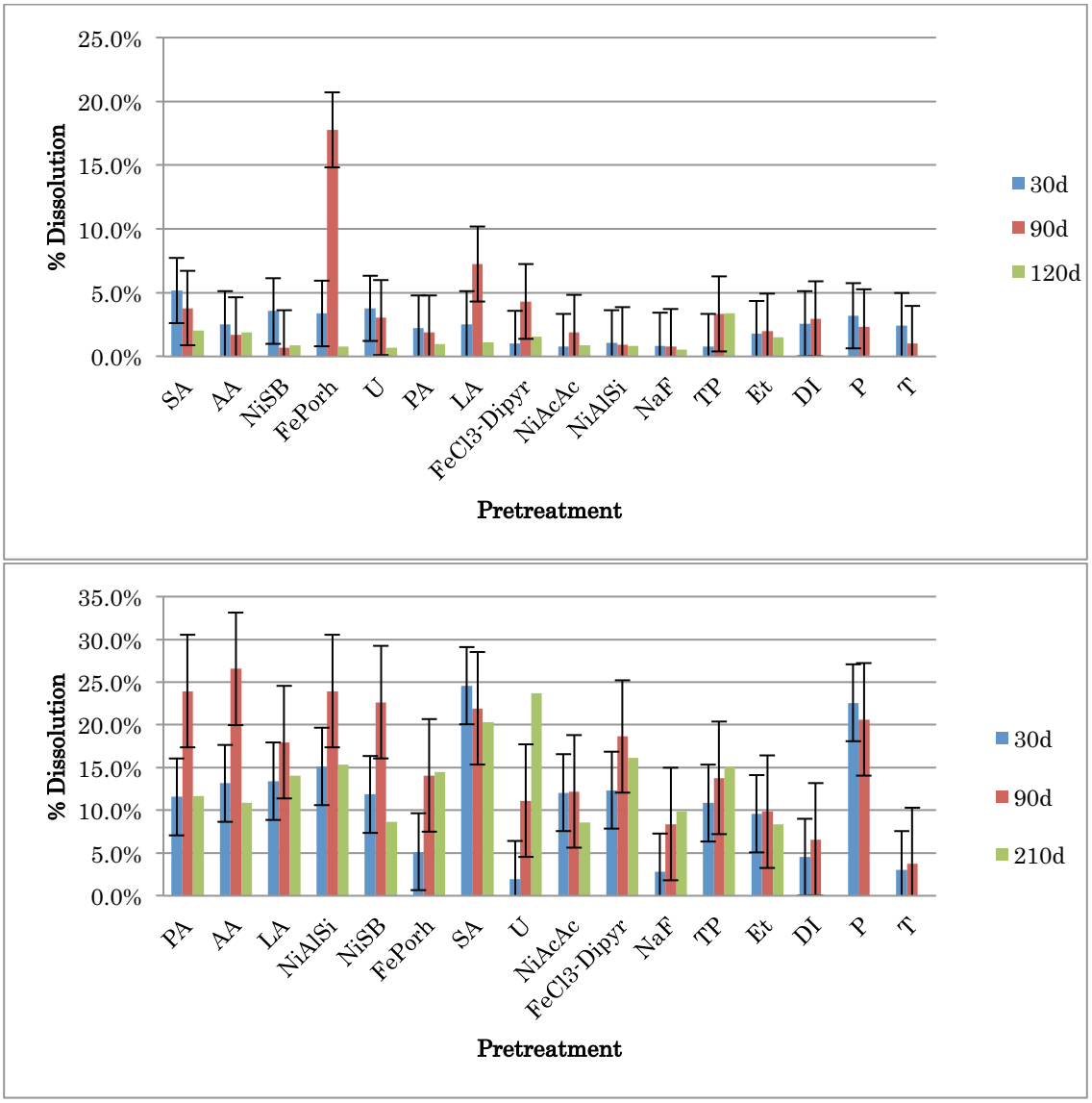

Figure 24. Chemical dissolution of bituminous coal waste (upper graph) and lignite (lower graph) over 30, 90, and 210 days. Error bars correspond to values obtained with control treatment (DI water). All reagents contain 3% H_2O_2 and 0.5% Tween20 unless noted otherwise. **AA** – 0.1M acetic acid; **DI** – deionized water (no Tween20 or peroxide added); **Et** – 1% ethanol (no Tween20 or peroxide added); **FeCl3-Dipyr** – 25mg/L dipyridyl + 100mg/L $FeCl_3$; **FePorph** – 40mg/L iron porphine (iron (III) meso-tetraphenylporphine-mu-oxo-dimer); **LA** – 0.1M lactic acid; **NaF** – 25mg/L sodium fluoride; **NiAcAc** – 60mg/L nickel acetyl acetone; **NiAlSi** – 30mg/L $NiCl_2$ + 20mg/L Al_2O_3 + 100mg/L SiO_2; **NiSB** – 60mg/L nickel Shiff base N,N'Bis(salicylidene)ethanediamino nickel II); **P** - 3% H_2O_2; **PA** – 0.1M phosphoric acid; **SA** – 0.1M sulfuric acid (no Tween20 added); **T** - Tween20; **TP** – Tween20 + H_2O_2; **U** – 100mg/L urea.

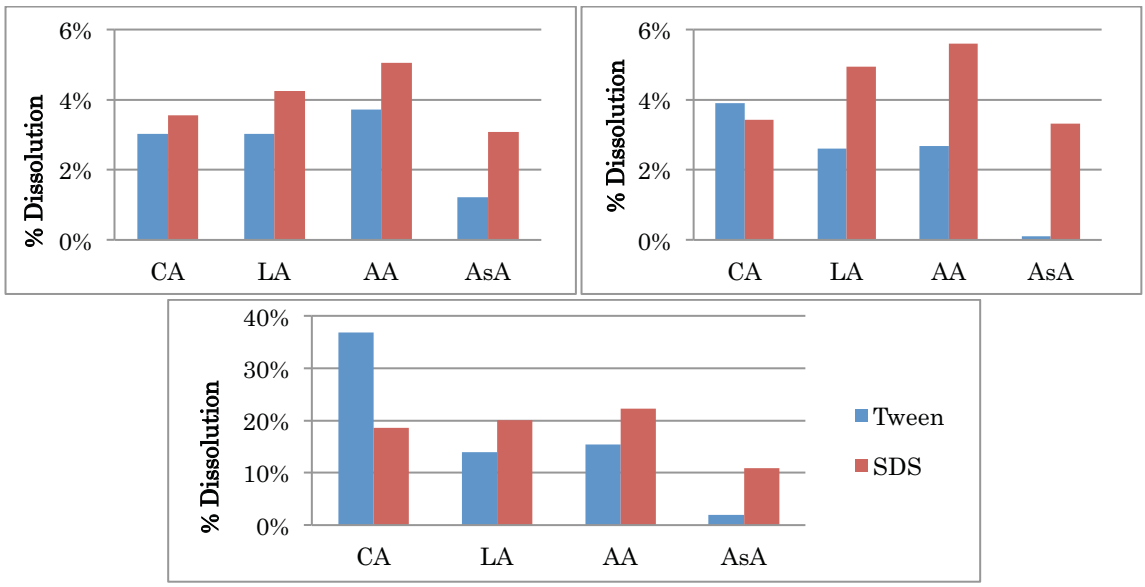

Figure 25. Influence of the type of the surfactant on the dissolution of bituminous coal (upper, left), bituminous coal waste (upper, right), and lignite (bottom). Every treatment included 3% H_2O_2 and 0.5% Tween20 or 0.001M SDS. CA-citric acid, LA-lactic acid, AA-acetic acid, AsA-ascorbic acid. All the organic acids were added at a concentration of 0.1M.

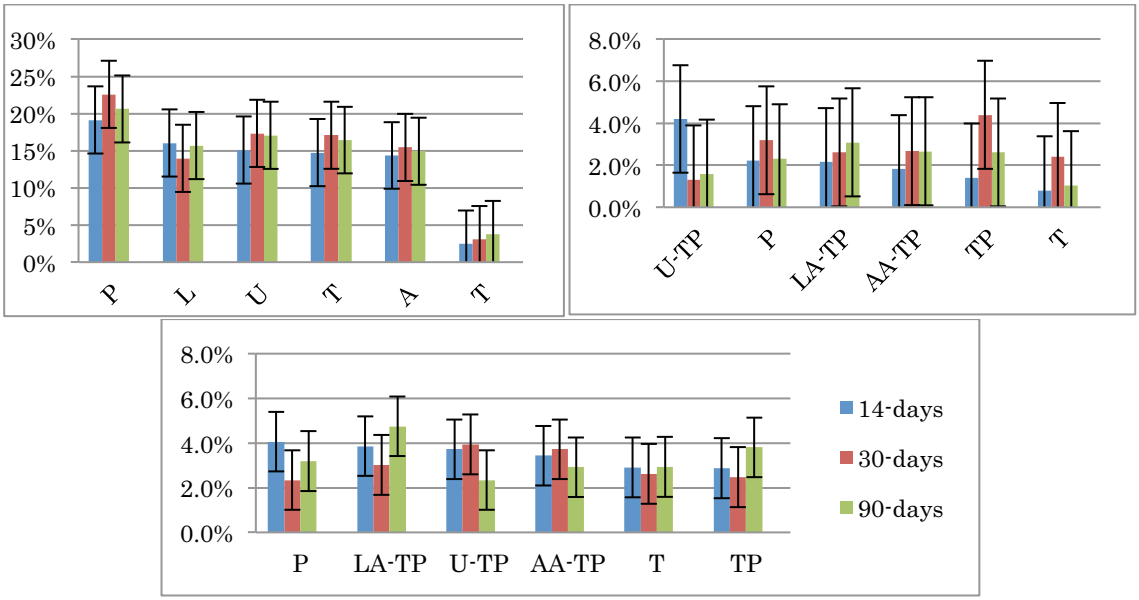

Figure 26. Chemical dissolution of lignite (upper, right), bituminous coal waste (upper, right), and bituminous coal (bottom) over 14, 30, and 90. Error bars correspond to values obtained with control treatment (DI water). **P** – 3% hydrogen peroxide; **LA-TP** – 0.1M lactic acid, 0.5% tween20, 3% hydrogen peroxide; **U-TP** – 100mg/L urea, 0.5% tween20, 3% hydrogen peroxide; **AA-TP** – 0.1M acetic acid, 0.5% tween20, 3% hydrogen peroxide; **T** – 0.5% tween20; **TP** – 0.5% tween20, 3% hydrogen peroxide.

Most tested chemical pretreatments contained hydrogen peroxide. Hydrogen peroxide is a strong oxidizer and a highly reactive oxygen species, capable of oxidizing organic matter. Moreover, at low concentrations (below 3%), it is harmless to most microorganisms, since they have catalase peroxidases (enzymes decomposing hydrogen peroxide into water and oxygen). Figures 24 and 26 show that 3% hydrogen peroxide achieved similar coal dissolutions as other chemical pretreatments, suggesting that oxidation might be a primary mechanism of coal dissolution. This indicates the need for developing coal biodegrading and methanogenic microbial consortia that are capable of biological oxidation and/or survival under low oxygen concentrations.

As in all experiments, the results obtained are potentially biased due to methods used. Coal breakdown into smaller molecules, not resulting in complete coal dissolution, would not be registered by this technique. Furthermore, the formation of chemical precipitates or extent of microbial growth may have influenced these results significantly. There is a high probability of producing inorganic cations (e.g., metals) and anions (e.g., carbonate, hydroxide, sulfide) during chemical digestion of complex structures as coal. Binding of this species could occur, leading to formation of chemical precipitates retained during filtration, which would negatively influence the results presented. Figure 27 shows a dramatic example of lignite sample pretreated with 1M sulfuric acid, where a chemical reaction occurred, leading to a formation of crystals. Since the crystals were not observed immediately after the lignite dissolution, formation of such a precipitate could be avoided to circumvent possible detrimental effects on reservoir permeability, by controlling water chemistry. Moreover, many of the tested chemical pretreatments are also microbial

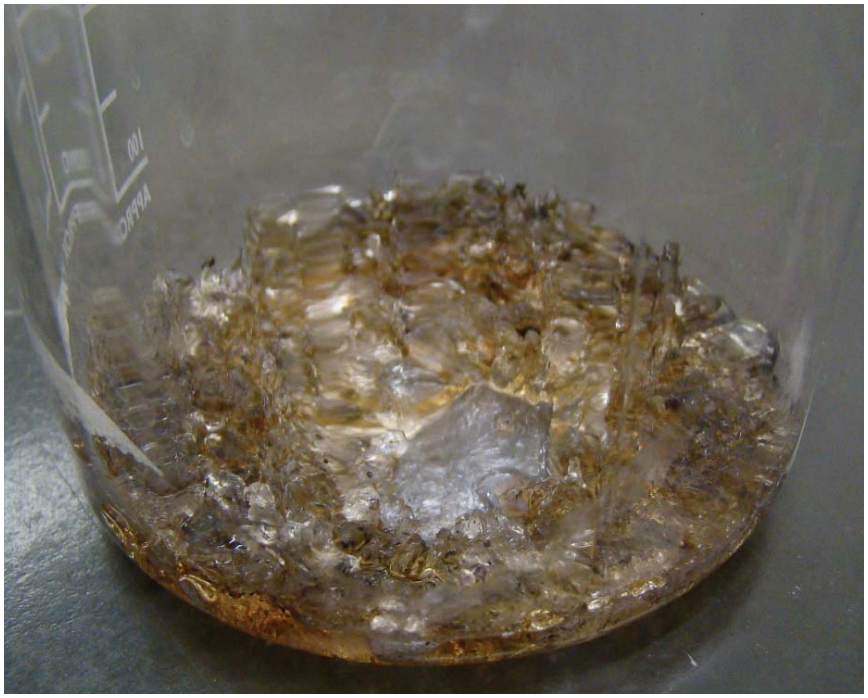

Figure 27. Chemical precipitation remaining at the bottom of the sample container after a complete dissolution of lignite with concentrated sulfuric acid. The solution was allowed 30 days of reaction, even though after 1 day all lignite was dissolved.

nutrients. Reagents like acetic acid, lactic acid or ethanol provide an easy carbon source for indigenous coal degrading and metabolizing microorganisms, potentially boosting their growth and abundance. Most microbes are larger than 1.2µm and would also be retained by the filter, potentially influencing the results. The high uncertainty of results is also reflected by the large error bars in Figures 24 and 26.

5.2.2. Raman Spectroscopy

A total of 30 Raman spectra were obtained. The findings and shortcomings of the Raman analysis will be discussed on one example only; the remaining data is provided in Appendix F. Figure 28 shows the spectra of the coarse waste coal

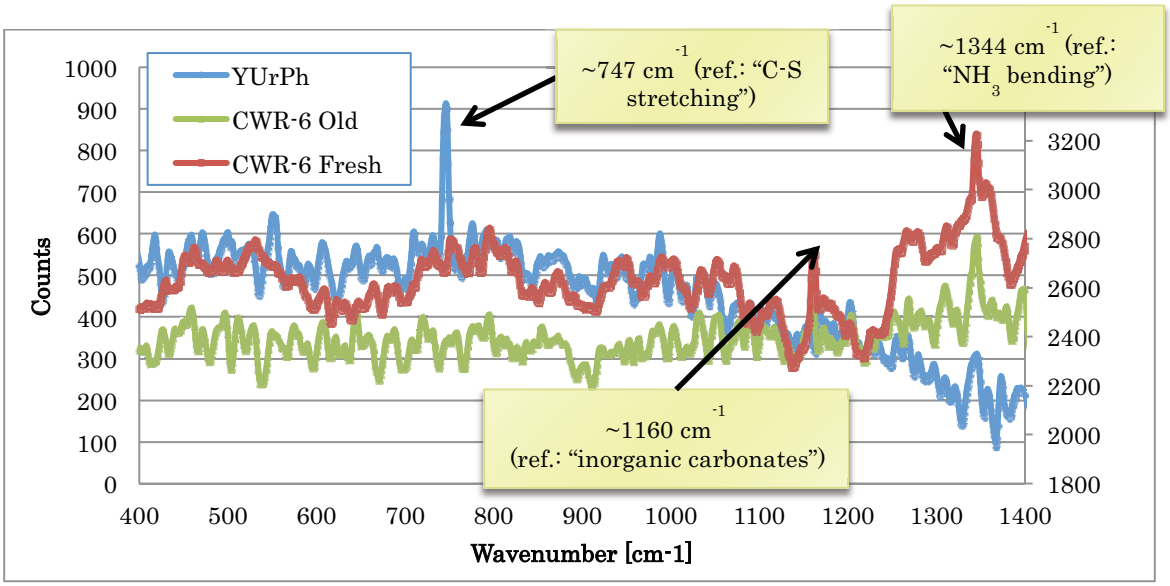

Figure 28. Spectra of bituminous coal waste sample immersed in yeast, urea and phosphate medium for 48 hours (CWR-6 Fresh) and for six months (CWR-6 Old), and of the yeast, urea phosphate medium (YUrPh).

immersed in yeast, urea and phosphate medium. All spectra were normalized with respect to the DI water spectrum.

The blue line, representing the yeast, urea and phosphate medium, has one strong peak at 747 cm^{-1}, caused by the S-S and C-S region [Qian and Krimm, 1992]. Spectra obtained from coal waste immersed in the medium for 48 hours (red line; secondary y-axis) and for six months (green line; primary y-axis) do not contain this peak. Two new peaks are visible on the red plot: 1160 cm^{-1} and 1344 cm^{-1}. The first one is caused by the inorganic carbonates [Socrates, 2004], while the latter one – by the NH_3 bending [Centeno and Collery, 2000]. Spectra of coal waste immersed in the medium for six months shows only the 1344 cm^{-1} peak. Such results strongly indicate that the medium is being utilized. The presence of the inorganic carbonates can be a result of decomposition of the yeast extract, whereas the ammonia is primarily a product of utilization of urea. However, these results do not indicate the decomposition of coal nor do they disprove it.

LC-MS analysis of hydrocarbons present in the liquid phase is the only technique capable of providing a solution fingerprint and therefore proving that coal decomposition was occurring. The largest shortcoming of chromatography techniques are the columns, which are used for the detection of very specific groups of hydrocarbons. For instance, a detection of alkenes or PAHs could be performed; however, it would still not provide a complete picture of the liquid phase composition.

5.3. Gas Generation Tests in Serum Bottles

5.3.1. Microbes in Liquid Media

5.3.1.1. Jordan River Sediments

Samples were incubated at about 23°C for a month and a half. Nick Dahdah from the Energy and Goescience Institute (EGI) at the University of Utah provided GC training. Figure 29 gives gas concentrations generated from Jordan River sediments in various media. Only methane and carbon dioxide were produced at significant levels (above 10,000 ppm). Carbon dioxide is an important indicator of carbon biodegradation as well as a direct methane precursor. The selected microbial consortia were the most active in 50% TSB medium (a solution prepared at 50% recommended strength), producing the largest amount of both methane (896,036 ppm) and carbon dioxide (126,006 ppm). Tryptic Soy Broth medium, having the largest quantity of carbon sources and providing a balanced mixture of other nutrients, was expected to generate the best results. On the other hand, deionized water contains no carbon sources or other nutrients and was expected to result in the smallest productivity, if the sediments did not contain significant amounts of

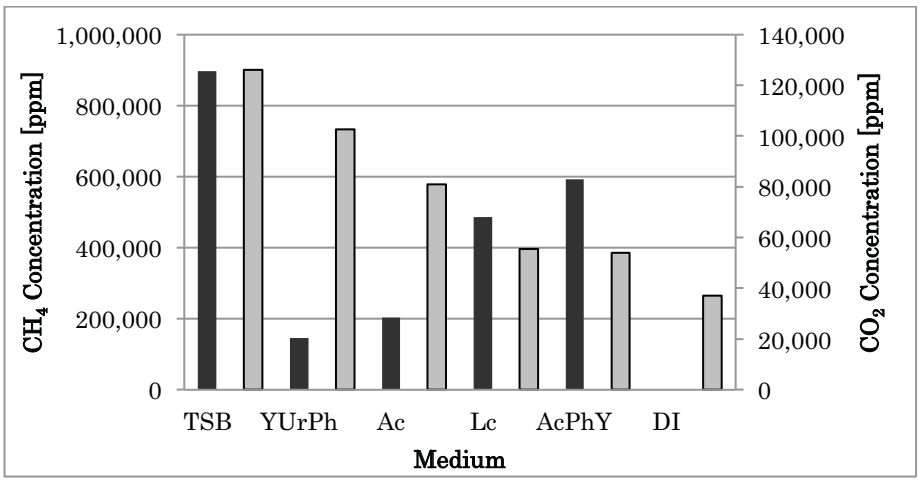

Figure 29. Methane (black bars) and carbon dioxide (gray bars) generation from the Jordan River sediments in various media.

organic matter. Only 10.6-ppm CH_4 (almost five orders of magnitude less than in case of the 50% TSB medium) and 37,063-ppm CO_2 were generated from Jordan River sediment samples incubated with DI water. Deionized water was used as a control medium and it was expected that little gas generation would be observed. All other media produced larger amounts of both CH_4 and CO_2. Since minute amounts of methane were generated from samples incubated in DI water and methane amounts increased significantly when additional carbon sources were provided, it was concluded that the collected Jordan River sediments contained low concentrations of easily biodegradable organic matter or lacked other essential nutrients for methanogenesis. Had the sediments contained all the necessary nutrients, microbial populations would have converted the carbon present into methane. High methane and carbon dioxide concentrations obtained from the Jordan River sediment samples incubated with media indicate that the methanogenic populations were present in these samples and that the produced gases were at least partially the result of metabolic conversion of carbon compounds present in the provided media.

5.3.1.2. Digester Sludge

The analysis of the gases generated by the digester sludge under various conditions show a completely different pattern from the Jordan River sediments (Figure 30). Only minute amounts of carbon dioxide (3,799 ppm) and methane (1,547 ppm) were produced and no other gases were detected after incubation in 50% TSB. The highest production of methane (509,678 ppm) was achieved from a mixture of yeast, urea and phosphate.

Surprisingly, methanogenic bacteria were also very active in the DI water sample and produced 432,020 ppm of methane. This indicates that the digester sludge sample was rich in nutrients and organic matter that could be easily broken down to simple degradation products. That was to be expected from municipal wastewater sludge. Addition of various media with high carbon content to a sludge containing many simple hydrocarbons might have been growth inhibiting. Finally, regardless of the media type used, heavier hydrocarbons were only detected in insignificant amounts in GC analysis.

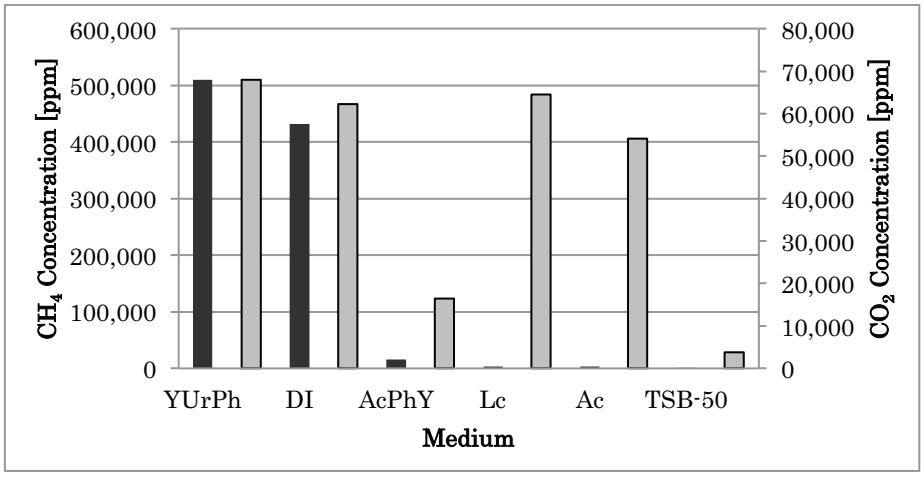

Figure 30. Methane (black bars) and carbon dioxide (gray bars) generation from the digester sludge in various media.

5.3.1.3. Great Salt Lake Sediments

The largest amounts of CH_4 and CO_2 obtained from the Great Salt Lake's wetland sediments (707,340 ppm and 84,270 ppm, respectively) were produced from the incubation in 50% TSB solution, while the lowest concentration of methane (13.5 ppm) was obtained from the control DI sample (Figure 31). This is not surprising, as tryptic soy broth provides a balanced mixture of nutrients as well as carbon sources. Deionized water on the other hand does not provide any nutrients or carbon sources. Therefore, gases produced from inoculation in DI water were most likely a result of decomposition of organic matter from wetlands remaining within microbial cells and the decomposition of dead microbial cells.

The sediments from the Great Salt Lake produced lower concentration of gases than the samples from the wetlands (Figure 32). However, they followed similar trend and generated the largest amount of methane when tested in a 50% TSB solution. The Great Salt Lake wetlands are an environment that sustains a diverse ecosystem. Plants growing in the wetlands fix carbon, nitrogen, and other nutrients from the atmosphere and soil. After the plants die, these elements are returned into the environment. On the other hand, the Great Salt Lake sediments do not sustain any plant growth and consist of largely inorganic sands. Therefore, it was expected that a higher gas generation would be obtained from the wetland sediments than the lake sediments, due to the higher availability of the essential nutrients. Figures depicting gas generation by microbes cultured from the coal, oil seep, and natural gas well samples are given in Appendix G.

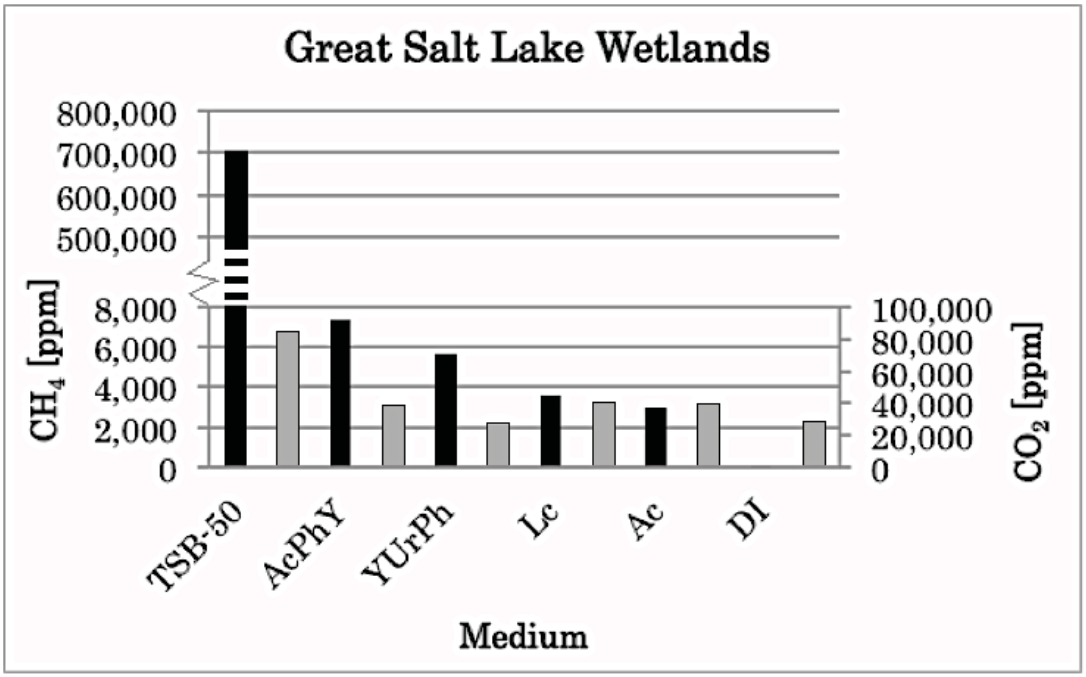

Figure 31. Methane (black bars) and carbon dioxide (gray bars) generation from the Great Salt Lake wetland sediments in various media.

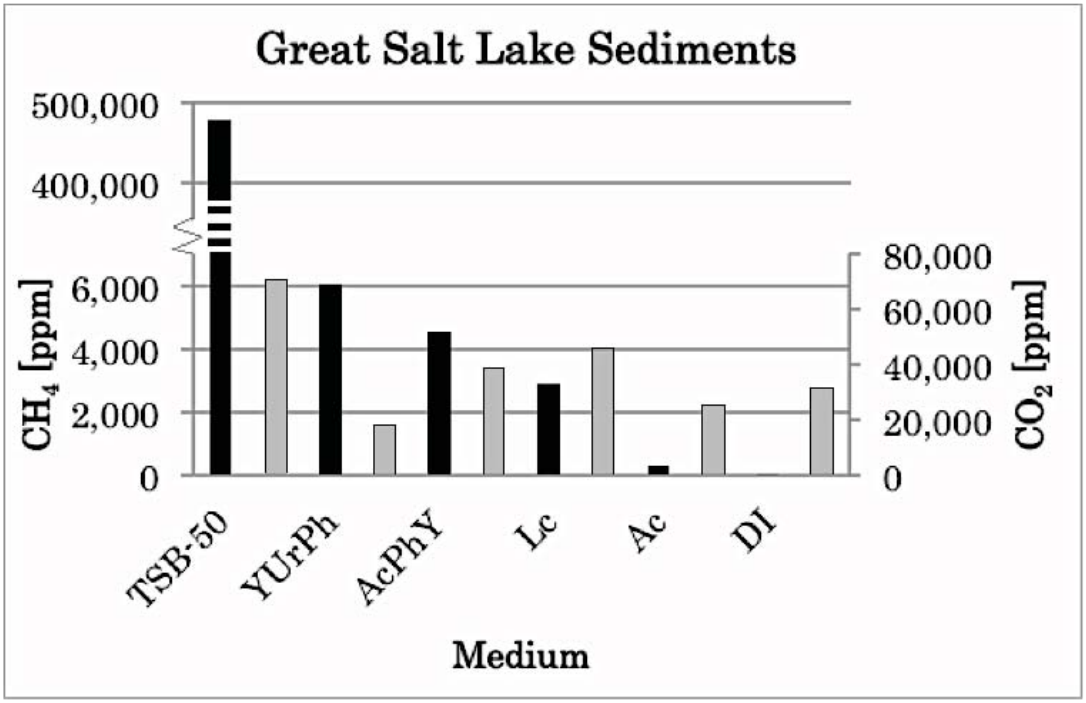

Figure 32. Methane (black bars) and carbon dioxide (gray bars) generation from the Great Salt Lake sediments in various media.

5.3.2. Microbes in Coal Samples with and without Nutrient Amendment

The main aim of this part of the research was to screen for microbial consortia capable of utilizing complex carbon compounds as a main food source and survive exposures to the atmospheric gases. The main expected gaseous byproducts of metabolism were carbon dioxide and methane. The samples were analyzed after a 30-day reaction period at about 23°C and were not agitated.

Control samples were created by adding normal saline solution to pulverized samples without the addition of external microbial population, and are represented on the following graphs by solid black and dashed gray lines for methane and CO_2, respectively. Any gases detected from control samples were a result of desorption from coal under atmospheric conditions and generations by native microbial populations present in coal.

Additional control samples included the same concentration of microbial inocula suspended in normal saline solution (0.85% NaCl) without any coal material. Gases generated from these controls most likely came from degradation of the dead cells by the remaining populations. Depending on a microbial inoculum source, 3.89 ppm to 5.40 ppm methane and 1,210 ppm to 1,597 ppm carbon dioxide were generated. These amounts are insignificant in comparison to the results obtained from the samples being tested and are not included in the figures.

Pulverized bituminous coal and bituminous coal waste materials, kept in closed containers, were tested for outgassing after six months. Approximately 10-ppm methane and 4,900-ppm CO_2 were generated in the bituminous coal containers, while bituminous coal waste produced about 13-ppm methane and 3,000-ppm CO_2 through outgassing.

A matrix with over 650 samples consisting of coal materials, microbial consortia from various environments, and saline or nutrient solution amendments, was developed and tested. For nutrient composition, see Appendix A. This matrix represents a large microbial screening test in which many of the microbial samples did not generate higher methane and/or carbon dioxide concentrations than the control samples, and are therefore not included in the results and discussion presented. For a complete set of results, please refer to Appendix H.

5.3.2.1. No Nutrients Added

Generation of methane or CO_2 from coal samples with no nutrient amendment is significant in this study, since it indicates microbial breakdown of coal and subsequent utilization of the intermediate compounds. Up to 5.4-ppm methane and 1,600-ppm carbon dioxide were generated from "no coal" controls, i.e., most likely from microbes utilizing carbon present in the dead microbial cells. Small amounts of additional gases were desorbed from coal matrix and produced by native microbial populations, as represented by horizontal lines on Figures 33 – 41.

The highest concentration of methane produced from bituminous coal samples was nearly 300 ppm, while carbon dioxide exceeded 6,000 ppm (Figure 33). Generation of both methane and carbon dioxide from lignite was considerably higher, reaching nearly 450 ppm and over 100,000 ppm, respectively (Figure 34). Bituminous coal waste samples generated less methane than either coal or lignite (over 250 ppm) but generated over 14,000-ppm CO_2 (Figure 35).

The highest concentrations of methane were obtained from the samples inoculated with consortia cultured from coal (C) or waste coal (WC) environments.

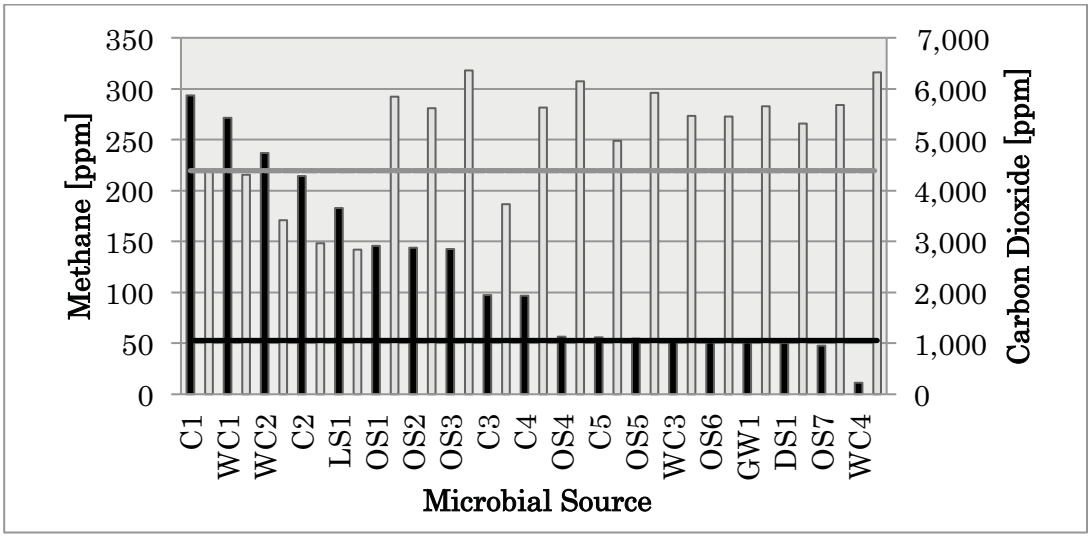

Figure 33. Methane (black bars) and carbon dioxide (gray bars) generated from bituminous coal samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with no nutrient amendment. Solid black and gray lines represent the DI control samples of methane and carbon dioxide, respectively.

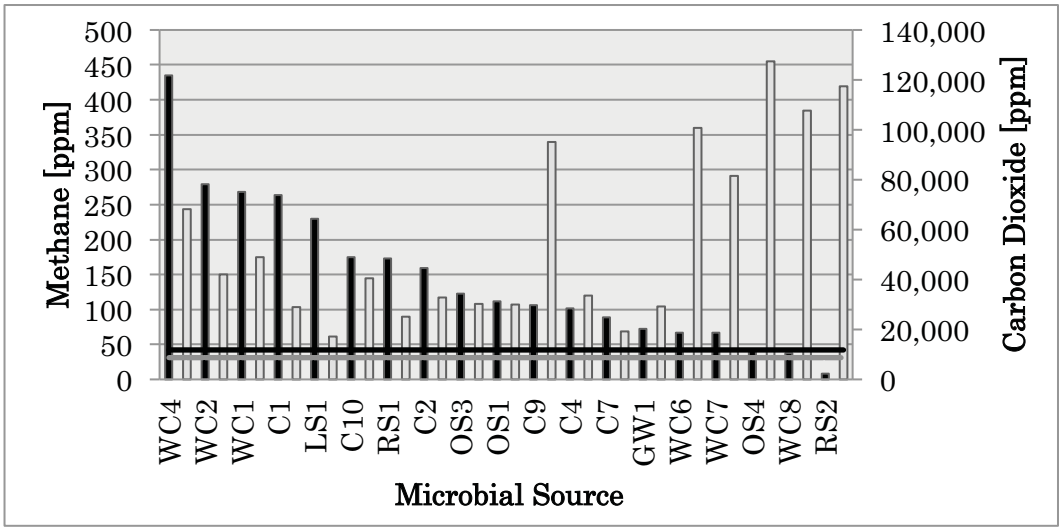

Figure 34. Methane (black bars) and carbon dioxide (gray bars) generated from lignite samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with no nutrient amendment. Solid black and gray lines represent the DI control samples of methane and carbon dioxide, respectively.

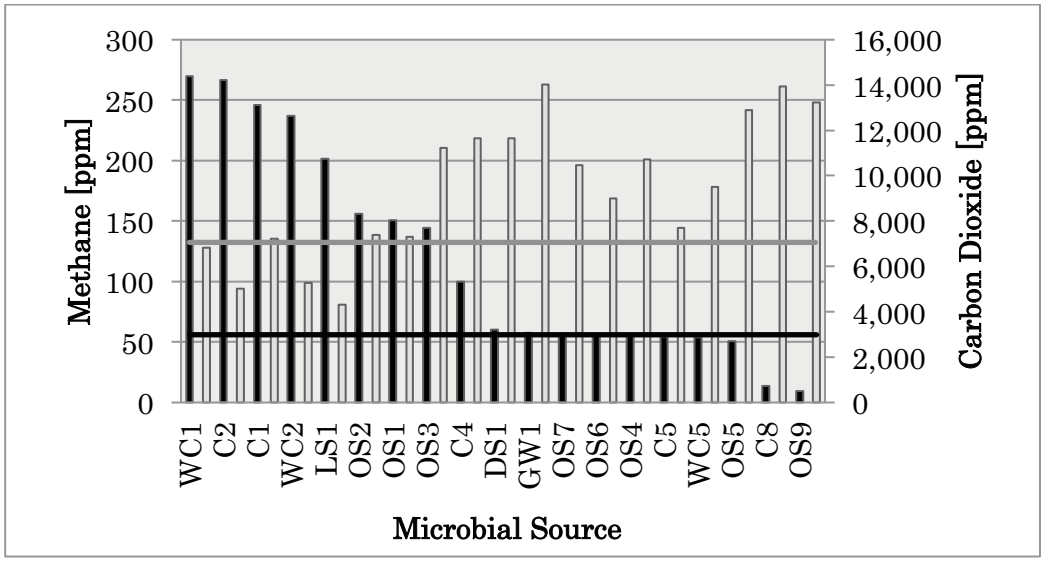

Figure 35. Methane (black bars) and carbon dioxide (gray bars) generated from bituminous coal waste samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with no nutrient amendment. Solid black and gray lines represent the DI control samples of methane and carbon dioxide, respectively.

This was expected, since coal populations contain native methanogens that are adapted to this environment. The highest concentrations of carbon dioxide were produced from samples inoculated with consortia cultured from other environments. These included oil seep (OS), natural gas wells (GW), lake sediments (LS), digester sludge (DG), and river sediments (RS). This is an important finding, indicating that introduction of non-native species could increase the rate of hydrocarbon biodegradation, with CO_2 as an end product. It can be assumed that under proper environmental conditions (e.g., sufficient amount of hydrogen ions, appropriate temperature, etc.), a part of generated carbon dioxide would ultimately be converted to methane.

Moreover, concentrations of both methane and carbon dioxide obtained from samples with no nutrient amendments, containing an inoculated consortia, were

higher than in control samples containing only normal saline solution and lower concentrations of native microbial populations. This strongly indicates that introduction of non-native species and/or higher concentrations of native species into solid hydrocarbon materials could potentially enhance the rates of gas production. Production of methane directly from coal sources showed a two- to seven-fold increase over control samples.

5.3.2.2. 10% Nutrients Added

Initial nutrient addition might be necessary to stimulate the methanogenic population, i.e., stimulate microbial growth to a point that a sufficient number of microbes would be present to produce readily measurable results. Amendment with 10% nutrient solution did not result in significant additional amounts of methane production from bituminous coal, with one exception generating over 550 ppm (Figure 36). However, carbon dioxide concentrations in all coal samples were significantly increased, ranging from 5,000 to 30,000 ppm. Similar results were observed with lignite and bituminous coal waste samples. Gases analyzed in the headspace of lignite samples reached about 300-ppm methane (with one exception of 2,000 ppm), while CO_2 generation was stimulated in all samples to 20,000-90,000 ppm (Figure 37). Bituminous coal waste material produced up to 400-ppm CH_4 and 10,000-30,000 ppm CO_2 (Figure 38).

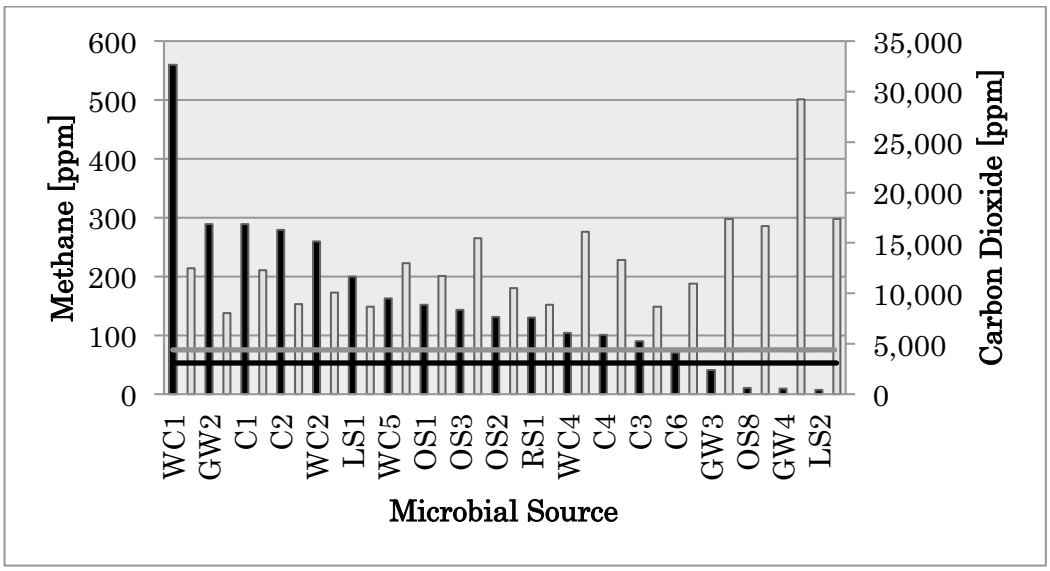

Figure 36. Methane (black bars) and carbon dioxide (gray bars) generated from bituminous coal samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with 10% nutrient amendment. Solid black and gray lines represent the DI control samples of methane and carbon dioxide, respectively.

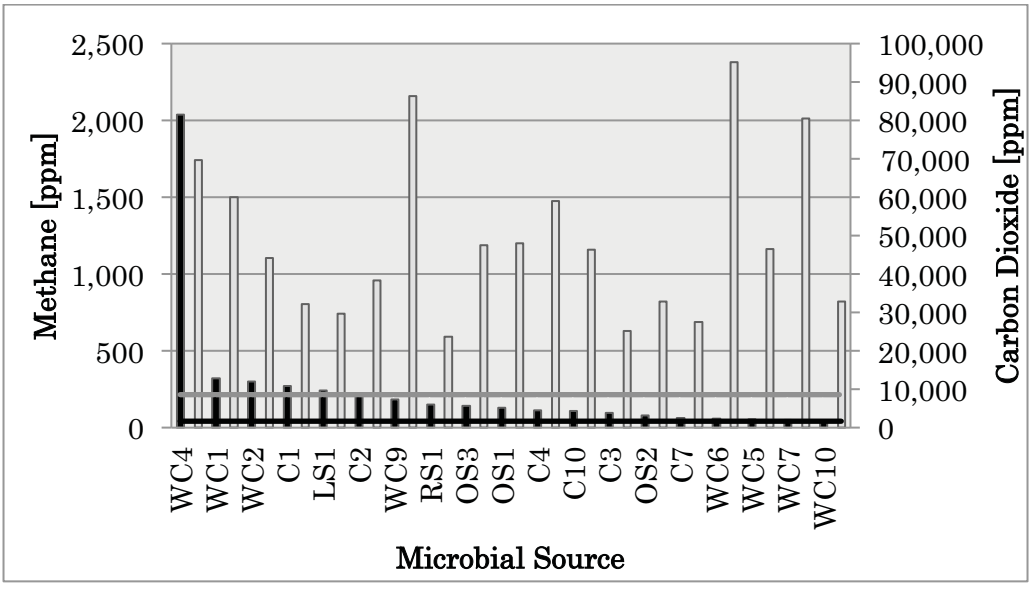

Figure 37. Methane (black bars) and carbon dioxide (gray bars) generated from lignite samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with 10% nutrient amendment. Solid black and gray lines represent the DI control samples of methane and carbon dioxide, respectively.

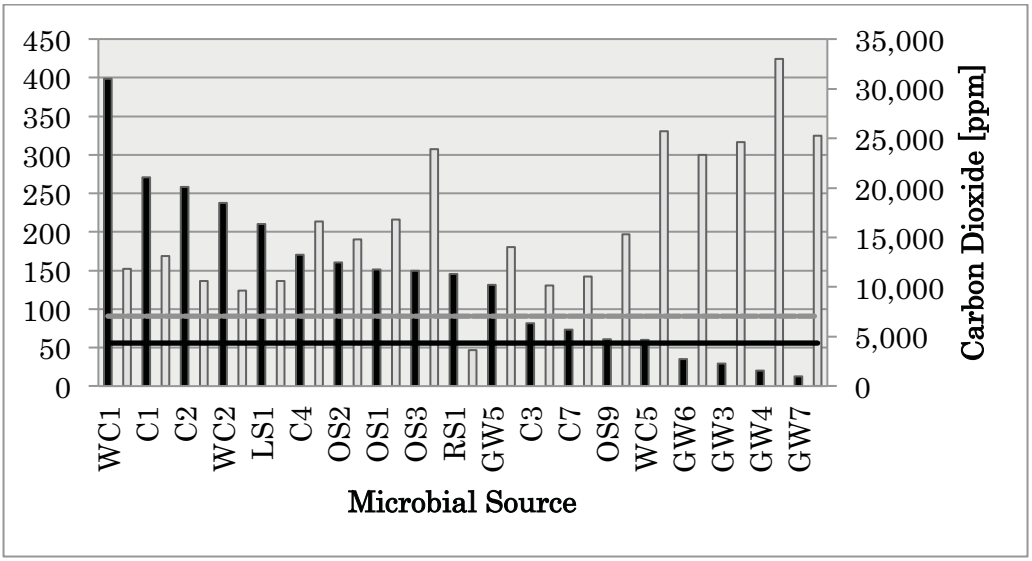

Figure 38. Methane (black bars) and carbon dioxide (gray bars) generated from bituminous coal waste samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with 10% nutrient amendment. Solid black and gray lines represent the DI control samples of methane and carbon dioxide, respectively.

5.3.2.3. 50% Nutrients Added

Nutrient amendments at 50% levels act as both stimulation for methanogenic consortia growth as well as a carbon source for methanogenesis. Increase of nutrients to 50% resulted in significant increase of generated gases from bituminous coal materials; where up to 200,000 ppm of methane and 50,000-ppm carbon dioxide were produced (Figure 39). One microbial consortium was stimulated enough, with 50% nutrient solution, to produce 110,000 ppm of CH_4 from lignite, while most lignite samples did not generate above 500-ppm CH_4 (Figure 40). CO_2 was produced in the range of 40,000 to 120,000 ppm from lignite. Good microbial stimulation was achieved in bituminous coal waste samples, where 120,000-ppm methane and up to 60,000-ppm carbon dioxide were produced (Figure 41).

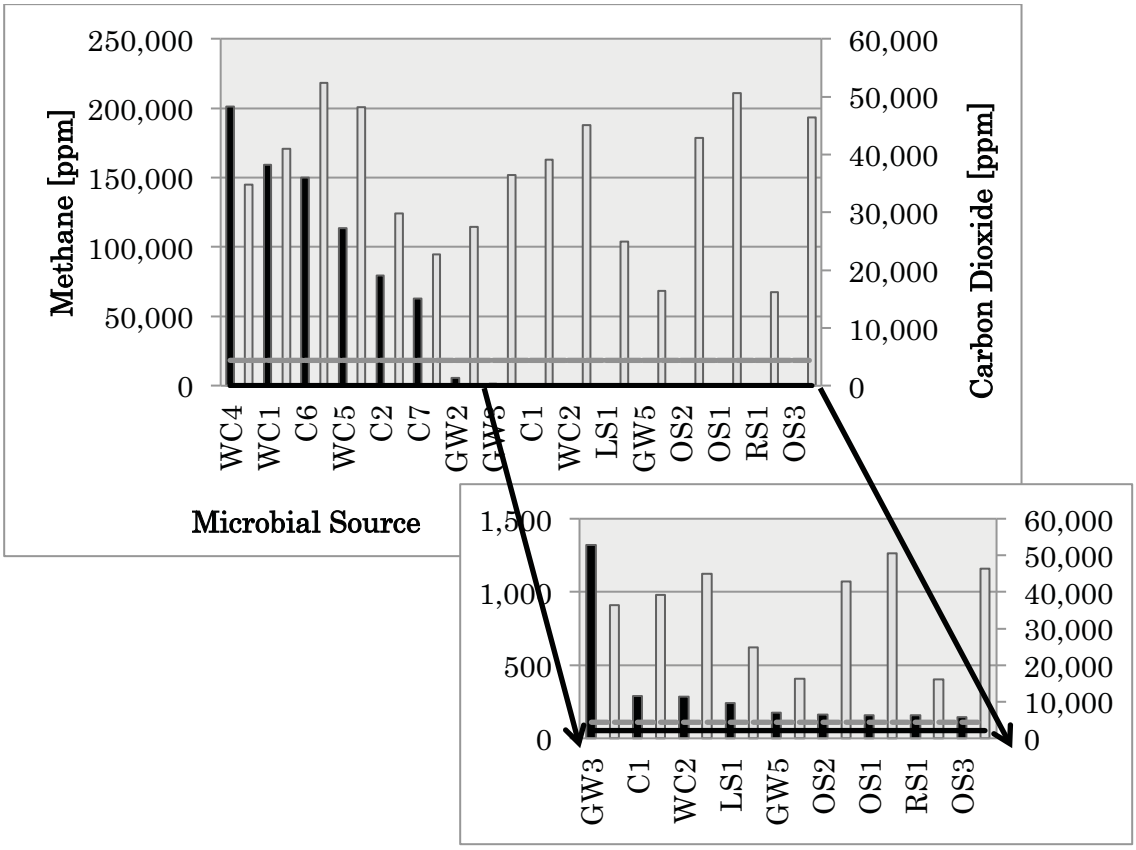

Figure 39. Methane (black bars) and carbon dioxide (gray bars) generated from bituminous coal samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with 50% nutrient amendment. Solid black and gray lines represent the DI control samples of methane and carbon dioxide, respectively.

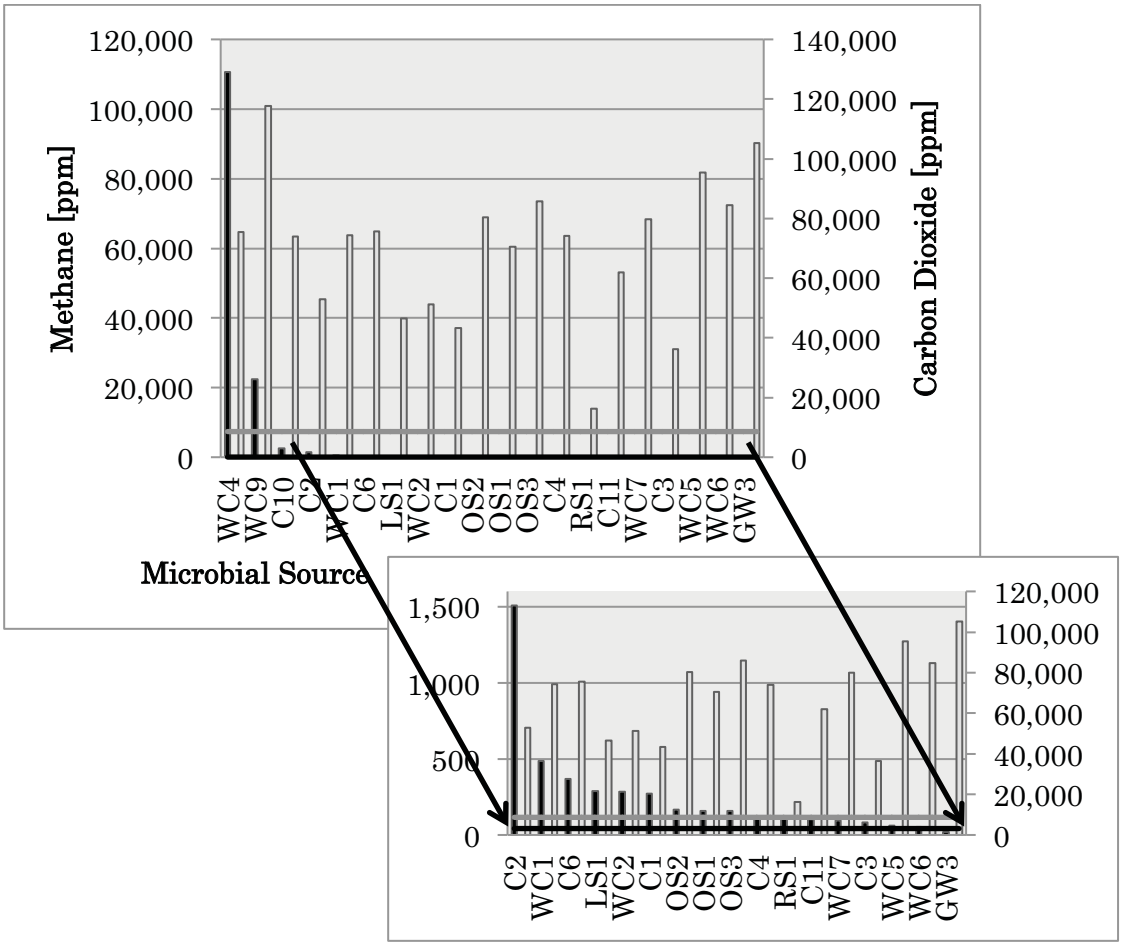

Figure 40. Methane (black bars) and carbon dioxide (gray bars) generated from lignite samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with 50% nutrient amendment. Solid black and gray lines represent the DI control samples of methane and carbon dioxide, respectively.

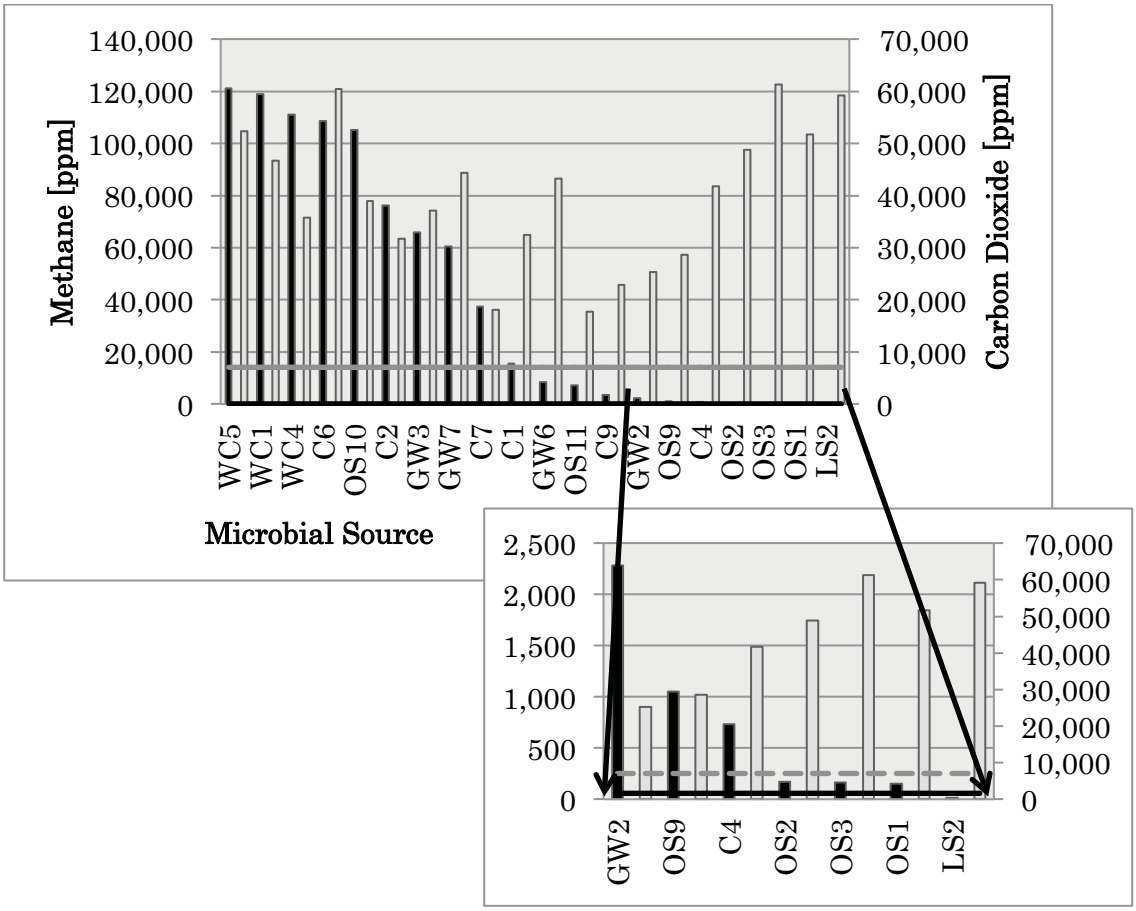

Figure 41. Methane (black bars) and carbon dioxide (gray bars) generated from bituminous coal waste samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with 50% nutrient amendment. Solid black and gray lines represent the DI control samples of methane and carbon dioxide, respectively.

5.3.2.4. Significance

The results obtained represent a one-time measurement after a 30-day testing period. Consequently, it is not known whether the maximum conversion to methane or CO_2 was achieved or whether partial gas pressures in the reaction vessels limited the amount produced. Furthermore, only limited conclusions on conversion kinetics can be drawn from the results obtained.

It is known that particle size will influence the extent of methanogenesis [Green et al., 2008]. The smaller the coal particle size, the larger the surface area accessible to the microbes; particle size and surface area is a cubic function relationship. Therefore, the results presented here represent a best-case scenario. High surface areas and high permeability can be obtained in the subsurface environments through hydraulic fracturing, but fractured materials would produce a large variation of particle sizes.

Analysis of duplicate samples showed standard deviation in methane concentration of 0.02 ppm at low concentrations to 20.82 ppm at high concentrations, or a deviation of about 0.16% to 8.34%. Carbon dioxide measured from duplicate samples indicated a standard deviation between 1.2% to 20.4%.

Using an equation from Buswell (1930), the maximum theoretical conversion of nutrient organic content to methane and carbon dioxide can be calculated (Equation 9).

$$C_cH_hO_oN_nS_s + yH_2O \rightarrow xCH_4 + (c - x)CO_2 + nNH_3 + sH_2S \quad (9)$$

$$x = 0.125(4c + h - 2o - 3n + 2s) \quad (9a)$$

$$y = 0.25(4c - h - 2o + 3n + 2s) \quad (9b)$$

Addition of nutrient solution increased the degradation of coal materials and subsequent methanogenesis. Higher nutrient concentration should have also resulted in a larger microbial population, generating larger quantities of gases. Only small gas concentrations were generated from “no coal” saline controls (3.89 ppm to 5.40 ppm methane and 1,210 ppm to 1,597 ppm carbon dioxide), representing gas

produced from decomposition of microbial cells. Larger gas amounts would be expected to come from cell degradation when samples were incubated in the supplied carbon-based nutrient media, where microbial populations were able to establish a higher cell density. Only a small fraction of generated methane (1-2%) and carbon dioxide (1-3%) was a direct result of nutrient conversion from the samples amended with 50% nutrients, based on a maximum theoretical nutrient conversion calculated from Equation 9 (Table 12). The highest carbon dioxide concentrations were generated from lignite samples at all nutrient amendment levels. Lignite is the lowest metamorphosed and the softest hydrocarbon rock used in this study. High concentrations of carbon dioxide indicate the faster biodegradation of lignite, compared to other coal samples. Methane concentrations detected at the highest levels from lignite samples amended with no and 10% nutrients could be a result of a direct CO_2 conversion.

Regardless of the nutrient amendment level, methane generated from bituminous coal waste material was at the same order of magnitude as that produced from bituminous coal. This indicates that a similar microbial population was established within each tested samples. With no additional nutrients, carbon dioxide concentrations detected from bituminous coal waste samples were over twice as high as those produced from bituminous coal. These results suggest that culm, gob, and potentially other waste hydrocarbon materials could be converted to useful fuel. Larger volumes of coal waste material would be necessary to generate the same methane concentration as the equivalent coal material, since the coal waste samples were upgraded in these tests, excluding larger inorganic rocks from the matrix. Where available space is not a limitation, a design similar to landfill heaps could be

Table 12. The best CH_4 and CO_2 production [ppm] from test results for lignite, bituminous coal, and bituminous coal waste, compared to the maximum theoretical gas generation from nutrient amendments.

		Nutrient	Bituminous Coal	Lignite	Coal Waste
0% nutrient	CH_4	0	290	440	270
	CO_2	0	6,100	130,000	14,000
10% nutrient	CH_4	428	580	2,000	400
	CO_2	286	29,000	95,000	33,000
50% nutrient	CH_4	2,143	200,000	110,000	120,000
	CO_2	1,429	50,000	120,000	60,000

used for methane generation from coal waste. The materials would be piled on an impermeable liner, with gas collection pipes placed throughout the heap, and a liquid distribution system on the surface (for the application of chemical pretreatment, microbial inoculum, and nutrient solution). Presence of inorganic material within such a heap would not be a problem and could actually increase the permeability of the system. In cases when space is not readily available, methane could efficiently be produced from upgraded coal waste materials using above ground bioreactors, in a similar manner that agricultural biogas is generated.

A novel approach presented in this experiment is based on the assumption that methanogenic microorganisms are not strict anaerobes. None of the microbial cultivation or testing was performed under anaerobic conditions. Agar plates were exposed to the atmosphere and the air present in the headspace of serum bottles was not evacuated prior to experiments. All other studies examining the potential of microbially enhanced coal-bed methane recovery (MECBM), as well as the microbiology textbooks, decisively state that methanogens cannot survive in the

presence of oxygen [Barker, 2010; Ulrich and Bower, 2008; Jones et al., 2008]. If that were true, designing a full scale MECBM operation and delivering the microorganisms to the subsurface would prove difficult if not impossible. The same would be true for organic carbon sources present on the surface, such as waste coal. By consciously designing the test in order to select methanogenic microorganisms capable to survive in the presence of oxygen, the approach presented in this study offers a simpler and refreshing look at the prospects of MECBM.

5.3.3. Microbial Consortia in Pretreated Coal Samples

5.3.3.1. Chemical Pretreatments

Chemical pretreatments generated significant amounts of gases from all coal samples (Figure 42). The best gas generation was obtained with lactic acid chemical pretreatment. This was expected, as lactic acid is a carbon source, which stimulated native methanogens and acted as an electron donor in methanogenesis. Under lactic acid pretreatment, approximately 170-ppm, 240-ppm, and 500-ppm methane was generated from bituminous coal, bituminous coal waste, and lignite, respectively. About 35,000-ppm and 110,000-ppm CO_2 was generated from the bituminous coal and bituminous coal waste pretreated with lactic acid pretreatment. High concentrations of carbon dioxide (above 200,000 ppm) obtained from lignite pretreated with catalytic reagent (Ni) and 3% hydrogen peroxide (HP) indicate that this coal matrix contains much higher amounts of carbonates than bituminous coals. Moreover, it is also the least metamorphosed coal sample used in this study and is therefore more readily oxidized and/or biodegraded.

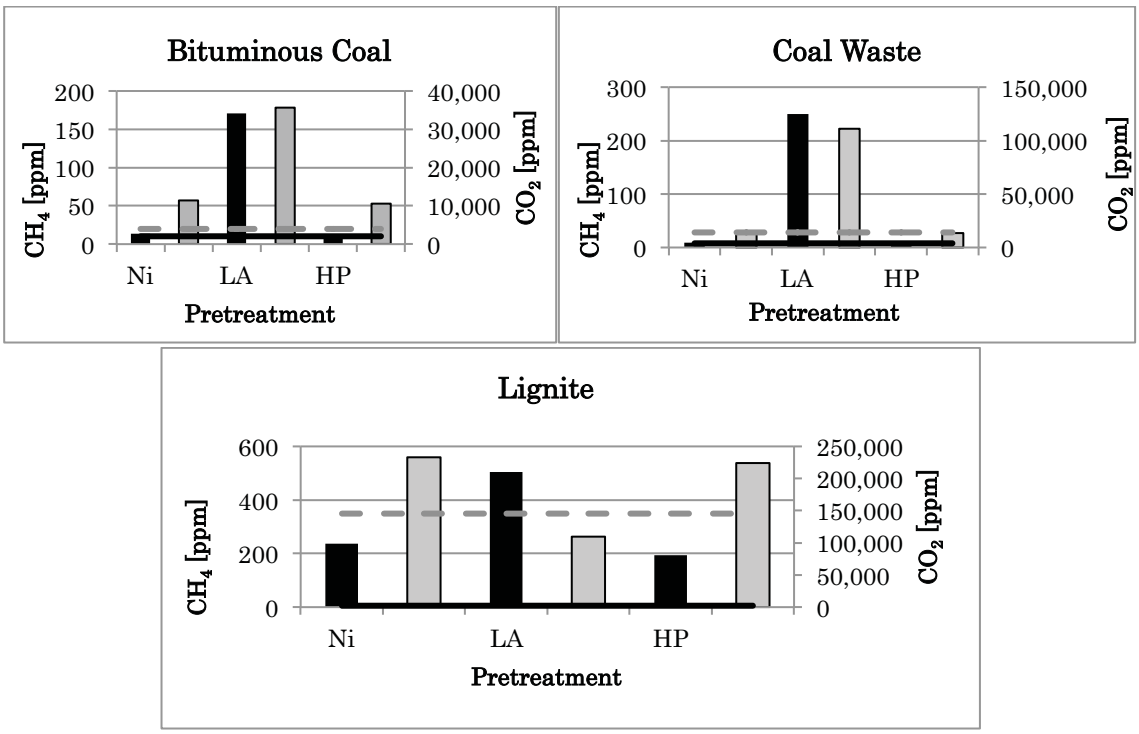

Figure 42. Methane (black bars) and carbon dioxide (gray bars) generated from chemically pretreated bituminous coal, bituminous coal waste, and lignite samples after 14 days incubation period. Solid black line and dashed gray line represent the DI control samples of methane and carbon dioxide, respectively.

According to the Buswell equation (Equation 9), no microbial methane or carbon dioxide should have been generated from the pretreated samples, due to a high oxygen component from an addition of 3% hydrogen peroxide. Oxygen is the preferred electron acceptor, because it is the most electronegative species. Before it is depleted, no other species can become reduced.

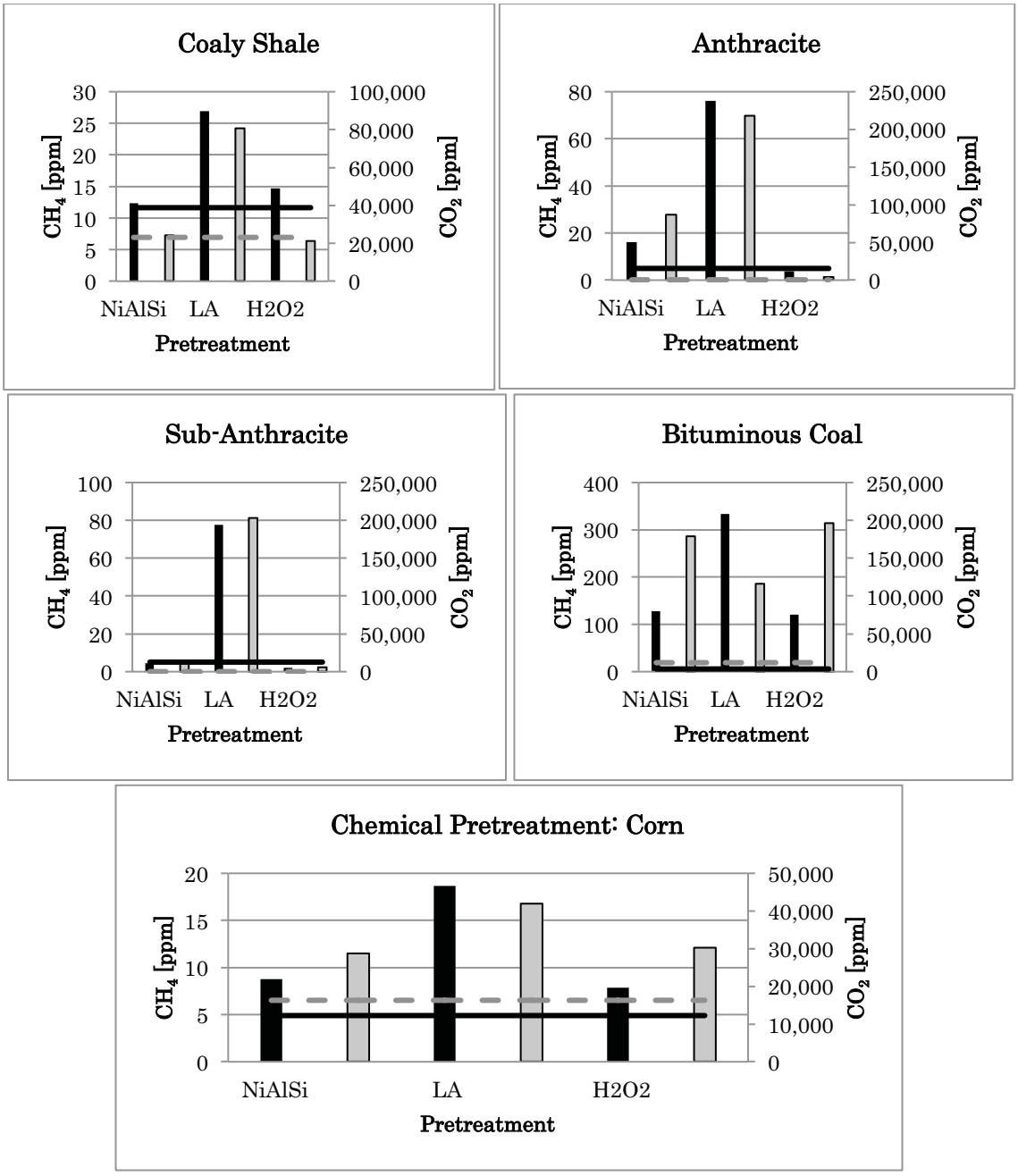

Figure 43. Methane (black bars) and carbon dioxide (gray bars) generated from chemically pretreated coaly shale, anthracite, sub-anthracite, bituminous coal, and corn samples after 14 days incubation period. Solid black line and dashed gray line represent the DI control samples of methane and carbon dioxide, respectively.

5.3.3.2. Other Carbonaceous Materials

Results obtained from four additional coal sources and corn pretreated with three chemical reagents, are shown in Figure 43. These samples followed the same general trends as the main coal materials tested, i.e., the highest concentration of both methane and carbon dioxide were obtained from the lactic acid pretreatment. Moreover, the less metamorphosed the coal, the more methane it generated when pretreated chemically. This is an interesting finding, since it is generally accepted that the higher the coal rank, the larger the amount of adsorbed natural gas content. This means that the main source of methane in these chemical pretreatment tests was not gas desorption from coal matrix but rather microbial methane production.

Corn samples, on the other hand, did not generate a lot of methane or carbon dioxide when pretreated chemically, indicating that the plant material did not contain a large native methanogenic population. This was to be expected. Coal samples were extracted from underground where conditions appropriate for methanogenesis exist (e.g., low oxygen content) and where the native microbial populations were evolving for millions of generations and adapting to this environment. Corn plants, however, grow fast, are harvested and processed, and are constantly exposed to high oxygen atmospheric conditions, environments where methanogens are not normally present in significant numbers.

5.3.3.3. Microbial Pretreatments

Approximately 30% and 40% more methane was generated from bituminous coal and bituminous coal waste samples inoculated with microbial consortia after a 14-day reaction time as compared to control samples. Up to 50% and 25% more carbon

dioxide was generated from the same bituminous coal and coal waste samples when inoculated with the microbial consortia. Lignite samples tripled the amount of methane, when inoculated with microbial consortia. However, CO_2 detected from microbially pretreated samples was lower than that of the control samples. This could indicate that the microbial reduction of carbon dioxide to methane was occurring, if the proper environmental conditions were present (e.g., availability of hydrogen ions and electrons). Figures are attached in Appendix I.

5.3.3.4. Combined Chemical and Microbial Pretreatments

Results obtained with bituminous coal waste are shown as an example in Figure 44, the remaining data is given in Appendix I. No significant methane production was observed after the 14-day incubation period in any of the samples tested. However, increased CO_2 concentrations were observed in bituminous coal and bituminous coal waste samples. The best gas production was achieved with consortia 1 and 3 for bituminous coal waste, consortia 3 and 4 for bituminous coal, and consortia 1, 4, and 5 for lignite. Moreover, lactic acid pretreatment generated the highest gas amounts.

After additional 30 days, a significant increase of gas generation was observed. Up to 300 ppm more CH_4 and 70,000 ppm more CO_2 were generated from bituminous coal waste samples (Figure 44). Gases produced from saline control samples were 7.9-ppm CH_4 and 5,040-ppm CO_2. Methane concentration increase after an additional 30 days was expected; methanogenesis is a slow process and requires times longer than 30-50 days in most biogas production facilities (e.g., municipal wastewater treatment facilities, landfills, agricultural biogas, etc.).

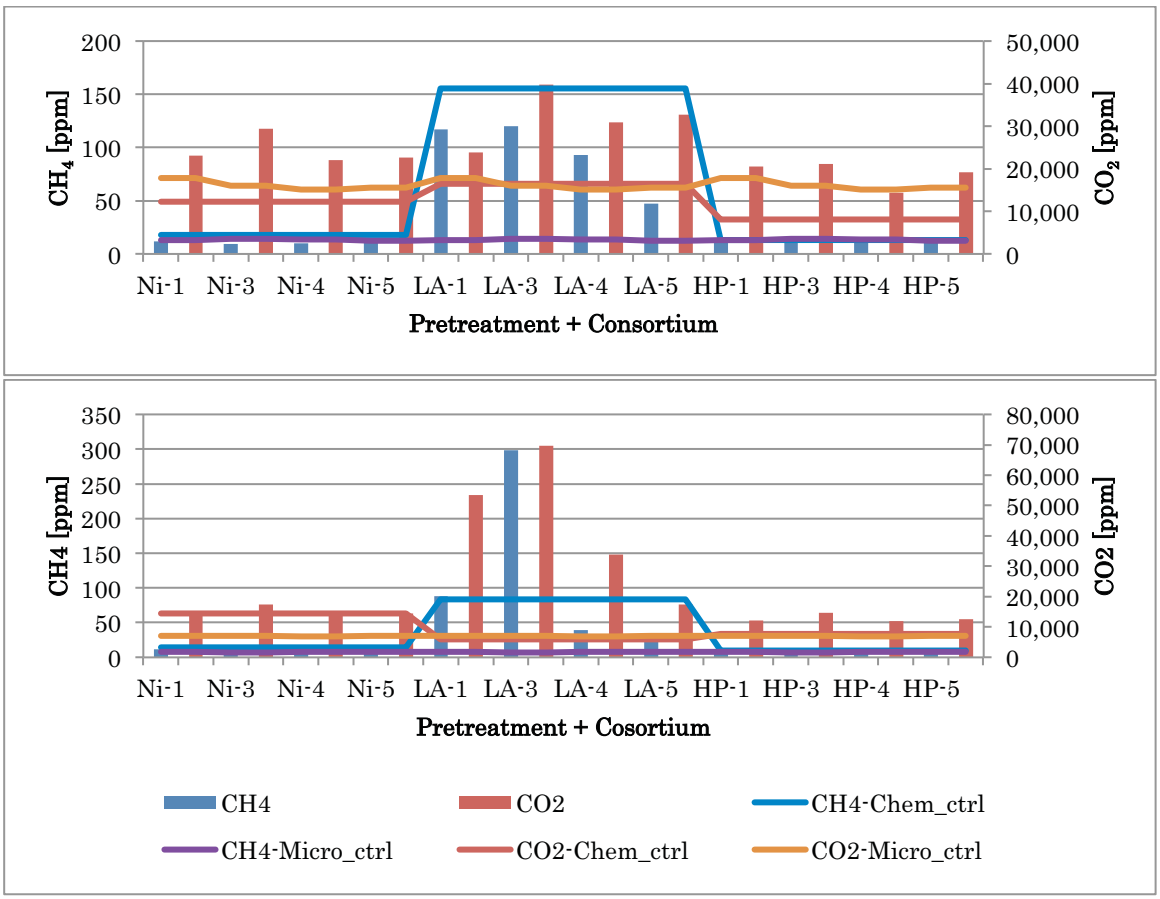

Figure 44. Methane and carbon dioxide generated from chemically pretreated bituminous coal waste samples, inoculated with four microbial consortia. Upper graph – 14-day incubation, bottom graph – 44-day incubation. Solid blue and red lines represent the DI control samples of methane and carbon dioxide, respectively. Violet and orange lines represent microbial control samples of methane and carbon dioxide, respectively.

Moreover, the best results obtained from combined chemical and microbial pretreatments represent a 3.5-fold improvement in methane generation and a 11.5-fold improvement in CO_2 generation over the gases produced from chemical pretreatments alone (marked by a blue and red line for methane and carbon dioxide on Figure 44). Results obtained with bituminous coal samples followed the same trend; however, lignite samples did not show any significant increase of gases after 44-days incubation. One possible explanation might be that lignite, being the least

metamorphosed of the tested coals, underwent a more complete dissolution under chemical pretreatment, yielding toxic compounds, such as metals, certain inorganics, or even high concentrations of organic compounds. Moreover, it is possible that lignite dissolution changed the physicochemical properties of the solution (e.g., pH, ORP, etc.) to less favorable ones for microbial activity.

Results obtained here suggest that a large initial release of gases can be obtained by applying a chemical pretreatment containing an oxidant, a surfactant, and an organic acid to the coal. Following adjustment to a suitable environment, sustained gas generation can be achieved by inoculating pretreated coals with selected microbial consortia. Microbial gas generation is characterized by a slower kinetics than the chemical release of these gases, however, significant concentrations can be achieved in a time frame as short as 30 days.

5.3.3.5. Physicochemical Parameters

Since a large discrepancy of results was obtained between the bituminous coal and lignite samples, the liquid phase from the tested samples was analyzed (Figure 45). It was discovered that while the pH of bituminous coal and coal waste samples was circum-neutral (6.8-7.8), regardless of the pretreatment, the pH of all lignite samples was below 4.0. Lignite materials generated high carbon dioxide concentrations (above 15%), even from samples incubated only with saline solution. This indicates that the lignite matrix either contains a high concentration of adsorbed CO_2 or that carbonate species are present in high concentrations, acidifying the solution. High carbonate content is common in sub-bituminous coals, where humic acid salts do not metamorphose into coal but decompose into

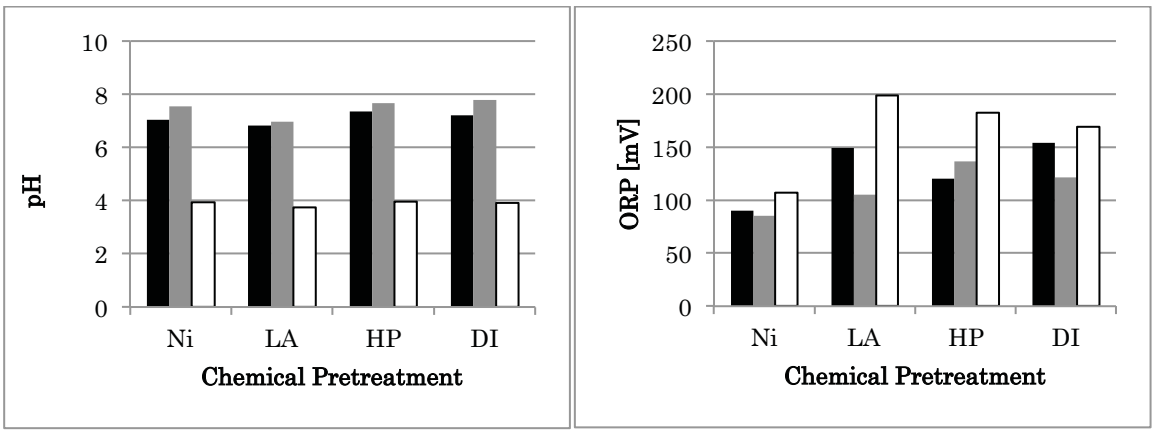

Figure 45. pH and ORP (Ag/AgCl reference electrode) of the liquid phase collected from chemically pretreated bituminous coal waste (black bars), bituminous coal (gray bars), and lignite (white bars) samples.

carbonates instead. Methanogenic organisms prefer neutral pH range (Table 3), as do the consortia created in this research (Figure 11). Therefore, low pH generated in the lignite samples was not optimal for the microbial population used in these tests.

Furthermore, lactic acid pretreatment acidified all the coal samples to the greatest extend. It was expected, since lactic acid has an acid dissociation constant, pKa, of 3.86. Moreover, cell metabolism might have been stimulated upon addition of carbon-based nutrient (lactic acid), resulting in microbial acid production.

Moreover, the electrochemistry of methanogenesis requires a low redox potential, one that provides a suitable reduction environment. None of the samples developed negative ORP values (measured with a Ag/AgCl reference electrode) (Figure 45). The lignite samples showed the highest redox potential among all coal sources. Lignite contained 28% oxygen, while the bituminous coal and bituminous coal waste samples contained only 9% and 11% oxygen, respectively. With the highest chemical and microbial degradation of lignite material, large quantities of oxygen would have been released, causing high ORP values. Furthermore, it was expected that the

lactic acid pretreatment would generate the lowest oxidation-reduction potential, since it acts as an electron donor. However, the lowest ORP values from coal sources were obtained with the Nickel/Alumina/Silica (Ni) pretreatment (20 to 90 mV lower than lactic acid pretreatment). Both nickel and aluminum ions are low in the electrochemical series, meaning that they are reducing agents. Moreover, it was expected that all chemical pretreatments would generate environments with higher potential than that found in normal saline control samples, since all the pretreatments contained 3% v/v hydrogen peroxide, which is a strong oxidizing agent. However, this was not the case. For instance, coal waste immersed in normal saline solution resulted in the highest ORP (154 mV), lactic acid pretreatment resulted in ORP of 149 mV, while 3% hydrogen peroxide created an environment with the ORP of 120mV. The lowest ORP of coal waste sample (90 mV) was achieved with Nickel/Alumina/Silica pretreatment. High ORP values indicate the environment suitable for oxidation reactions, such as degradation of organic matter. Such an environment could be beneficial in the initial stages of the methane production, when the main goal is degradation of complex carbon sources. However, an additional step would be necessary to decrease the potential to levels suitable for methanogenesis (generally, negative ORP values with -400 mV being an optimum [Khanal, 2008]).

5.3.3.6. Solid vs. Liquid Phase

Methane and CO_2 generation from solid (coal re-suspended in a normal saline solution) and liquid phase (chemical pretreatment) was investigated separately (Appendix I). Generally, solids produced more methane and carbon dioxide than

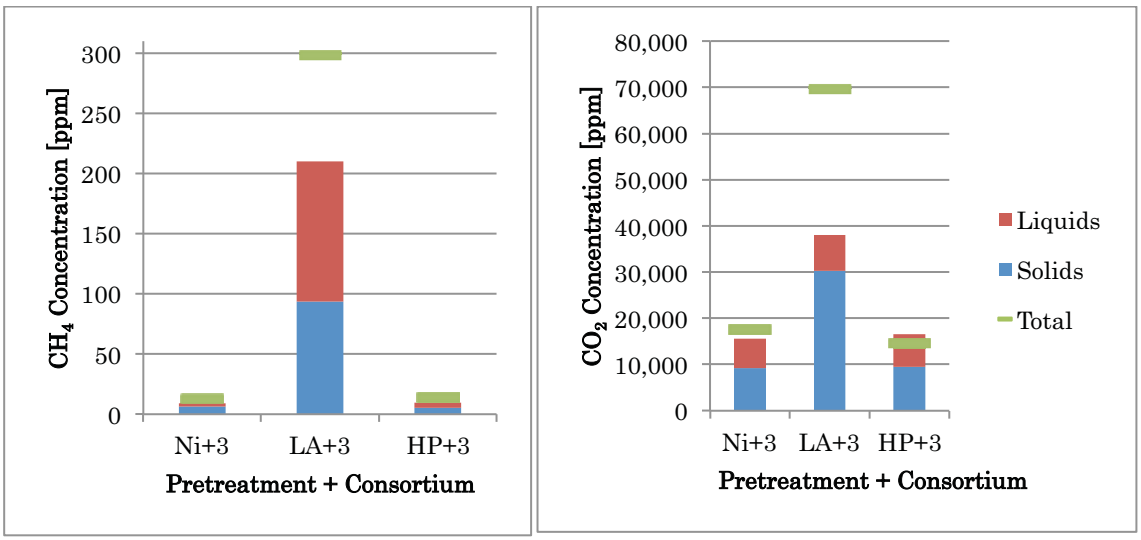

Figure 46. Methane and carbon dioxide generated from the solids and liquid phase of the bituminous coal waste sample after 58 days of combined chemical and microbial pretreatment.

liquid phases did (results obtained with the bituminous coal waste sample are shown as an example in Figure 46). This can be explained by the fact that centrifuged coal particles contained higher carbon concentration than was dissolved in the liquid phase. Moreover, the native microbial population was concentrated with the solids during the coal washing procedure, involving centrifugation. Native populations contain microbes that are the best equipped for degradation of a given coal and subsequent production of gases. Moreover, combined concentrations of gases produced from solid and liquid phase alone were generally lower than those produced when the two phases were combined. There are many potential reasons for that phenomenon; e.g., inadvertently, a fraction of dissolved carbon in the solution as well as a fraction of the native microbes might have been lost with the discarded solution during the washing procedure.

5.3.3.7. Immobilized Microbial and Enzyme Pretreatments

No increase in gas production was observed in the samples pretreated with an enzyme extract. Samples pretreated with an immobilized enzyme extract generated more carbon dioxide, which could have been caused by a biodegradation of the alginate (Figure 47 shows results obtained with the bituminous coal waste as an example; the remaining results are given in Appendix I). Immobilized microbial consortia produced insignificant amounts of methane and less carbon dioxide than the non-immobilized consortia over a 28-day incubation period (Figure 48 shows results obtained with the bituminous coal waste as an example; the remaining results are given in Appendix I). This indicates that the diffusion through the immobilization material was not occurring. Some possible solutions to this problem include: use of a less dense alginate preparation, application of alginate degrading reagents (e.g. citrate), use of different immobilization materials (e.g., carrageenans, poly-vinyl alcohols, high-surface porous materials, such as activated carbon, etc.).

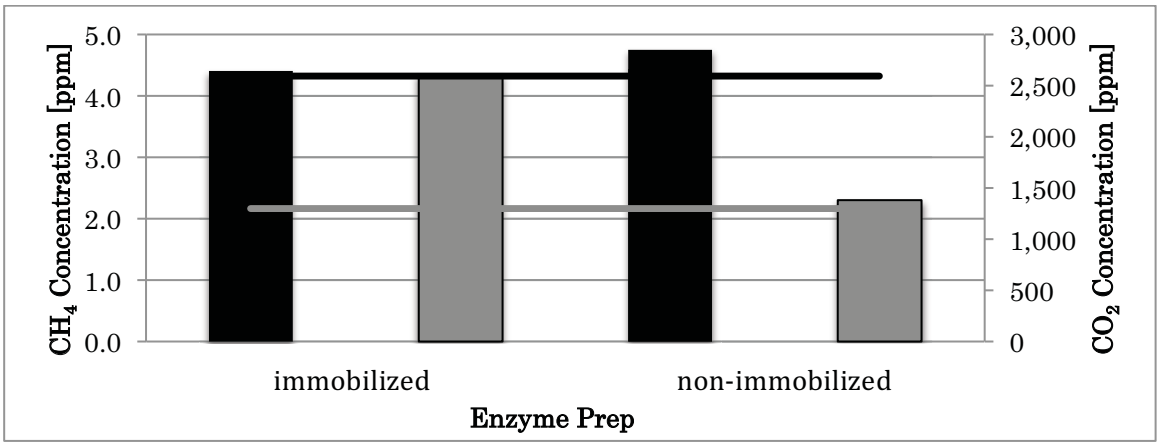

Figure 47. Methane (black bars) and carbon dioxide (gray bars) generated from bituminous coal waste samples after 14 days incubation period with immobilized and non-immobilized enzyme preparation. Black and gray lines represent the DI control samples of methane and carbon dioxide, respectively.

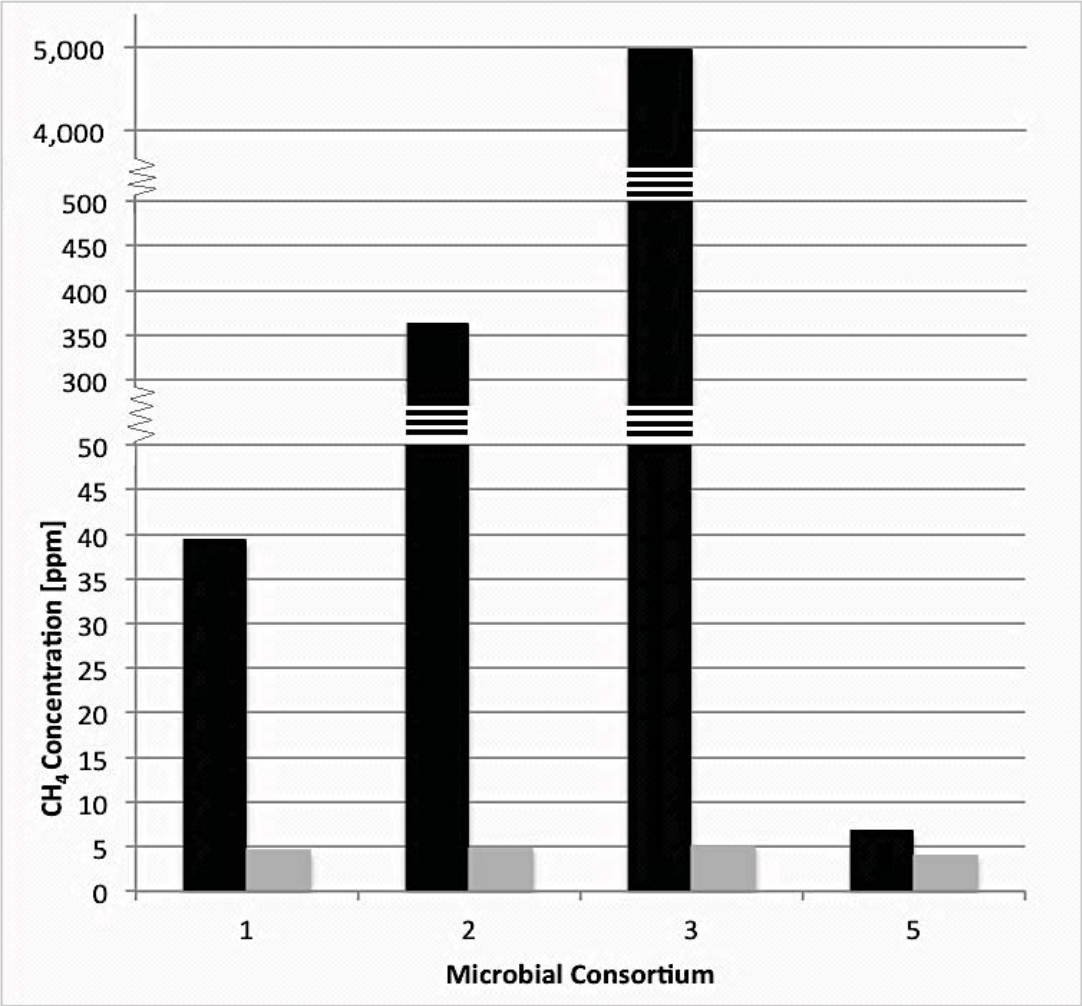

Figure 48. Methane generated from bituminous coal waste samples after 28 days incubation period with non-immobilized (black) and immobilized (gray) microbial consortia.

5.3.3.8. Statistical Significance of the Results

For all GC analyses a standard gas with a known concentration of methane and CO_2 was run with each 40 samples tested. Table 13 shows a summary of results obtained using three methane standards and two CO_2 standards over 18 measurements. Standard deviations depended on gas concentration and were generally higher at higher concentrations. Moreover, 14 samples were analyzed twice (Table 14). The second analysis showed generally a lower result, which was expected on such a small scale. Finally, a set of 12 duplicate samples was prepared and analyzed, for repeatability of results (Table 15). The standard deviation between the duplicates was generally lower than 10%.

Table 13. Average standard deviation obtained from 18 measurements of methane and carbon dioxide standard gases.

Concentration of a standard	Average standard deviation
Methane [ppm]	
14.9	0.21
102	0.81
995	8.86
Carbon Dioxide [ppm]	
1,010	13.4
10,000	100.1

Table 14. Results of methane and carbon dioxide concentrations obtained from duplicate measurements of 14 samples.

CH_4 [ppm]		CO_2 [ppm]	
240.81	240.67	242,333	242,097
506.57	506.86	103,734	103,648
189.27	189.17	207,723	207,647
8.27	8.26	10,053	10,070
10.43	10.24	12,141	12,108
11.49	11.33	7,635	7,605
12.08	12.11	10,934	10,890

Table 15. Average results of methane and carbon dioxide concentrations obtained from 12 duplicate samples.

CH_4 [ppm]	CO_2 [ppm]
7.89	10,556
8.77	11,404
8.84	13,781
10.99	13,928
11.84	28,692
13.64	30,314
18.68	35,711
171.08	41,934
194.75	109,806
236.74	111,289
249.66	223,991
505.26	233,479

5.4. Bench Scale Bioreactor Tests

Large quantities of gas were released after application of chemical pretreatments. The reactors became over-pressurized and the gasses were evacuated immediately after the initial GC measurement (four hours after preparation) for the safety reasons.

A significant increase of methane generation was observed from all coal samples after 14 days, as compared to "no microbes" saline controls and "no coal" microbial controls (Figure 49 shows results obtained with bituminous coal; for a complete set of results, please refer to Appendix J). Significant improvement of carbon dioxide production was observed in comparison to "no microbes" saline controls. However, the CO_2 results obtained were four times lower than the gas produced from "no coal" lactic acid microbial controls. This could be an indication that a part of produced carbon dioxide was converted to methane. Another possible explanation would be that the coal environment provided suitable conditions for microbial transformation of the nutrient to other compounds, instead of CO_2. Finally, there is also a possibility

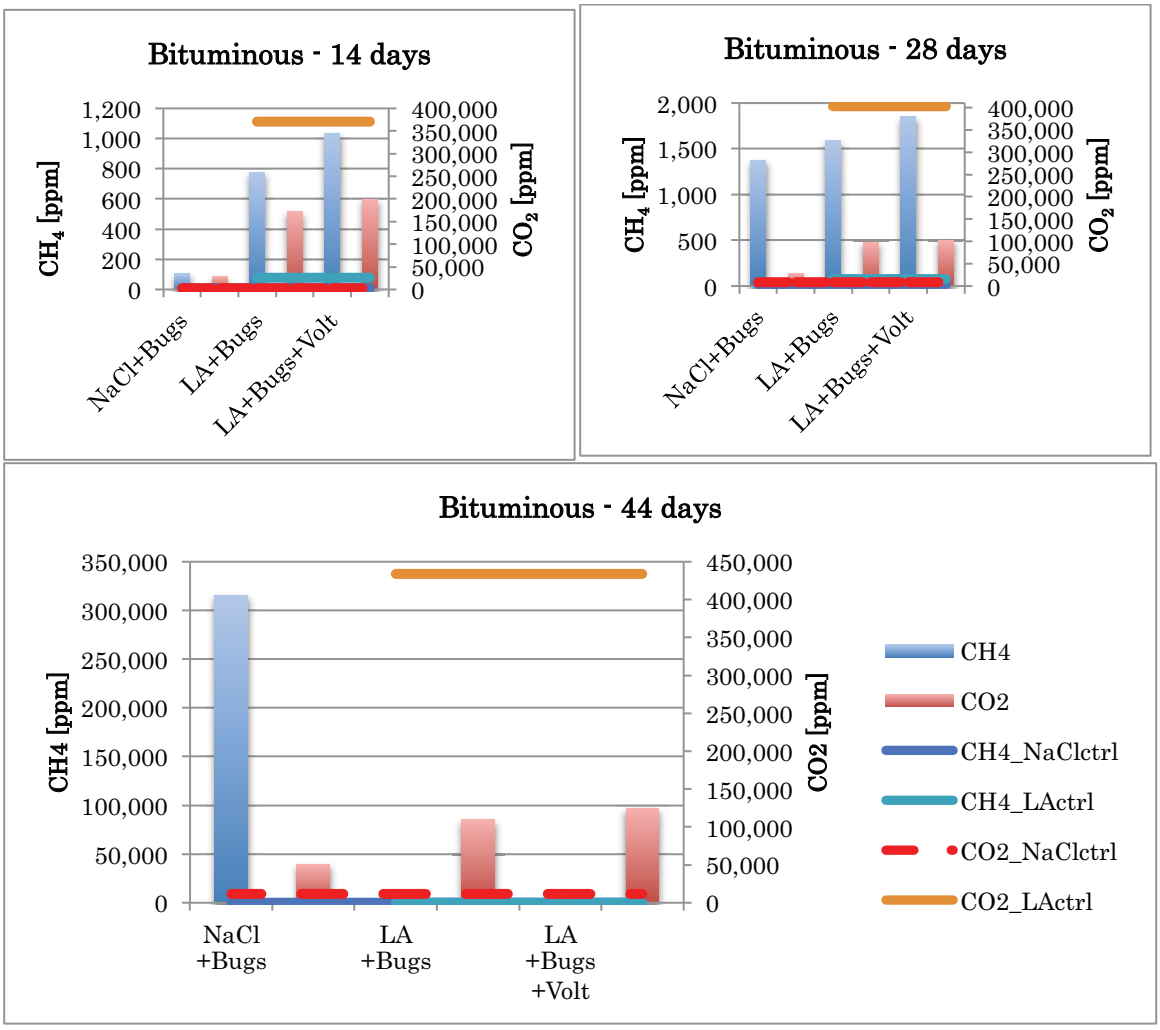

Figure 49. Methane (blue bars) and carbon dioxide (red bars) generated from microbiochemically treated bituminous coal samples after 14, 28, and 44 days. Blue and red lines represent "no microbes" saline controls of methane and CO_2, respectively. Light blue and orange lines represent "no coal" microbial controls of methane and CO_2, respectively.

that a large portion of the nutrient was absorbed on the pulverized coal and was less available to the microbes.

Bituminous coal and bituminous coal waste materials produced the same order of magnitude of both methane and carbon dioxide. Bituminous coal waste generated more carbon dioxide than the bituminous coal sample, perhaps because the coal waste material was exposed to the atmosphere and was therefore more easily

biodegradable. Lignite samples produced significantly different results than the bituminous coal and bituminous coal waste samples (Appendix J). Nearly 100% CO_2 was produced from lignite amended with lactic acid treatment and microbial inoculum, which also resulted in methane concentration as low as 150 ppm. Lignite was also the only coal sample that produced more carbon dioxide when bio-chemically amended as compared to "no coal" lactic acid control. The gas generation from the bituminous coal, bituminous coal waste, and lignite followed the same trends as in the previous gas generation tests with smaller quantities of coal materials, indicating reproducibility and scalability of the results.

After the initial increase of methane concentration in the samples amended with nutrients, the methane concentrations decreased (Figure 50). Surprisingly, after 44 days, coal samples containing only the microbial amendment produced over a 100 times more methane than the samples containing additional carbon nutrients. Based on the common sense and the results obtained from the smaller scale gas generation tests, it was expected that samples amended with carbon-based chemical pretreatment/nutrient and microbes would generate the highest methane concentrations. It is possible that environmental conditions created by the chemical reagents (i.e., pH and ORP) were not the most suitable for methanogenesis, as experienced with the lignite samples in the smaller scale tests (Section 5.3.3.5). Samples amended with a lactic acid treatment generated considerably higher CO_2 concentrations, which might have caused acidification of the liquid phase below the optimal methanogenic conditions. The dissociation constant of lactic acid is 3.86, while that of carbonic acid is 6.35.

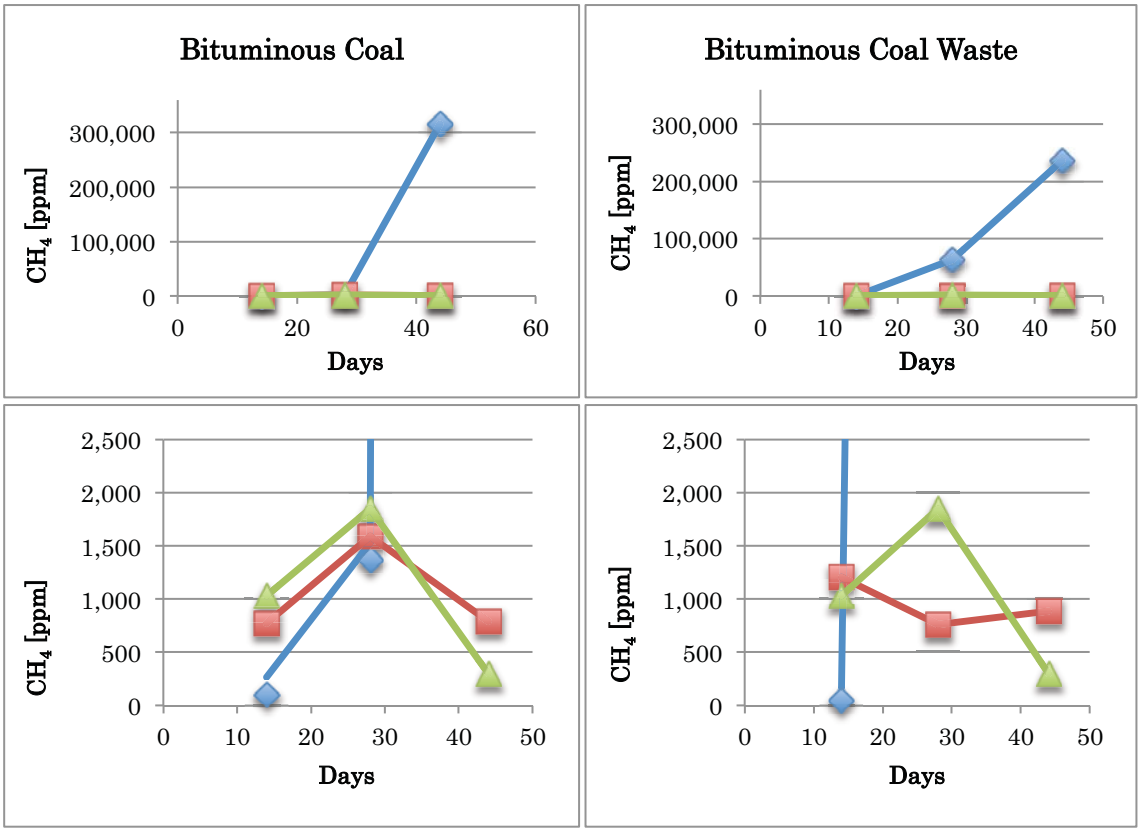

Figure 50. Methane generated from microbiochemically pretreated bituminous coal and bituminous coal waste samples after 14, 28, and 44 days. Blue line shows samples immersed in normal saline solution, red lines shows samples immersed in lactic acid treatment, and green lines show samples immersed in lactic acid treatment and placed in electro-biochemical reactors. Bottom figures show the results on a smaller y-axis.

Two-fold improvement in CO_2 generation was observed in bituminous coal samples, and over a four-fold improvement was observed in bituminous coal waste samples. Lactic acid pretreatment contained 3% hydrogen peroxide, which might have resulted in fast oxidation of coal materials but might have also generated large quantities of oxygen, inhibiting methanogenesis. Such a treatment would be preferential in locations where carbon dioxide is as valuable as methane. In other

cases, hydrogen peroxide would have to be balanced with an organic carbon nutrient to reduce ORP to the region suitable for methanogenesis. Eventually, aerotolerant microbes would also consume the oxygen present in the solution and in headspace through metabolism of carbon-based nutrient to CO_2, allowing methanogens to function.

Furthermore, there might have been a negative feedback created in the samples amended with the lactic acid treatment. Substances such as VFA (volatile fatty acids), hydrogen, and hydrogen sulfide can act as methanogenesis inhibitors, if present at high enough concentrations. One of the more important methanogenic inhibition mechanisms is that of weak acids and bases, changing pH to unsuitable levels for methanogens (indirect pH inhibition). It is caused by a passive transfer of the free acid or base across the cell membrane and subsequent dissociation. Microorganisms such as methanogens, which use reactions with a low energy yield or use proton motive forces, are especially sensitive to this type of inhibition. For instance, volatile fatty acids (such as acetate) are methane precursors with pKa values of 4.7-4.9 that can inhibit methane generation at elevated levels. The toxicity of VFA depends on the pH of the environment, since at low pH a larger portion of the acid exists as an undissociated form, much more toxic than the ionic form, due to its higher membrane permeability. Moreover, acetate toxicity is microbial species dependent. Slow growing *Methanothrix* does not tolerate acetate concentrations above 1mM, while fast growing *Methanosarcina* prefers acetate concentrations above that threshold. On the other side of the pH spectrum, ammonia (pKa of 9.3) causes methane generation inhibition due to increased pH. Ammonia concentration should not be high in the tested bioreactors, since no additional nitrogen was

provided with the growth medium nor was it present at high concentrations in the coal matrix. Hydrogen sulfide acts as another inhibitor, while its pKa is 6.95 and thus it should act as a buffer. The mechanism of hydrogen sulfide inhibition is still debated. Sulfides are toxic to methanogens at levels higher than about 200 mg/L, and should not have reached that threshold in the tested bioreactors. Hydrogen acts also as an indirect methanogenic inhibitor, although the mechanism of hydrogen inhibition varies from the pH inhibition. Methanogens are strongly dependent on the proton reducing or hydrogen producing bacteria that oxidize fatty acids under anaerobic conditions, since hydrogen ions are necessary in most methanogenic pathways (see Figure 3 and Table 4). However, hydrogenase (an enzyme responsible for hydrogen metabolism in hydrogen producing bacteria) is inhibited by high hydrogen levels. Thus if methanogenic transformations do not keep up with hydrogen production, hydrogen concentrations build up and inhibit the entire system.

Nevertheless, not only was the generated methane concentration stabilized at some point in the bioreactors supplied with lactic acid pretreatment (that is to be expected from a batch reactor), it actually decreased with time. The generated gases were not withdrawn from the reactors, causing them to be over-pressurized. If methane was no longer being generated, perhaps because of one of the inhibition mechanisms, there is a possibility that the produced gas was being dissolved in the liquid phase. Moreover, the created consortium was a combination of several populations collected from various environmental samples; i.e., there is no guarantee that it did not contain small percent of methanotrophic microorganisms. Methanotrophs are a specialized subgroup of methylotrophs, which utilize C1

compounds, and are common in environments where methane is naturally formed. Microbial methane oxidation can be carried out under aerobic and anaerobic conditions. Formaldehyde formation through methanol is an aerobic methane oxidation pathway (Equation 10). The mechanism of anaerobic oxidation of methane is still debated; however, it is believed that it is coupled with reduction of other species, with sulfate being the most common identified electron acceptor (Equation 11).

$$CH_4 \rightarrow CH_3OH \rightarrow HCHO \rightarrow HCOOH \rightarrow CO_2 \quad 10)$$

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \quad 11)$$

Both aerobic and anaerobic methylotrophs are less sensitive to pH changes. Some of the anaerobic *Archaea* methylotrophs are extremophiles, able to carry out metabolism at pH as low as 1-2 [Pol et al., 2007] and as high as 11.

As expected, the fastest initial methane generation was obtained from coal samples immersed in lactic acid treatment and placed in electro-biochemical reactors (Figures 48 and 49). Lactic acid is a carbon-based pretreatment that stimulated microbial growth and methanogenesis. It should have also caused ORP decrease, since lactic acid is an organic electron donor. Availability of electrons might be a limiting parameter in CO_2 reduction to methane, as seen in Figure 3.

The Electro-Biochemical Reactor (EBR) is a new technology based on a recent discovery that certain microorganisms can utilize electrons supplied via electrodes [Thrash and Coates, 2008; Adams et al., 2011]. It is currently used in the wastewater treatment for removal of metals and inorganics, where electron

availability is crucial. However, after the initial advantage, methane concentration present in the Electro-Biochemical Reactors started decreasing and after 44 days was the lowest among all samples. In fact, methane concentration in the EBRs was lower after 44 days than it was after 14 days, and it was even lower in the EBR than in the equivalent conventional reactors containing the same lactic acid pretreatment and no electrodes (Figure 50). Anaerobic methane oxidation is strongly dependent on the availability of electron accepting species, such as sulfate (Equation 11), which should have been sparse in these reactors. There is a possibility that the provision of electron acceptors in in the EBR gave an advantage to the methanotrophic population, once the conditions changed to unfavorable for methanogenesis in the lactic acid pretreated samples.

6. CONCLUSIONS

This research showed that methane and/or carbon dioxide can be produced from various carbonaceous materials using selected microbial populations and chemical pretreatments. A summary of conclusions, significance of the results, and implications for future R&D are listed below.

- Methane and carbon dioxide generating microbial consortia capable of surviving under aerobic conditions were adapted through selective collection and screening.
- These facultative methane and CO_2 producing consortia represent a significant contribution to potential application of microbial enhanced methane production, as all known methanogens are strict anaerobes. The use of strict anaerobes for methane production outside laboratory could be problematic. High risk of oxygen exposure could exist during inoculum preparation, within above ground bioreactors, during injections into subsurface, and within biochemically degraded coal seams.
- Methane and CO_2 generation screening of over 150 different populations has been performed in this study. This work reduces the microbial screening requirement for future bench- and pilot-scale tests.
- The microbial consortia cultivated for this research preferred temperature range of 20-30^{o}C, pH of 7.0-9.5, and slight addition of salt (2g/L NaCl).

- In certain cases, specialized consortia will have to be developed. For instance, microbial methane production from deep coal seams, characterized by high temperatures (generally, over 35°C) might be possible with cultivation of thermophilic microorganisms from deep coal reservoirs, geothermal reservoirs, or hydrothermal vents. Similar possibilities exist for high salinity coal-bed methane fields.
- Subsurface or bioreactor chemistry may need to be monitored and adjusted, if necessary. For instance, well injection of a carbon-based nutrient might result in a decrease of pH to values below 4.0. In such a case, a buffer would need to be delivered into the subsurface, prior to or with microbial injections.
- The microbial environment must be maintained within the range tolerated by the microbes. In order to apply microbial generation of carbon dioxide and methane to specific applications scenarios, suitable microbial population, nutrients, and chemical pretreatments must be selected. Laboratory bench-scale tests are needed to identify appropriate environmental conditions prior to any field work.
- To select the best performing microbial consortia, screening should be conducted within a given subsurface biogeochemistry range. Ideally, microbial screening would be performed in coal-bed methane well production water or waste coal pile infiltration waters. This will ensure that microbial consortium is selected based on a given water chemistry and ability to augment the indigenous microbial population. Nutrient composition and chemical pretreatments need to be based on coal geochemistry and on availability of macro-nutrients (carbon, nitrogen, phosphorus, sulfur) and other important elements (oxygen, hydrogen) in the

solution after chemical pretreatment. For example, coal with low nitrogen content will need to be supplemented with higher urea containing nutrients.

- Similar methane and higher CO_2 production were obtained from bituminous coal and bituminous coal waste, demonstrating that coal waste materials could be utilized for methane generation.
- Larger volumes of coal waste material would be necessary to generate the same methane concentration as the equivalent coal material, since the coal waste samples were upgraded in these tests, excluding larger inorganic rocks from the matrix.
- Extent of coal chemical dissolution is dependent on coalification stage of the sample and concentration of the chemical reagent. Lignite was generally dissolved more readily than bituminous coal.
- The extent of coal dissolution did not correlate well with methane and/or CO_2 generation. However, lactic acid pretreatment (containing carbon based nutrient, oxidizing agent, and a surfactant) increased methane generation from bituminous coal and bituminous coal samples significantly.
- Microbial carbon dioxide generation can be a relatively fast process. Methane generation by microorganisms is a slower process than carbon dioxide generation. The differences in microbial generation of carbon dioxide and methane will need to be taken into account when planning for bench- and pilot-tests.
- Direct provision of electrons and electron acceptors influenced methane generation in a negative manner. Availability of electron acceptors is a limiting step in methylotrophic utilization of methane under anaerobic conditions.

Provision of electron acceptor environment within a subsurface or reactor environment might have given an advantage to the methanotrophic population.

6.1. Future Research

The following items were identified as future research tasks:

1. Conduct larger-scale batch tests, until methane generation stops increasing to define the maximum coal to methane and/or CO_2 conversion.
2. Continue to study microbial immobilization, population viability, and methane and CO_2 production in subsurface environments. Certain aspects of this task are currently being developed by Dr. John McLennan's student, Joshua Sewell (Department of Chemical Engineering, The University of Utah).
3. Further examination of enzymatic biodegradation of coal is warranted as is examination of enzymatically enhanced methanogenesis from coal.
4. Testing of flow-through bench-scale methane and carbon dioxide generation from coal with continuous gas analysis is needed for ex situ methane generation on large scale.
5. An on-site pilot study for enhanced methane and carbon dioxide generation from coal-bed methane wells is needed to validate laboratory testing as is a pilot study for above ground hydrocarbon waste conversion to methane.

APPENDIX A

MEDIA COMPOSITION

1. Acetate medium: 3.5 g/L acetate (suggested reagent: sodium acetate)
2. Acetate, yeast, and phosphate medium: 2.5 g/L acetate (suggested reagent: sodium acetate), 0.75 g/L yeast extract, 0.5 g/L phosphate (suggested reagent: potassium phosphate monobasic)
3. 50% TSB: 15 g/L tryptic soy broth
4. Lactate medium: 1 g/L yeast extract, 6.667 mL/L sodium lactate, 1.23 g/L sodium acetate, 0.5 g/L ammonium chloride, 1 g/L potassium phosphate, 0.2 g/L magnesium sulfate, 0.1 g/L calcium chloride, 0.5 g/L sodium sulfate
5. Yeast, urea, phosphate: 1.25 g/L yeast extract, 0.15 g/L urea, 0.5 g/L phosphate (suggested reagent: potassium phosphate monobasic)

APPENDIX B

STANDARD OPERATING PROCEDURES

University of Utah

Environmental Microbiology Laboratory

Jack Adams

Standard Operating Procedure

Cultivation of Microorganism for Gas Generation Tests

Author: Ola Opara

Revision record

Revision	Date	Author	Description
1	7/8/2010	Ola Opara	Initial release
2	7/9/2010	Ola Opara	Added references and attachment sections

1. Scope and Application

This method is used to cultivate microorganisms present in environmental samples in the best conditions allowing for methane production and/or coal degradation.

2. Method Summary

Environmental samples are immersed in various media (see SOP: *Sampling of Microbial Populations*). Six tenfold dilutions of the environmental samples are prepared with saline solution and plated on TSA plates (see section 10.1.6.). After three days, grown colonies are characterized and counted. The most representative plate is chosen from each set and 2-5 mL of a medium that was used in a given environmental sample is added to it. Liquid is collected from the plate and placed in a sterile 15 mL graduated centrifuge tube containing 10 mL of the appropriate medium. Tubes are vortexed and stored in room temperature. After 2-8 days microbes are washed with saline solution and used to inoculate coal samples (see SOP: *Methane Generation Tests*).

3. Health and Safety

3.1. Microbiological testing involves potentially pathogenic microorganisms. Standard laboratory biosafety precautions should be followed.
3.2. No drinking or eating is allowed in the laboratory.
3.3. Mouth pipetting is prohibited.
3.4. All microbiologically contaminated materials should be autoclaved prior to disposal.

4. Interferences

There are no known interferences.

5. Equipment and Materials

5.1. Analytical balance
5.2. Petri dishes
5.3. Autoclave
5.4. Vortex mixer
5.5. Disposable borosilicate glass sterile pipettes, separately wrapped (1, 5, 10 mL)
5.6. Pipetters
5.7. "Hockey sticks" (L-shaped bacteria spreaders)

5.8. Graduated plastic centrifuge tube (15 mL)

6. Reagents and Chemicals

6.1. Deionized water from laboratory tap
6.2. 10% Chlorox solution (for disinfection purposes)
6.3. Lactate media
6.4. TSB
6.5. Acetate
6.6. Urea
6.7. Agar
6.8. Sodium chloride (NaCl)

7. Procedure

7.1. Disinfect sample stations (media and microbiology hood) before and after work.
7.2. Prepare TSA plates (see section 10.1.6.).
7.3. Prepare saline solution (0.85% w/v NaCl).
7.4. Autoclave saline solution, dilution tubes with caps, and "hockey sticks".
7.5. Using environmental samples, prepare sets of six tenfold dilution tubes with saline solution.
7.6. Plate 10^{-1} to 10^{-6} dilutions of environmental samples on TSA plates.
7.7. Store plates in room temperature for three days.
7.8. After three days take pictures of plates, characterize colony morphology and count the colonies.
7.9. Prepare and autoclave the media.
7.10. Place 5 mL of appropriate media into sterile 15 mL graduated centrifuge tubes.
7.11. Choose the most representative plate from each set. Under sterile conditions, inject 2-5 mL of a medium that was used in a given environmental sample into each chosen plate and mix it with a "hockey stick".
7.12. Using a sterile pipette, draw the liquid off the plate and place it in a sterile 15 mL graduated centrifuge tube containing the appropriate medium. Vortex the tube.
7.13. Store tubes in room temperature for 2-8 days.
7.14. Wash the microbes present in 15 mL tubes with normal saline solution in order to reduce the amount of available nutrients.
7.15. Inoculate coal samples with microbes, adding a mixture of urea and phosphate in normal saline (see SOP: *Methane Generation Tests*).

8. Data Reporting

8.1. Take pictures of the plates after three days.
8.2. Characterize colony morphology.
8.3. Count colonies.

9. References

9.1. SOP: *Sampling of Microbial Populations,* A. Opara, April 2010
9.2. SOP: *Methane Generation Tests*, A. Opara, June 2010

10. Attachment

10.1. Media composition
10.1.1. Acetate medium: 3.5 g/L acetate (suggested reagent: sodium acetate)
10.1.2. Acetate, yeast, phosphate medium: 2.5 g/L acetate (suggested reagent: sodium acetate), 0.75 g/L yeast extract, 0.5 g/L phosphate (suggested reagent: potassium phosphate monobasic)
10.1.3. TSB: 15 g/L tryptic soy broth
10.1.4. Lactate medium: 1 g/L yeast extract, 6.667 mL/L sodium lactate, 1.23 g/L sodium acetate, 0.5 g/L ammonium chloride, 1 g/L potassium phosphate, 0.2 g/L magnesium sulfate, 0.1 g/L calcium chloride, 0.5 g/L sodium sulfate
10.1.5. Yeast, urea, phosphate: 1.25 g/L yeast extract, 0.15 g/L urea, 0.5 g/L phosphate (suggested reagent: potassium phosphate monobasic)
10.1.6. TSA plates: 15 g/L agar, 30 g/L tryptic soy broth

University of Utah

Environmental Microbiology Laboratory

Jack Adams

Standard Operating Procedure

Oxygen Requirement Test with Thioglycollate

Author: Aleksandra Opara

Revision record

Revision	Date	Author	Description
1	08/17/2011	Aleksandra Opara	Initial release

1. Scope and Application

This method is used in determination of microbial oxygen requirement. Growth medium used in the test is thioglycollate broth, containing a mixture of nutrients (dextrose, yeast extract, digest of casein), compounds removing oxygen from the medium (L-cystein and sodium thioglycollate), agar slowing down the return of oxygen to the sample and thus creating an oxygen gradient and oxygen indicator (resazurin). It supports the growth of common, easily grown bacteria; however, many organisms are not being able to grow in this medium.

2. Method Summary

Microbial liquid cultures are centrifuged. Working under sterilized microbial hood, microbial culture is collected on a sterile loop and the loop is pushed almost to the bottom of the thioglycollate tube. The cap is placed loose on the tube, allowing for oxygen introduction into the headspace. Growth in the tubes is analyzed after 1-2 days incubation in the dark.

3. Health and Safety

3.1. No drinking or eating is allowed in the laboratory.
3.2. Personal protective equipment (i.e., lab coat, gloves, and goggles) is required at all times.
3.3. Mouth pipetting is prohibited.

4. Equipment and Materials

4.1. Autoclave
4.2. UV hood
4.3. Analytical balance
4.4. Bacti-Cinerator
4.5. Microbial loop

5. Reagents and Chemicals

5.1. Deionized water
5.2. 10% Clorox solution
5.3. Thioglycollate broth tubes (BD BBL)

6. Procedure

6.1. Disinfect work hood with Clorox solution before work.
6.2. Turn UV light under the hood and leave it on for 30 minutes.
6.3. Centrifuge liquid microbial cultures for 15 minutes.
6.4. Discard the liquid phase, leaving microbial cells at the bottom of the centrifuge tube.
6.5. Sterilize a loop in Becti-Cinerator.
6.6. Collect a loop-full of culture and carefully place it almost at the bottom of thioglycollate tube.
6.7. Slowly remove the loop from the tube, making sure the tube is not mixed and no oxygen is allowed in the broth.
6.8. Sterilize the loop.
6.9. Place the thioglycollate tube in a rack, making sure the cap is loose to allow for oxygen transfer into the headspace.
6.10. Clean and sterilize the work area.
6.11. Incubate the tubes for 1-2 days in darkness.

7. Data Reporting

After incubation period, observe the location of microbial growth:

1. Growth at the bottom of the medium: obligate anaerobes
2. Growth throughout the medium: aerotolerant anaerobes
3. Growth throughout with higher concentration at the top: facultative anaerobes
4. Growth just below the surface: microaerophililes
5. Growth at the very top of the medium: obligate aerobes

8. Attachments

8.1. Composition of Thioglycollate growth medium

8.1.1. 15.0 g Pancreatic Digest of Casein
8.1.2. 0.5 g L-Cystein
8.1.3. 5.0 g Dextrose
8.1.4. 5.0 g Yeast Extract
8.1.5. 2.5 g Sodium Chloride
8.1.6. 0.5 g Sodium Thioglycollate
8.1.7. 0.001 g Resazurin
8.1.8. 0.75 g Agar

University of Utah

Environmental Microbiology Laboratory

Jack Adams

Standard Operating Procedure

Community Level Physiological Profiling

Author: Aleksandra Opara

Revision record

Revision	Date	Author	Description
1	08/20/2011	Aleksandra Opara	Initial release

1. Scope and Application

This method is used in community level physiological profiling with BIOLOG EcoPlates. It enables a fast assessment of metabolic diversity and metabolic fingerprinting of consortia by providing them with a set of 31 carbon sources.

2. Method Summary

Working under sterile conditions, a serial dilution of microbial liquid cultures is prepared in order to obtain similar concentrations of CFUs in all tested cultures (10^5 CFU per 100µL). Prepared diluted inocula are vortexed and a 100µL is transferred into each well of the Biolog Ecoplates. The Biolog plates are incubated at the optimal growth temperature defined for each culture with a moist paper towel to minimize evaporation.

3. Health and Safety

3.1. No drinking or eating is allowed in the laboratory.
3.2. Personal protective equipment (i.e., lab coat, gloves, and goggles) is required at all times.
3.3. Mouth pipetting is prohibited.

4. Equipment and Materials

4.1. Autoclave
4.2. UV hood
4.3. Glass culture tubes
4.4. Metal spreaders
4.5. Biolog Ecoplates
4.6. Pipettes

5. Reagents and Chemicals

5.1. 0.85% saline solution
5.2. 10% Clorox solution

6. Procedure

6.1. Disinfect work hood with Clorox solution before work.

6.2. Turn UV light under the hood and leave it on for 30 minutes.

6.3. Prepare a microbial dilution series with normal saline solution to obtain approximately 10^5 CFU per 100µL in each culture.

6.4. Label the Biolog Ecoplates with the name of the culture, the date, and your initials.

6.5. Transfer 100µL of the diluted culture into each one of 32 Biolog wells. Make sure to vortex the tube each time prior to transferring.

6.6. Clean and sterilize the work area.

6.7. Incubate the plates for 1-4 days at the optimal growth temperature with a moist paper towel to minimize evaporation.

University of Utah

Environmental Microbiology Laboratory

Jack Adams

Standard Operating Procedure

Small Scale Coal Crushing

Author: Aleksandra Opara

Revision record

Revision	Date	Author	Description
1	01/03/2011	Aleksandra Opara	Initial release
2	01/03/2011	Jack Adams	Corrections in the procedure; Added point 7.4

1. Scope and Application

This method is used to pulverize small quantities of coal or other carbonaceous materials under sterile environment. The procedure has been developed for small laboratory scale and is applicable to material weighing up to approximately 20 grams.

2. Method Summary

Small quantity of rock (less than 1 g) is placed in a clean, sterilized mortar under sterilized hood. Using a sterilized pestle, rock is crushed until completely pulverized. More material is added and crushing continues. Pulverized material is placed in sterile containers and its weight is calculated.

3. Health and Safety

3.1. No drinking or eating is allowed in the laboratory.
3.2. Personal protective equipment (i.e., lab coat, gloves, and goggles) is required at all times.
3.3. Mouth pipetting is prohibited.

4. Interferences

There are no known interferences.

5. Equipment and Materials

5.1. UV sterilization station
5.2. Small stainless steel mortar and pestle set; 4.1 x 4.1 x 2.4 inches

6. Reagents and Chemicals

6.1. Deionized water
6.2. 10% Clorox solution
6.3. 10 % hydrogen peroxide

7. Procedure

7.1. Disinfect work hood with Clorox solution before work.
7.2. Turn UV light under the hood and leave it on for 30 minutes.
7.3. Carefully wash mortar and pestle with hydrogen peroxide and place under UV light in the hood. Let it dry and be exposed to UV light for 30 min.
7.4. Turn off UV light.
7.5. Working under the sterilized hood, place small quantity of material in a mortar (less than 1 g).
7.6. Using a pestle, carefully crush the rock until it is completely pulverized.
7.7. Add more material to the mortar and continue crushing.
7.8. When done, place pulverized material in sterilized containers under the hood (e.g., autoclaved glassware or sterile centrifuge tubes).
7.9. Wash the mortar and pestle with DI water and hydrogen peroxide.
7.10. Disinfect work hood with Clorox solution after work.
7.11. Turn UV light under the hood and leave it on for 30 minutes.

8. Data Reporting

8.1. Weigh containers before and after placing pulverized material inside.
8.2. Calculate the weight of pulverized material stored in each container.

University of Utah

Environmental Microbiology Laboratory

Jack Adams

Standard Operating Procedure

Chemical Dissolution of Coal - Sample Preparation

Author: Aleksandra Opara

Revision record

Revision	Date	Author	Description
1	03/21/2011	Aleksandra Opara	Initial release

1. Scope and Application

This method is used for chemical dissolution of pulverized carboniferous samples. The weight of sample used can vary between 1 and 5 grams, depending on the carbon content. The volume of chemical reagent used for dissolution is 20mL. The method was designed to minimize introduction of foreign microbial population into the sample.

2. Method Summary

Chemical reagent is prepared and filtered into a sterile flask. Desired amounts of ground samples are weighed under the sterilized hood and placed in 50mL centrifuge tubes. A 20mL aliquot of the prepared chemical reagent is added into the sample. Tubes are stored at room temperature for the desired period of time and shook thoroughly once a week. After the desired reaction time, samples are filtered accordingly to an SOP: "Chemical Dissolution of Coal – Filtration".

3. Health and Safety

3.1. No drinking or eating is allowed in the laboratory.
3.2. Personal protective equipment (i.e., lab coat, gloves, and goggles) is required at all times.
3.3. Mouth pipetting is prohibited.
3.4. Samples must be prepared under a hood, due to a reactive character of some of the reagents.

4. Equipment and Materials

4.1. UV sterilization station
4.2. Autoclave
4.3. Analytical balance
4.4. Weighing dishes
4.5. 50mL sterile centrifuge tubes
4.6. Erlenmeyer flasks
4.7. Aluminum foil
4.8. Sterile syringes
4.9. 0.22µm sterile syringe filters

5. Reagents and Chemicals

5.1. Deionized water
5.2. 10% Clorox solution

6. Procedure

6.1. Disinfect work hood with Clorox solution before work.
6.2. Turn UV light under the hood and leave it on for 30 minutes.
6.3. Autoclave an empty Erlenmeyer flask.
6.4. Prepare a solution of the required chemical reagent in another Erlenmeyer flask.
6.5. Working under a sterilized hood, filter the reagent into an autoclaved flask, using a syringe and a 0.22µm sterile syringe filter.
6.6. Working under a sterilized hood, weigh a desired amount of previously ground sample into a sterile 50mL centrifuge tube. The desired accuracy is ±5mg. (See SOP: "Coal Crushing – Small Scale" for crushing method)
6.7. Using a sterile pipette, add 20mL of chemical reagent into the 50mL centrifuge tube containing ground sample.
6.8. Thoroughly shake the 50mL centrifuge tube until the complete wetting of the sample occurs.
6.9. Disinfect work hood with Clorox solution after work.
6.10. Turn UV light under the hood and leave it on for 30 minutes.
6.11. Leave the prepared samples in the room temperature for a desired period of time. Shake the samples vigorously twice a week, until no sediment is attached to the bottom of the tubes.
6.12. After the desired reaction time, filter the samples accordingly to an SOP: "Chemical Dissolution of Coal – Filtration".

7. Data Reporting

7.1. See SOP: "Chemical Dissolution of Coal - Filtration ".

University of Utah

Environmental Microbiology Laboratory

Jack Adams

Standard Operating Procedure

Chemical Dissolution of Coal - Filtration

Author: Aleksandra Opara

Revision record

Revision	Date	Author	Description
1	03/21/2011	Aleksandra Opara	Initial release

1. Scope and Application

This method is based upon the standard method 2540D *Total Suspended Solids Dried at 103-105°C*. It is used to determine the amount of solids remaining after the chemical dissolution of carboniferous materials (see SOP: "Chemical Dissolution of Coal – Sample Preparation").

2. Method Summary

Using a vacuum filtration apparatus, glass microfiber filters are washed with DI water and dried at 105°C for an hour (or until a stable weight is achieved). Dried filters are weighed, placed in the filtration apparatus and a small aliquot of DI water is added to create a seal. Sample is mixed thoroughly and poured onto the filter and the suction is applied. The sample container and its cap are washed with DI water and detergent, if necessary, until all the solids are on the filter. Suction is applied until all the water is evacuated. Filter papers with solids are dried at 105°C for 24 hours (or until a stable weight is achieved), cooled in a desiccators, and weighed. The amount of solids is calculated as a difference between the final and initial weight of the filter.

3. Health and Safety

3.1. No drinking or eating is allowed in the laboratory.

3.2. Personal protective equipment (i.e., lab coat, gloves, and goggles) is required at all times.

3.3. Mouth pipetting is prohibited.

4. Interferences

Large amounts of solids (usually above 2g) may lead to clogging of the filter pores. In such case, divide the volume of the sample and use several filters, as necessary.

5. Reagents and Chemicals

5.1. Deionized water

5.2. Laboratory detergent

6. Equipment and Materials

6.1. Filtration apparatus (Büchner flask, vacuum pump, magnetic filter holder)
6.2. Aluminum foil
6.3. Metal tray
6.4. Glass microfiber filters, 1.2µm
6.5. Metal tweezers
6.6. Metal spatula
6.7. Wash bottle
6.8. Analytical balance
6.9. Desiccator
6.10. Drying oven

7. Procedure

7.1. Prepare the filtration apparatus.
7.2. Place a sheet of aluminum foil in the metal tray. Using a permanent marker, divide it into equal squares, each the size of the filter paper used, and number them.
7.3. Using clean metal tweezers grab a glass fiber filter and weigh it. (To avoid leaving any residual oils on the filter, never touch it with bare hands. Always handle filters with clean metal tweezers.)
7.4. Insert the glass fiber filter in the filtration apparatus, apply vacuum and wash with 50mL of DI water.
7.5. Dry filters at about 105°C for an hour.
7.6. Weigh the glass fiber filter and compare this weight to the initial one. If the difference is larger than 0.5mg, repeat steps 7.2 through 7.4.
7.7. Store the filters in the desiccator until needed.
7.8. Note the initial weight of the filter.
7.9. Place the filter on the filtration apparatus and add a small volume of DI water to create a seal and avoid sample loss.
7.10. Mix the sample thoroughly, until the sediment at the bottom of the tube is agitated. Pour it onto the filter and apply vacuum.
7.11. If the filtration lasts longer than 5 minutes, reduce the sample size by half.
7.12. Wash the sample tube and its cap with a small amount of DI water to remove any remaining solids and pour them onto the filter.
7.13. If the sample is hydrophobic and is not completely removed from the tube with DI water, use laboratory detergent and repeat step 7.12.
7.14. Wash the inside of the filter holder with DI water until all the solids are on the filter.

7.15. Continue suction to remove all traces of water, turn the vacuum off, and discard the washings.
7.16. Carefully dismantle the upper part of the magnetic filter holder.
7.17. Using a clean metal spatula, remove the filter from the apparatus and place it on the aluminum foil that is spread in the metal tray.
7.18. Leave the metal tray at room temperature for about an hour to dry the filters. If the filters are too wet when placed in the oven, they will stick to the aluminum foil and will be problematic to remove.*
7.19. Place the tray in the oven at 105°C for 24 hours.
7.20. Take the tray out of the oven and place the filters in the desiccator for cooling.
7.21. Weigh the filter.
7.22. Place the filters back onto the metal tray and in the oven at 105°C for 1 hour.
7.23. Take the tray out of the oven and place the filters in the desiccator for cooling.
7.24. Weigh the filter. If the weight varies by 0.01g from the previous measurement, repeat steps 7.22-7.24.
7.25. Clean the work station.

**As an improvement to the technique, it is suggested that filter papers are placed inside individual aluminum weighing dishes.*

8. Data Reporting

8.1. Note the initial weight of the filter paper.
8.2. Note the final weight of the filter paper.
8.3. Calculate the weight of the solid material collected on the filter by subtracting the initial weight of the filter paper from the final weight.

University of Utah

Environmental Microbiology Laboratory

Jack Adams

Standard Operating Procedure

Gas Chromatography

Author: Ola Opara

Revision record

Revision	Date	Author	Description
1	9/11/2010	Ola Opara	Initial release

1. Scope and Application

This method is used to analyze the concentration of organic and inorganic gases using gas chromatography (GC). Chromatograph used in this procedure was HP 6890 but the method can be adapted and used with other GC units.

2. Method Summary

Procedure begins with turning on the computer and flaming the GC unit. The column and gastight syringe are cleaned with fresh air. The tip of the gastight syringe is placed in the sample vial through an opening made in the septum. The gases are allowed to fill the syringe up to the 500µL mark, the plunger is pushed down to the 200µL mark, and excess gases are evacuated into DI water. After preparing the GC unit, sample is injected into the injection port. Using calibration data, integrated peak areas are recalculated into gas concentrations.

3. Health and Safety

3.1. No drinking or eating is allowed in the laboratory.

3.2. Many parts of the GC equipment are dangerously hot and touching them with bare skin or placing paper or other flammable materials nearby should be avoided.

4. Interferences

This is a procedure for the gas phase and injection of liquid phase should be avoided as it can interfere with the analysis and damage the column.

5. Equipment and Materials

5.1. Hamilton gastight 500 µL syringe, model #1750

5.2. GC unit, model HP 6890

5.3. GC column: GS-GasPro PLOT

5.4. Detectors: FID (flame ionization detector) for hydrocarbons and TCD (thermal conductivity detector) for inorganic gases

5.5. 50 mL glass beaker

5.6. KimWipes

6. Reagents and Chemicals

6.1. Deionized water

6.2. Calibration gases, Scotty Analyzed Gases

7. Procedure

7.1. Start the computer and the software operating the GC unit.

7.2. On the panel of the GC unit, press "Det Control" and "On", in order to light the flame.

7.3. Use Method 2 that has the initial temperature set to 35°C and held for 3.8 minutes to allow for carbon dioxide, methane, and ethane elution. After 3.8 minutes, temperature is set to increase by 25°C/min up to 260°C to allow for analysis of longer-chain gases.

7.4. Following the procedure described below, prepare calibration curves of gases of interest. Use Calibration Gases (Scotty Analyzed Gases).

7.5. Prior to every set of samples, run a sample of fresh air in order to clean the coil from any remaining gases.

7.6. Using a sharp needle, make an opening in a sample's septum.

7.7. In order to clean the gastight syringe from any remaining gases, draw in fresh air, close the valve, apply pressure to the plunger and simultaneously open the valve to let the air out. Repeat this step at least five to ten times prior to sampling.

7.8. Gently insert the tip of the gastight syringe into the opening made in the septum. Special care needs to be applied in order not to bend or break a gentle needle.

7.9. Open the valve of the gastight syringe and slowly pull the plunger to a 500µL mark. Keep the valve open for about 10-15 seconds to allow the gases to fill the syringe.

7.10. Close the valve and, pressurizing the collected gases, lower the plunger to a 200µL mark.

7.11. Insert the tip of the gastight syringe into a beaker of deionized water and, keeping the plunger at the 200µL level, open the valve and release excess gases. Dry the needle with a KimWipe. This should minimize the contamination of gases with the outside air and eliminate the contamination of the needle with other materials that could interfere with the analysis (e.g. coal from the sample). It will also ensure that the syringe is working properly.

7.12. In the software's menu, insert the name of the sample under Sample Info and press Run Method.

7.13. Wait for the chromatograph to cool down to the initial temperature.

7.14. Press "Prep run" on the control panel and wait for the Status to change to Ready.

7.15. By introducing pressure to a plunger and simultaneously opening the valve, inject the gases into the injection port of the GC unit.

7.16. Press "Start" on the control panel, starting the analysis.

8. Data Reporting

8.1. Prepare calibration curves, plotting peak areas against concentration.

8.2. In the software's menu, go to "Data analysis" and load the chromatogram of the sample.

8.3. Using an integration tool, calculate peak areas for all the visible peaks.

8.4. Note the peak areas in the Excel spreadsheet.

8.5. Using calibration data, calculate concentrations of the analyzed gases.

University of Utah

Environmental Microbiology Laboratory

Jack Adams

Standard Operating Procedure

Gas Generation Tests

Author: Ola Opara

Revision record

Revision	Date	Author	Description
1	6/25/2010	Ola Opara	Initial release
2	7/8/2010	Ola Opara	Change of sample containers and volume; Included reference to an SOP: *Cultivation of Microorganisms for Methane Tests.*
3	7/14	Ola Opara	Changed/added detailed information in sections: 2; 7.1; 7.2; 7.3; 7.4; 8.1

1. Scope and Application

This method is used to investigate generation of natural gases from carbon sources by various microorganisms under different environmental conditions. Two gases of interest are methane and carbon dioxide but the method can also be used to investigate generation of other hydrocarbons. The procedure has been developed for small laboratory scale and is applicable (after scaling) for 20-450 mL containers. Microorganisms can be introduced with a solid sample and/or from isolated cultures.

2. Method Summary

4 g of solid carbon source is mixed with 5 mL of medium in a 20 mL glass serum bottle (see SOP: *Coal Preparation*). 1.5 mL of microbial culture from a selected environment is added to the bottle (see SOP: *Cultivation of Microorganisms for Methane Tests*). A Teflon silicone septum is placed on top of a bottle and it is sealed with an aluminum seal using a crimper. Samples are stored in room temperature. Volume of produced gases is measured with a 1 mL syringe. Prior to measurement, the bottom of the syringe's plunger is placed on a 0.1 mL mark in order to reduce the pressure necessary to move it. Volume of produced gases is measured 1, 3, 5, 7, 14, 21, and 28 days after sample preparation. Gases from the syringe are analyzed with a portable handheld gas detector by slowly releasing the gas from the syringe with the needle tip held at the tip of the detector to determine if they are organic or inorganic. If a significant volume of gas is collected in the headspace, a GC analysis follows (see SOP: *GC Method*).

3. Health and Safety

3.1. Microbiological testing involves potentially pathogenic microorganisms. Standard laboratory biosafety precautions should be followed.
3.2. No drinking or eating is allowed in the laboratory.
3.3. Mouth pipetting is prohibited.
3.4. All microbiologically contaminated materials should be autoclaved prior to disposal.

4. Interferences

There are no known interferences.

5. Equipment and Materials

5.1. Analytical balance
5.2. Autoclave
5.3. Vortex mixer
5.4. Disposable borosilicate glass sterile pipettes, separately wrapped (1, 5, 10 mL)
5.5. Pipetters
5.6. Sterile syringes (1 mL)
5.7. Syringe needles
5.8. Glass serum bottles (20 mL)
5.9. Teflon silicone septa (20 mm)
5.10. Aluminum seals (20 mm)
5.11. Portable gas detector
5.12. Crimper

6. Reagents and Chemicals

6.1. Deionized water from laboratory tap
6.2. 10% Clorox solution (for disinfection purposes)
6.3. Lactate media (yeast extract, sodium lactate, sodium acetate, ammonium chloride, potassium phosphate, magnesium sulfate, calcium chloride, sodium sulfate)
6.4. TSB
6.5. Acetate
6.6. Urea
6.7. 10 % hydrogen peroxide

7. Procedure

7.1. Disinfect sample stations with 10% Chlorox solution (media and microbiology hood) before and after work.
7.2. Under sterile conditions, disinfect 20 mL glass serum bottles and Teflon silicon septa with 10 % hydrogen peroxide solution.
7.3. Color code with laboratory tape and label the contents of all 20 mL glass serum bottles.
7.4. Weigh out 4 g of a ground solid carbon source (coal, waste coal, waste soil or lignite).
7.5. Place 4 g of a ground solid carbon source in a 20 mL glass serum bottle.

7.6. Add 6.7 mL of liquid media into a 20 mL glass serum bottle containing a ground solid carbon source.
7.7. Introduce microbial colonies into serum bottles under sterile conditions.
7.8. Place a Teflon silicone septum and an aluminum seal on top of the serum bottle and seal it with a crimper.
7.9. Shake the bottle thoroughly.
7.10. Store bottles in room temperature for a designated period of time.
7.11. Using a 1 mL syringe with the bottom of its plunger located at the 0.1 mL mark, measure the volume of gases collected in the headspace by making an opening in the bottle's septum. Remove the syringe from the bottle after 30 seconds.
7.12. Slowly empty the gases collected in the syringe into the portable gas detector.

8. Data Reporting

8.1. Record the position of the syringe's plunger 1, 3, 5, 7, 14, 21, and 28 days after sample preparation. Record the cumulative volume of gas collected.
8.2. Determine whether the collected gases are organic or inorganic with a portable gas detector.
8.3. Sample the gas from the headspace of samples generating more than 0.2 mL of gases (cumulative) and submit for GC analysis (see SOP: *GC Method*).

University of Utah

Environmental Microbiology Laboratory

Jack Adams

Standard Operating Procedure

Extraction and Immobilization of Enzymes

Author: Aleksandra Opara

Revision record

Revision	Date	Author	Description
1	03/25/2011	Aleksandra Opara	Initial release

1. Scope and Application

This method is used for immobilization of enzymes in alginate beads. Enzymes used for immobilization can be extracted directly from cells, as described in the procedure below. Purified enzymes that are available for purchase from biotech suppliers can also be used.

2. Method Summary

Liquid microbial cultures are prepared, grown to the top of the exponential log phase, and washed twice with normal saline solution. Concentrated cells are immersed in cold NP40 lysis buffer. The bead-beater chamber filled with cold glass beads and biomaterial is operated for three minutes under an ice-jacket. Immediately after, 1% of cold TSB solution is added to the cell homogenate, which is then mixed with 3% sodium alginate solution. Immobilized enzyme beads are formed by dripping alginate into calcium chloride solution, left to cure for 0.5 to 3 hours and stored in a fridge. Remaining biomaterial is plated in order to determine the extent of homogenization. Glass beads and bead-beater chamber are thoroughly cleaned.

One of the advantages of immobilization of cell homogenate is that multiple enzymes can be introduced to the reaction, eliminating the need for separate immobilization of multiple enzymes. Immobilized enzymes are more convenient to use, usually provide higher stability, and offer protection from the environment.

3. Health and Safety

3.1. No drinking or eating is allowed in the laboratory.
3.2. Personal protective equipment (i.e., lab coat, gloves, and goggles) is required at all times.
3.3. Mouth pipetting is prohibited.

4. Reagents and Chemicals

4.1. Deionized water
4.2. 10% Clorox solution
4.3. Calcium chloride ($CaCl_2$)
4.4. Sodium alginate ($NaC_6H_7O_6$)
4.5. Tryptic Soy Broth (TSB)
4.6. Agar
4.7. NP40 lysis buffer (see Attachment 8.2)
4.8. Sodium chloride (NaCl)

5. Equipment and Materials

5.1. Bead-beater with 15mL chamber
5.2. Autoclave
5.3. UV hood
5.4. Sterile centrifuge tubes
5.5. Beakers
5.6. Erlenmeyer flasks
5.7. Magnetic stirrer
5.8. Petri dishes
5.9. Glass beads
5.10. Sterile syringe
5.11. Sterile 18G 1½ needles
5.12. Analytical balance

6. Procedure

6.1. Disinfect work hood with Clorox solution before work.
6.2. Turn UV light under the hood and leave it on for 30 minutes.
6.3. Prepare a desired volume of liquid microbial culture in a fresh medium and leave to grow. (It is desired to extract the enzymes from the microbes at the top of their exponential log phase of growth. The actual time required for this to occur must be determined by microbial growth study of every consortium used).
6.4. Prepare a solution of sodium alginate (see Attachment 8.1)
6.5. Prepare a solution of 0.2M $CaCl_2$ and store it in a fridge until needed.
6.6. Prepare a 1% solution of TSB (tryptic soy broth) and store it in a fridge until needed.
6.7. Prepare a NP40 lysis buffer and store it in a fridge until needed (see Attachment 8.2).
6.8. Pour about 20mL of glass beads into a small beaker and place it in the freezer. (The correct diameter sizes of glass beads are 0.1mm for bacteria, 0.5mm for yeast, and 1.0-2.5mm for plant or animal tissue).
6.9. Wash the grown liquid cultures twice with normal saline solution (0.85% NaCl).
6.10. After the second centrifuging, replace the saline solution with the NP40 lysis buffer, thoroughly vortex the tubes, and store them in the fridge until needed.
6.11. Fill in the ice jacket of the bead-beater with crushed iced.
6.12. Fill in a bead-beater chamber ½ to ¾ full with ice-cold glass beads.
6.13. Add the biomaterial mixed with the lysis buffer to the chamber, until it is filled completely to the top, excluding as much air as possible.

6.14. Screw on the ice jacket and assemble the chamber on the bead-beater motor.

6.15. Operate the bead-beater for 3 minutes.

6.16. Immediately after the operation, add a small volume (about 1-3mL) of ice-cold 1% solution of TSB into the cell homogenate and place it in the fridge for further cooling.

6.17. Clean the glass beads and bead-beater chamber (see Attachments 8.3 and 8.4).

6.18. Plate the cell homogenate on TSA plates to determine the extent of cell breakage.

6.19. Mix the cell homogenate with sodium alginate solution.

6.20. Using a sterile syringe and needle, drip the biomaterial-alginate mixture from about 20cm into a beaker containing excess (at least 100mL) of cold $CaCl_2$ solution.

6.21. Leave the beads in the $CaCl_2$ solution to cure for 0.5 to 3 hours.

6.22. Store the beads in autoclaved DI water in a fridge until needed (preferably to be used within 2 days of immobilization).

6.23. Clean and sterilize the work area.

7. Data Reporting

7.1. Wait three days for the cells to grow on agar plate and count them. If the count of the surviving colonies is higher than 1,000, a longer homogenization time might be necessary.

8. Attachments

8.1. Preparation of sodium alginate solution

8.1.1. Prepare a 3% sodium alginate solution by slowly adding alginate to autoclaved, stirred DI water.

8.1.2. Continue to stir the solution until a complete dissolution of alginate is achieved.

8.1.3. Leave the solution undisturbed for at least 30 minutes to eliminate the air bubbles that can cause buoyancy of alginate beads.

8.1.4. If hardening is desired or required, mixing amines with the sodium alginate and cross-linking with glutaraldehyde can be performed.

8.1.5. Store the alginate solution in the fridge until needed.

8.1.6. Dripping of sodium alginate solution into calcium chloride will cause gelation, since depending on the associated salt alginic acid can be either water soluble or insoluble. Salts of ammonia and alkali metals,

such as sodium, are water soluble, while salts of divalent cations, such as calcium, are water insoluble. The exception is magnesium salt. The reaction of gelation proceeds as follows:

$2NaC_6H_7O_6 + CaCl_2 \rightarrow Ca(C_6H_7O_6)_2 + 2NaCl$

8.2. Components of NP40 lysis buffer

8.2.1. 1% Triton X-100
8.2.2. 0.15M NaCl
8.2.3. 50mM Tris-HCl, pH 7.2
8.2.4. 2mM EDTA (to prevent phosphorylation in the lysate)

8.3. Cleaning glass beads

8.3.1. Glass beads can be reused approximately ten times.
8.3.2. Soak the beads in laboratory detergent for 10 minutes.
8.3.3. Rinse repeatedly with DI water.
8.3.4. Soak the beads in 10% Clorox solution for 10 minutes, in order to ensure that the beads are clean of all nucleic acids and nucleases.
8.3.5. Rinse repeatedly with DI water.
8.3.6. Dry overnight at 50°C.

8.4. Cleaning the bead-beater chamber

8.4.1. Clean the chamber immediately after homogenate is prepared. Residual cell homogenate includes corrosive materials such as nucleic acids, etc. that can degrade the chamber materials.
8.4.2. Wash the chamber with tap water.
8.4.3. Dismantle the chamber and soak all the parts in laboratory detergent for 10 minutes.
8.4.4. Rinse repeatedly with DI water.
8.4.5. Soak in 10% Clorox solution for 2 minutes.
8.4.6. Rinse repeatedly with DI water.
8.4.7. Although not always necessary, lubrication of parts with mineral oil and silicone oil will allow for a smoother operation and a longer life-span of the bead-beater chamber.

University of Utah

Environmental Microbiology Laboratory

Jack Adams

Standard Operating Procedure

Immobilization of Microbial Cells

Author: Aleksandra Opara

Revision record

Revision	Date	Author	Description
1	03/25/2011	Aleksandra Opara	Initial release

1. Scope and Application

This method is used for immobilization of microbial cells in alginate beads.

2. Method Summary

Liquid microbial cultures are prepared, grown to the top of the exponential log phase, and washed twice with normal saline solution. Immobilized cell beads are formed by dripping alginate into calcium chloride solution and left to cure for 0.5 to 3 hours and stored in a fridge.

3. Health and Safety

3.1. No drinking or eating is allowed in the laboratory.
3.2. Personal protective equipment (i.e., lab coat, gloves, and goggles) is required at all times.
3.3. Mouth pipetting is prohibited.

4. Equipment and Materials

4.1. Autoclave
4.2. UV hood
4.3. Sterile centrifuge tubes
4.4. Beakers
4.5. Erlenmeyer flasks
4.6. Magnetic stirrer
4.7. Refrigerator and freezer
4.8. Sterile syringe
4.9. Sterile 18G 1½ needles
4.10. Analytical balance

5. Reagents and Chemicals

5.1. Deionized water
5.2. 10% Clorox solution
5.3. Calcium chloride ($CaCl_2$)
5.4. Sodium alginate ($NaC_6H_7O_6$)
5.5. Sodium chloride (NaCl)

6. Procedure

6.1. Disinfect work hood with Clorox solution before work.

6.2. Turn UV light under the hood and leave it on for 30 minutes.

6.3. Prepare a desired volume of liquid microbial culture in a fresh medium and leave to grow. (It is desired to use the microbes at the top of their exponential log phase of growth. The actual time required for this to occur must be determined by microbial growth study of every consortium used).

6.4. Prepare a solution of sodium alginate (see Attachment 7.1)

6.5. Prepare a solution of 0.2M $CaCl_2$ and store it in a fridge until needed.

6.6. Wash the grown liquid cultures twice with normal saline solution (0.85% NaCl).

6.7. Mix the cells with sodium alginate solution.

6.8. Using a sterile syringe and needle, drip the biomaterial-alginate mixture from about 20cm into a beaker containing excess (at least 100mL) of cold $CaCl_2$ solution.

6.9. Leave the beads in the $CaCl_2$ solution to cure for 0.5 to 3 hours.

6.10. Store the beads in autoclaved DI water in a fridge until needed (preferably to be used within 2 days of immobilization).

6.11. Clean and sterilize the work area.

7. Attachments

7.1. Preparation of sodium alginate solution

7.1.1. Prepare a 3% sodium alginate solution by slowly adding alginate to autoclaved, stirred DI water.

7.1.2. Continue to stir the solution until a complete dissolution of alginate is achieved.

7.1.3. Leave the solution undisturbed for at least 30 minutes to eliminate the air bubbles that can cause buoyancy of alginate beads.

7.1.4. If hardening is desired or required, mixing amines with the sodium alginate and cross-linking with glutaraldehyde can be performed.

7.1.5. Store the alginate solution in the fridge until needed.

7.1.6. Dripping of sodium alginate solution into calcium chloride will cause gelation, since depending on the associated salt alginic acid can be either water soluble or insoluble. Salts of ammonia and alkali metals, such as sodium, are water soluble, while salts of divalent cations, such as calcium, are water insoluble. The exception is magnesium salt. The reaction of gelation proceeds as follows:

$2NaC_6H_7O_6 + CaCl_2 \rightarrow Ca(C_6H_7O_6)_2 + 2NaCl$

APPENDIX C

LIST OF TESTED CHEMICAL PRETREATMENTS

- 0.1M Lactic Acid, 0.5% Tween-20, 3% Hydrogen Peroxide
- 0.1M Lactic Acid, 0.001M SDS, 3% Hydrogen Peroxide
- 1M Lactic Acid, 5% Hydrogen Peroxide
- 11M Lactic Acid, 30% Hydrogen Peroxide
- 0.1M Acetic Acid, 0.5% Tween-20, 3% Hydrogen Peroxide
- 0.1M Acetic Acid, 0.001M SDS, 3% Hydrogen Peroxide
- 1M Acetic Acid, 1M Phosphoric Acid, 5% Hydrogen Peroxide
- 1M Acetic Acid, 1% Ethanol, 5% Hydrogen Peroxide
- 17M Acetic Acid, 30% Hydrogen Peroxide
- 0.1M Sulfuric Acid, 3% Hydrogen Peroxide
- 5M Sulfuric Acid, 5% Hydrogen Peroxide
- 0.1M Phosphoric Acid, 0.5% Tween-20, 3% Hydrogen Peroxide
- 1M Phosphoric Acid, 1% Ethanol, 5% Hydrogen Peroxide
- 15M Phosphoric Acid, 30% Hydrogen Peroxide
- 0.1M Ascorbic Acid, 0.5% Tween-20, 3% Hydrogen Peroxide
- 0.1M Ascorbic Acid, 0.001M SDS, 3% Hydrogen Peroxide
- 0.1M Citric Acid, 0.5% Tween-20, 3% Hydrogen Peroxide
- 0.1M Citric Acid, 0.001M SDS, 3% Hydrogen Peroxide
- 100 mg/L $FeCl_3$, 3% Hydrogen Peroxide
- 100 mg/L $FeCl_3$, 3% Hydrogen Peroxide (pH 4)
- 100 mg/L $FeCl_3$, 25 mg/L Dipyridyl, 1% Ethanol, 3% Hydrogen Peroxide
- 60 mg/L Iron Citrate, 3% Hydrogen Peroxide
- 30 mg/L $NiCl_2$, 20 mg/L Al_2O_3, 100 mg/L SiO_2, 0.5% Tween-20, 3% Hydrogen Peroxide
- 30 mg/L $NiCl_2$, 20 mg/L Al_2O_3, 100 mg/L SiO_2, 30% Hydrogen Peroxide
- 60 mg/L Nickel Shiff Base, 0.5% Tween-20, 3% Hydrogen Peroxide
- 100 mg/L Nickel Shiff Base, 5% Hydrogen Peroxide
- 100 mg/L Nickel Shiff Base, 30% Hydrogen Peroxide
- 60 mg/L Nickel Aceteylacetone, 0.5% Tween-20, 3% Hydrogen Peroxide
- 40 mg/L Iron Porphine, 0.5% Tween-20, 3% Hydrogen Peroxide
- 100 mg/L Iron Porphine, 5% Hydrogen Peroxide
- 100 mg/L Iron Porphine, 30% Hydrogen Peroxide
- 20 mg/L Urea, 0.5% Tween-20, 3% Hydrogen Peroxide
- 100 mg/L Urea, 30% Hydrogen Peroxide
- 1% Ethanol
- 1% Ethanol, 60 mg/L NaF, 30% Hydrogen Peroxide
- 60 mg/L NaF, 0.5% Tween-20, 3% Hydrogen Peroxide
- 0.5% Tween-20, 3% Hydrogen Peroxide
- 0.5% Tween-20,
- 3% Hydrogen Peroxide

APPENDIX D

MORPHOLOGY CHARACTERIZATION RESULTS

Table D.1. Morphology information collected for each environmental sample on an example of the Jordan River Sediments.

	Sample	Jordan River Sediments					
	Medium	YUrPh	Ac	AcPhY	Lc	DI	TSB-50
A	Size	Medium	Medium	Big	Big	Large	Medium
	Color	Orange-white	Yellow	White	Red-orange	Gray	Yellow
	Form	Circular	Circular	Circular	Circular	Irregular	Circular
	Margin	Entire	Entire	Undulate	Entire	Undulate/ Erose	Entire
	Elevation	Convex	Raised	Flat	Raised	Flat	Raised
	Surface	Smooth	Smooth	Rugose	Smooth	Smooth	Smooth
	Count	1.2E+05	6.0E+04	1.0E+04	2.0E+03	2.0E+04	9.1E+06
B	Size	Tiny	Large	Tiny	Medium	Big	Small
	Color	White	White	White	Yellow	Pinkish	Pink
	Form	Punctiform	Irregular	Punctiform	Circular	Circular	Circular
	Margin	Entire	Undulate	Entire	Entire	Entire	Entire
	Elevation	Convex	Flat	Convex	Convex	Convex	Convex
	Surface	Smooth	Rugose	Smooth	Smooth	Smooth	Smooth
	Count	1.5E+05	1.2E+04	8.4E+04	5.0E+04	2.0E+04	7.7E+06
C	Size	Large	Medium	Medium	Large	Medium	Big
	Color	Whitish-gray	White	Yellowish	White	White	White
	Form	Irregular	Circular	Circular	Irregular	Circular	Irregular
	Margin	Undulate	Entire	Entire	Undulate	Entire	Undulate
	Elevation	Flat	Convex	Convex	Flat	Raised	Raised
	Surface	Rugose	Smooth	Smooth	Rugose	Concentric	Rugose
	Count	3.2E+04	6.0E+05	3.4E+04	3.0E+03	1.0E+04	2.8E+06
D	Size	Medium	Small	Medium	Big	Small	
	Color	Orange	Black	White	Whitish-gray	White	
	Form	Rhizoid	Circular	Circular	Circular	Circular	
	Margin	Raised	Entire	Entire	Entire	Entire	
	Elevation	Filamentous	Convex	Convex	Raised	Raised	
	Surface	Rugose	Smooth	Smooth	Rugose	Smooth	
	Count	2.0E+03	1.0E+04	2.8E+04	3.0E+04	1.0E+04	
E	Size		Small		Medium	X-Large	
	Color		White		Yellowish-pink	White	
	Form		Circular		Circular	Filamentous	
	Margin		Undulate		Entire	Filamentous	
	Elevation		Flat		Convex	Raised	
	Surface		Smooth		Smooth	Rugose	
	Count		3.4E+05		9.2E+05	1.0E+03	

Table D.2. A summary of total colony counts and number of distinguishable colonies obtained from each environmental sample cultured in five liquid media and DI water.

Medium	YUrPh	Ac	AcPhY	Lc	DI	TSB-50
	Jordan River Sediments					
Count	3.0E+05	1.0E+06	1.6E+05	1.3E+06	6.1E+04	2.0E+07
#Colonies	4	5	4	7	5	3
	Great Salt Lake Wetland Sediments					
Count	1.9E+06	6.7E+06	6.1E+08	2.6E+07	8.7E+04	4.5E+07
#Colonies	4	4	6	5	6	4
	Great Salt Lake Sediments					
Count	1.5E+06	1.1E+07	7.1E+06	3.8E+06	5.9E+05	1.3E+05
#Colonies	8	7	7	5	6	4
	Anaerobic Digester Sludge					
Count	1.6E+07	4.2E+06	1.2E+07	2.7E+07	2.1E+07	5.7E+06
#Colonies	6	6	6	6	6	5
	Coarse Bituminous Coal					
Count	2.2E+07	2.0E+05	5.9E+07	1.8E+08	5.8E+04	1.5E+07
#Colonies	5	3	4	3	3	5
	Coarse Bituminous Coal Waste					
Count	5.4E+05	3.5E+05	1.7E+06	7.0E+05	3.5E+05	3.2E+07
#Colonies	8	5	5	6	8	4
	Soil Sample from Bituminous Coal Waste					
Count	5.3E+05	7.3E+05	2.6E+06	8.4E+04	6.6E+05	NA
#Colonies	4	6	6	7	5	NA
	Grinded Bituminous Coal					
Count	4.9E+06	6.8E+05	2.5E+06	2.9E+06	NA	1.3E+06
#Colonies	5	5	5	8	NA	5
	Grinded Bituminous Coal Waste					
Count	2.7E+06	1.7E+06	7.5E+04	1.2E+06	1.0E+03	8.1E+05
#Colonies	2	2	5	7	1	3
	Water with Sediments from Oil Seep Area					
Count	1.3E+04	9.9E+03	1.5E+05	1.3E+04	1.3E+03	4.5E+03
#Colonies	3	4	1	4	7	1
	Sediments from Oil Seep Area					
Count	4.6E+04	7.6E+05	5.8E+03	2.0E+03	2.2E+04	1.4E+02
#Colonies	2	4	8	8	10	6
	Sediments from underneath an Active Oil Seep					
Count	6.1E+05	4.7E+03	7.1E+04	1.7E+03	1.0E+05	6.9E+02
#Colonies	6	9	5	9	5	7
	Tar with Sediments from Oil Seep					
Count	1.3E+06	1.8E+02	1.6E+02	3.1E+05	2.0E+05	1.1E+04
#Colonies	2	7	4	5	7	6
	Water with Sediments from underneath the Tar Mat					
Count	1.1E+06	8.8E+06	5.3E+04	7.5E+05	3.1E+03	1.8E+06
#Colonies	3	4	5	3	3	1
	Sediments from underneath the Water Sample from Oil Seep Area					
Count	8.3E+05	6.9E+05	3.4E+05	9.1E+03	1.6E+05	1.0E+04
#Colonies	4	6	5	6	6	10
	Oily Sediments from underneath the Water Sample from Oil Seep Area					
Count	4.6E+05	8.0E+01	6.5E+03	1.3E+03	2.1E+02	7.4E+05
#Colonies	2	1	5	3	2	3
	Surface Sediments from Oil Seep Area					
Count	1.7E+05	1.1E+03	3.4E+04	6.5E+03	6.3E+03	3.3E+04
#Colonies	8	7	4	5	3	5

Table D.2. continued

Medium	YUrPh	Ac	AcPhY	Lc	DI	TSB-50
	Natural Gas Well Production Water (Well 8-355)					
Count	7.50E+06	3.10E+06	2.75E+05	4.70E+05	3.22E+05	3.09E+06
#Colonies	3	1	4	2	4	3
	Natural Gas Well Production Water (Well 14-386)					
Count	9.61E+06	7.08E+05	3.61E+07	1.70E+07	1.90E+03	2.03E+07
#Colonies	4	3	4	4	2	3
	Natural Gas Well Production Water (Well 17-370)					
Count	1.28E+07	5.30E+04	1.15E+08	5.00E+05	9.80E+05	9.27E+07
#Colonies	1	1	1	1	2	11
	Natural Gas Well Production Water (Well 26-168)					
Count	3.40E+05	1.59E+06	2.30E+06	9.30E+06	4.29E+05	4.22E+07
#Colonies	4	2	4	7	2	9
	Natural Gas Well Production Water (Well 30-290)					
Count	7.11E+05	3.38E+06	2.60E+07	7.50E+07	5.48E+05	9.22E+06
#Colonies	4	6	3	5	4	6
	Natural Gas Well Production Water (Well 33-522)					
Count	1.20E+07	4.03E+05	1.53E+08	9.09E+07	3.80E+05	2.17E+07
#Colonies	8	3	3	5	2	2

APPENDIX E

ENVIROMENTAL INFLUENCES RESULTS

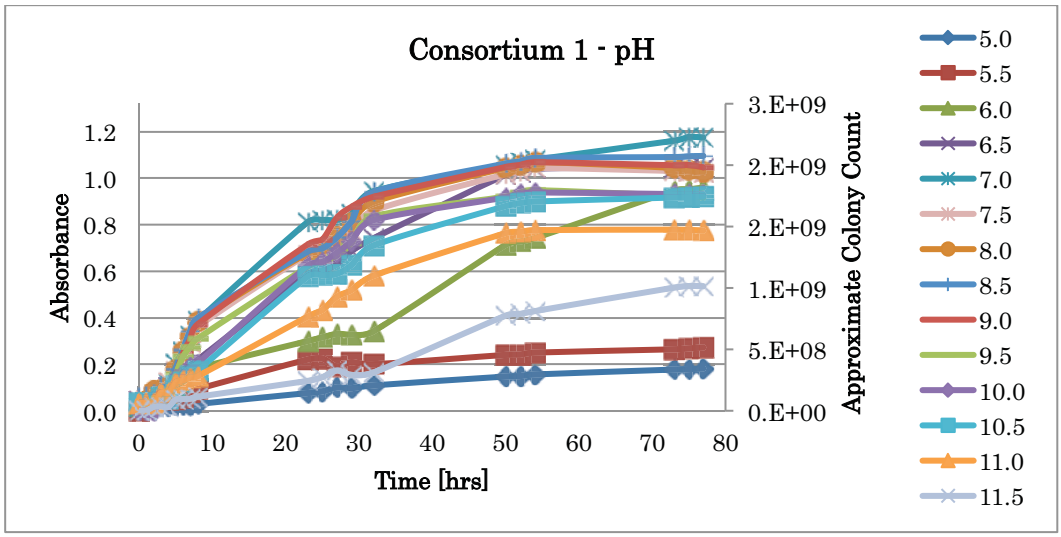

Figure E.1. Consortium 1 growth under starting pH conditions ranging between 5.0 and 11.5.

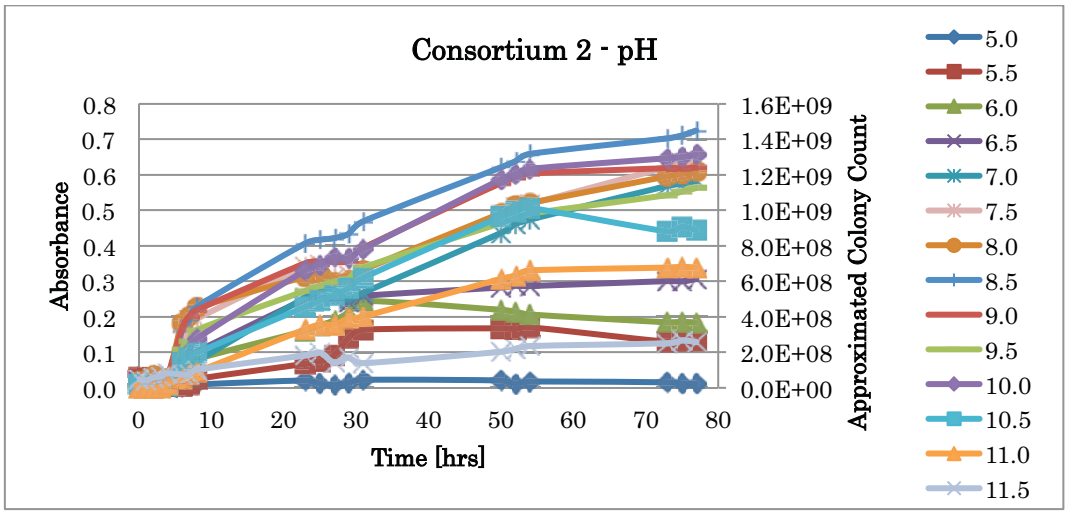

Figure E.2. Consortium 2 growth under starting pH conditions ranging between 5.0 and 11.5.

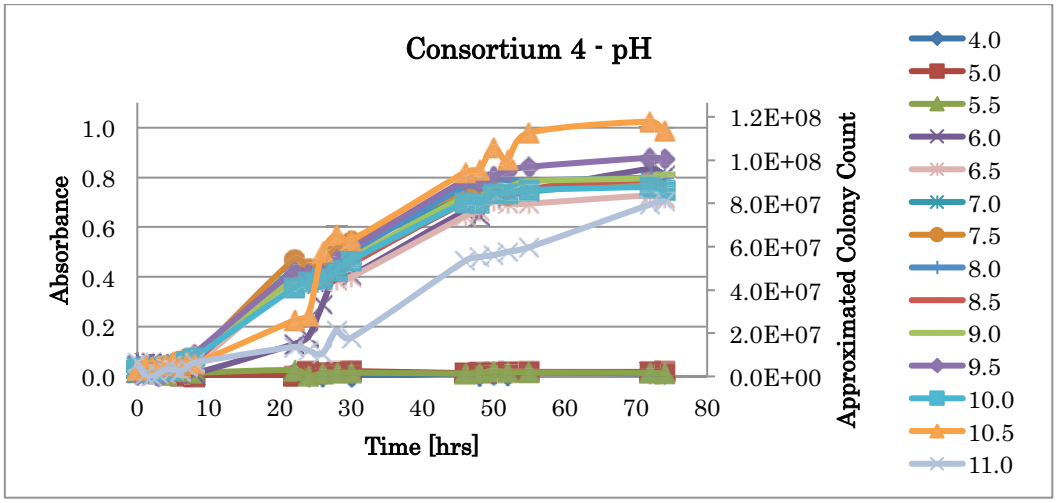

Figure E.3. Consortium 4 growth under starting pH conditions ranging between 4.0 and 11.0.

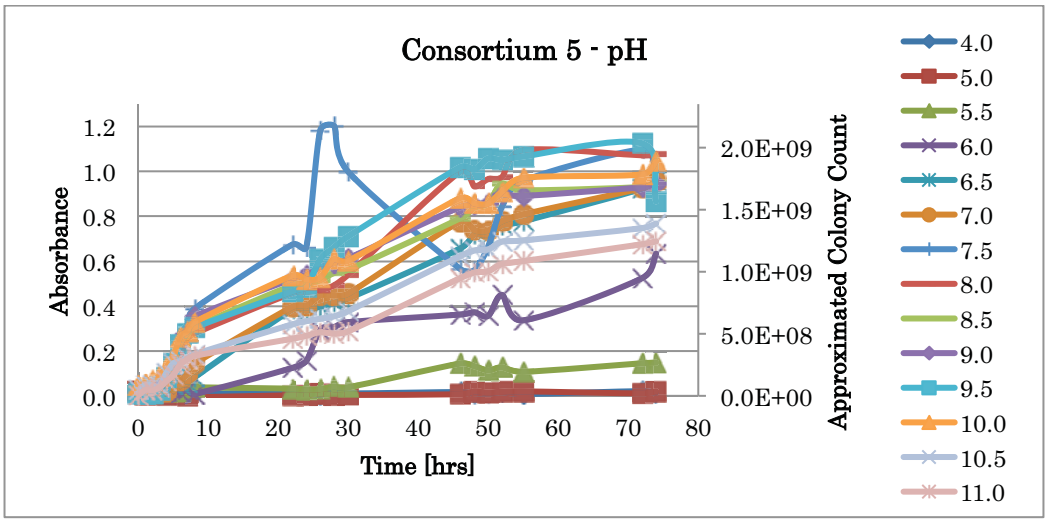

Figure E.4. Consortium 5 growth under starting pH conditions ranging between 4.0 and 11.0.

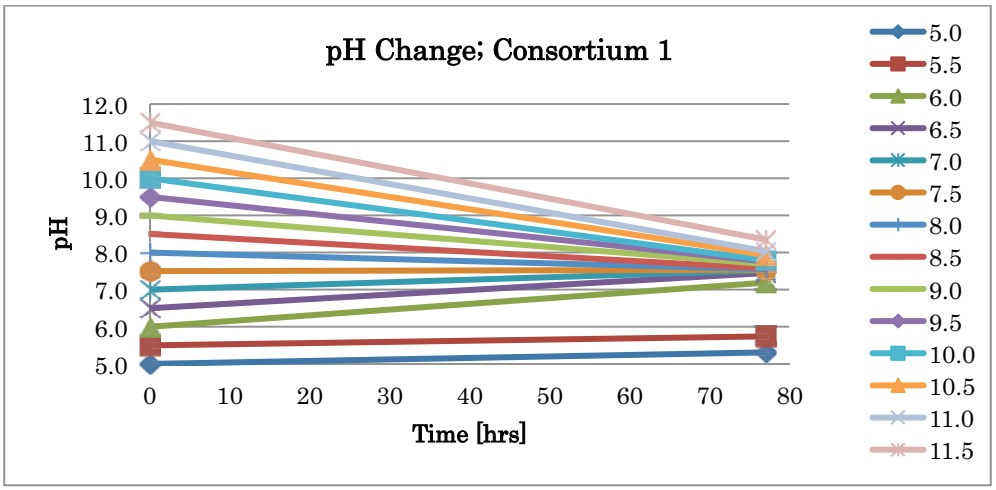

Figure E.5. Change of pH in consortium 1 after 77 hours.

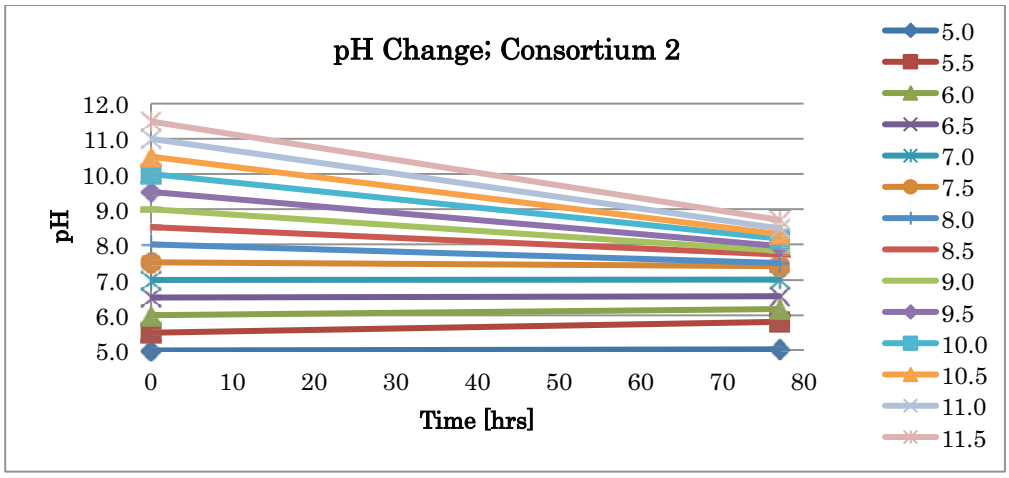

Figure E.6. Change of pH in consortium 2 after 77 hours.

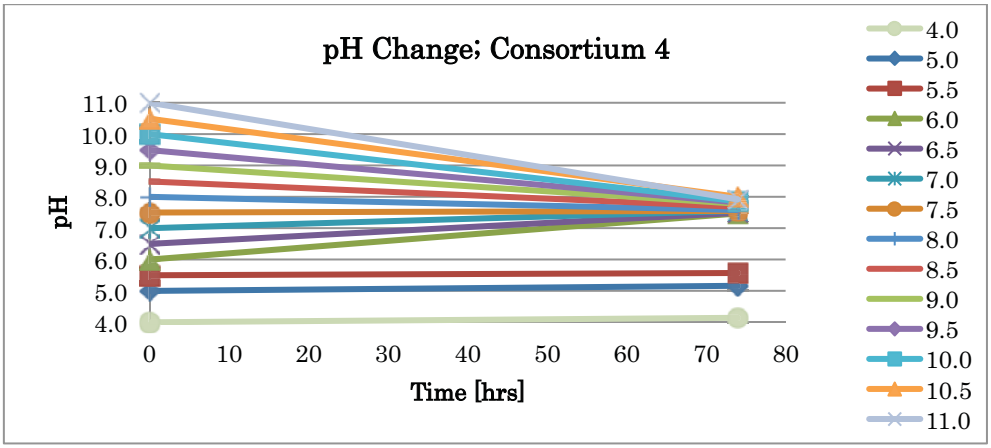

Figure E.7. Change of pH in consortium 4 after 77 hours.

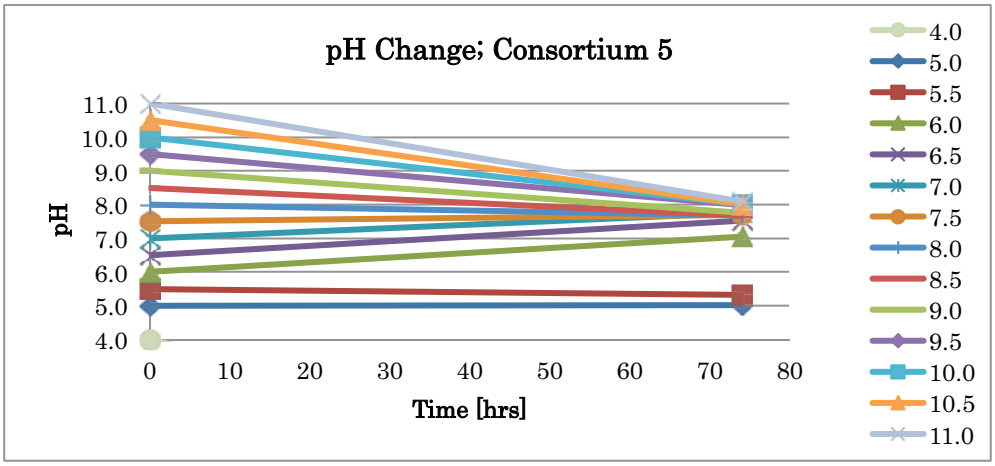

Figure E.8. Change of pH in consortium 5 after 77 hours.

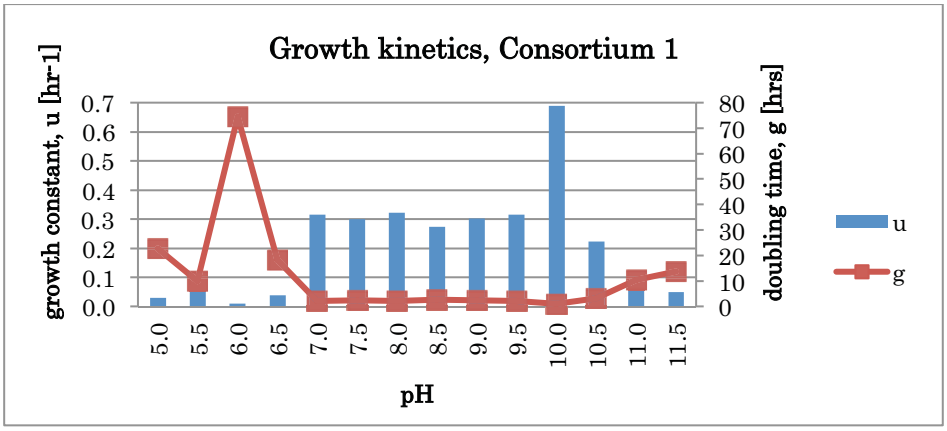

Figure E.9. Growth rate constant, μ, and doubling time, g, for consortium 1 under various pH conditions.

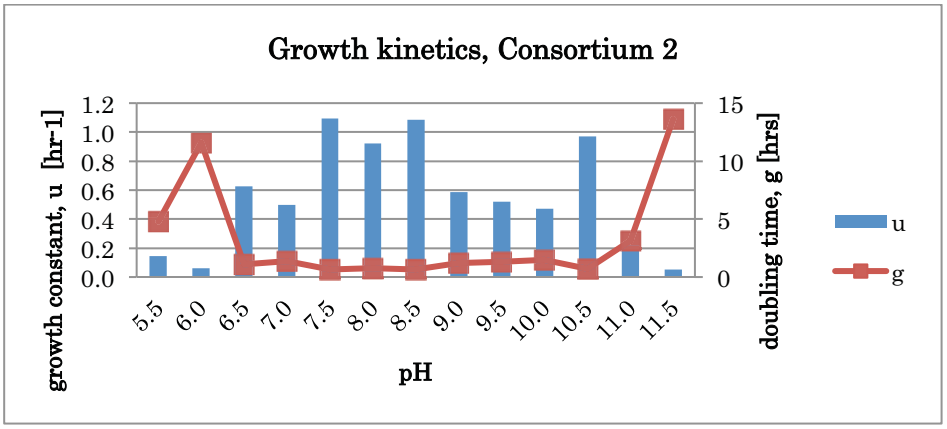

Figure E.10. Growth rate constant, μ, and doubling time, g, for consortium 2 under various pH conditions.

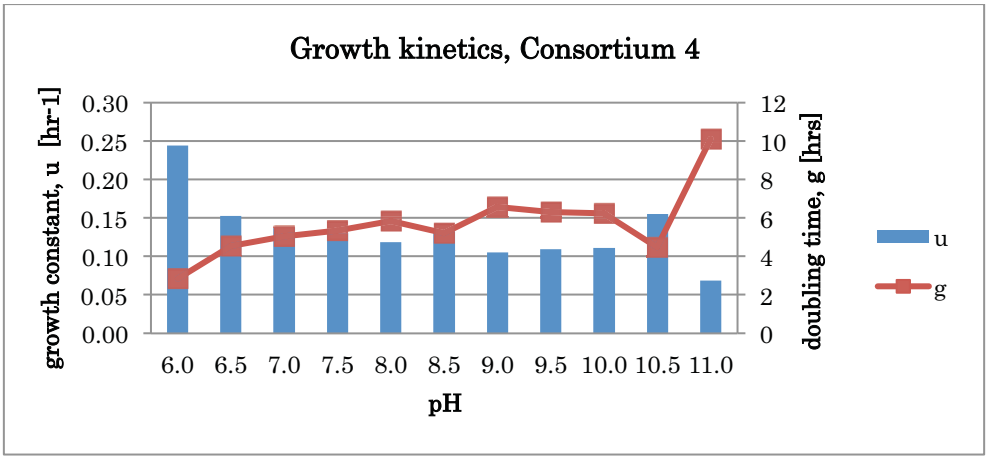

Figure E.11. Growth rate constant, μ, and doubling time, g, for consortium 4 under various pH conditions.

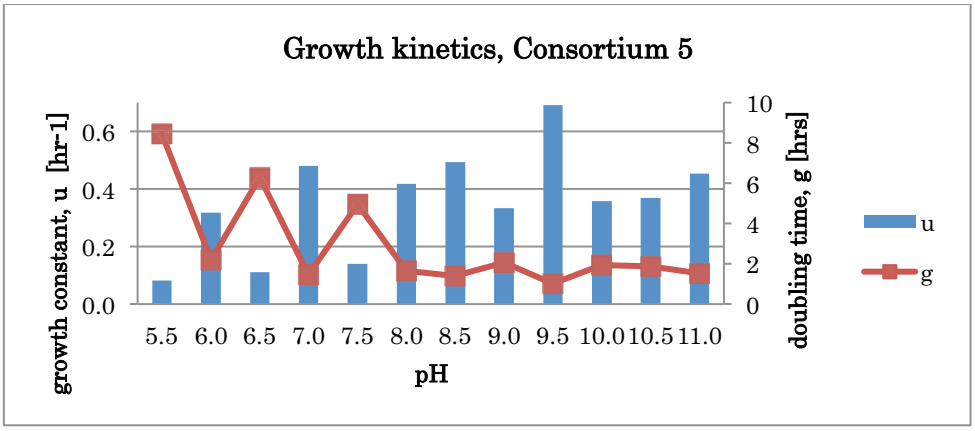

Figure E.12. Growth rate constant, μ, and doubling time, g, for consortium 5 under various pH conditions.

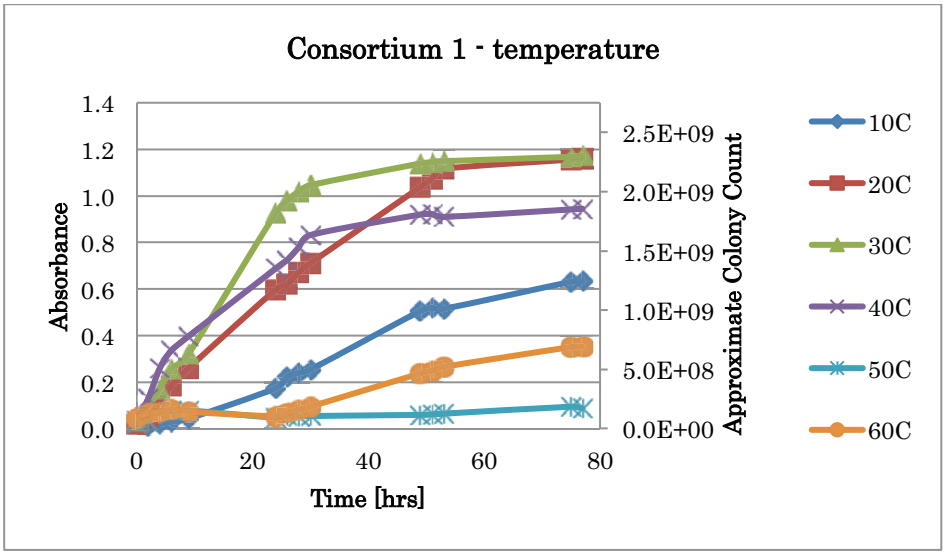

Figure E.13. Consortium 1 growth under temperature ranging between 10-60°C.

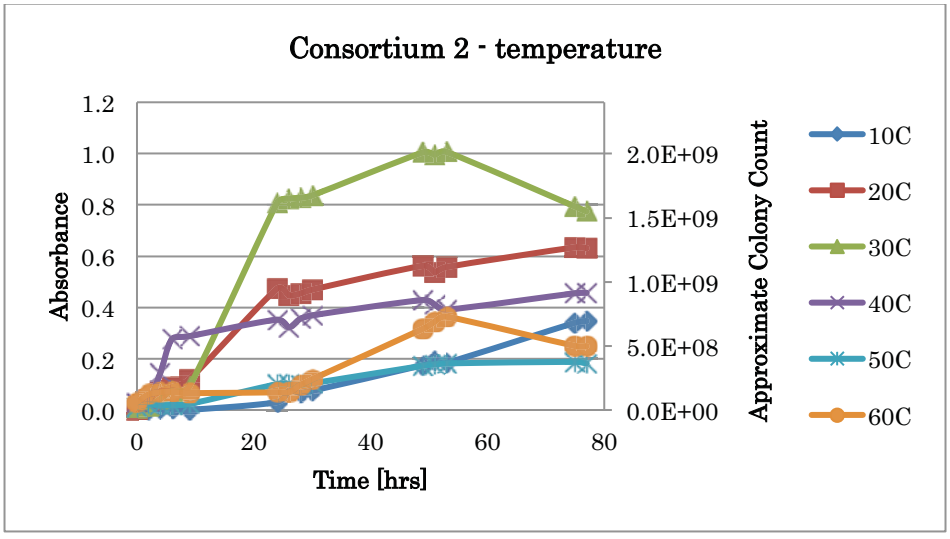

Figure E.14. Consortium 2 growth under temperature ranging between 10-60°C.

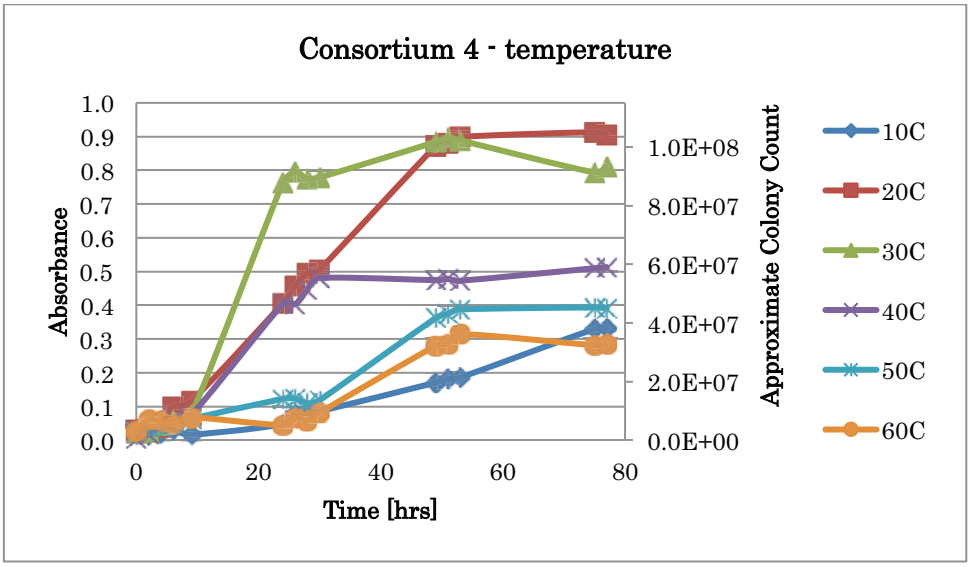

Figure E.15. Consortium 4 growth under temperature ranging between 10-60°C.

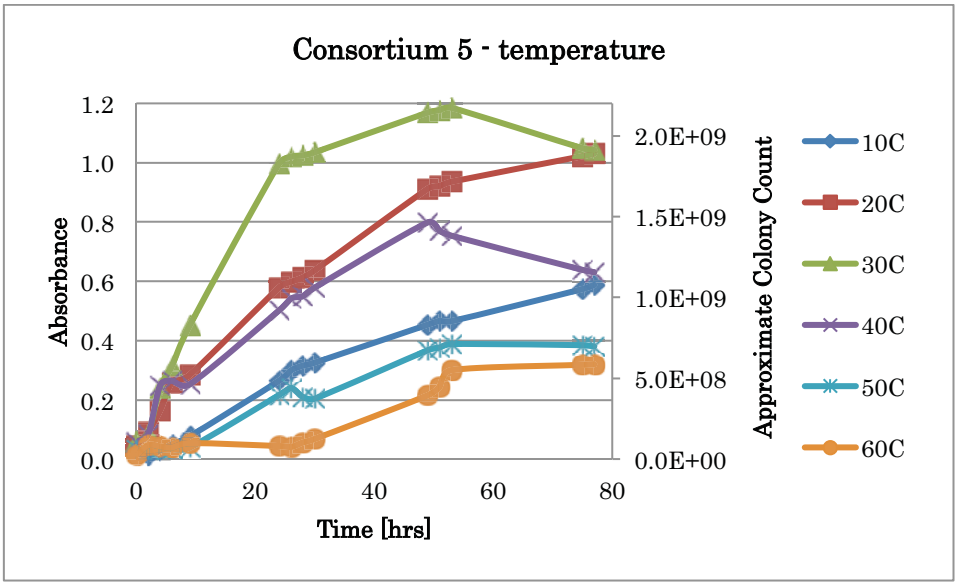

Figure E.16. Consortium 5 growth under temperature ranging between 10-60°C.

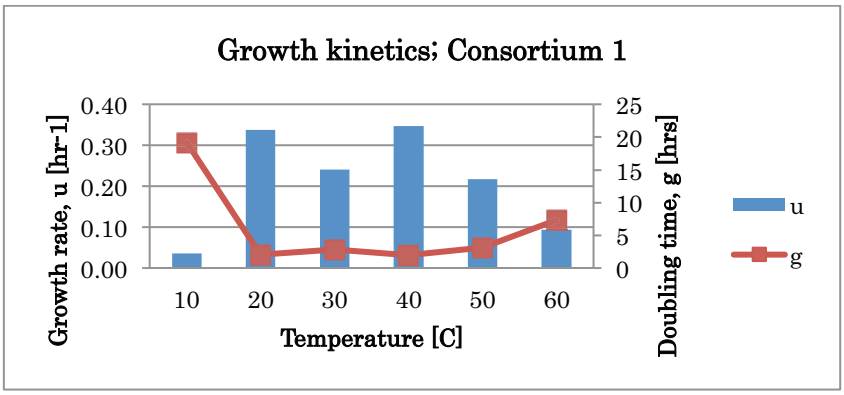

Figure E.17. Growth rate constant, μ, and doubling time, g, for consortium 1 under various temperature conditions.

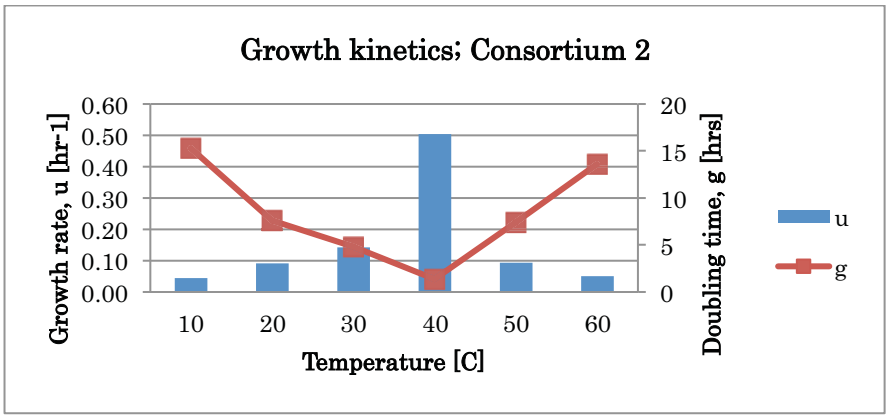

Figure E.18. Growth rate constant, μ, and doubling time, g, for consortium 2 under various temperature conditions.

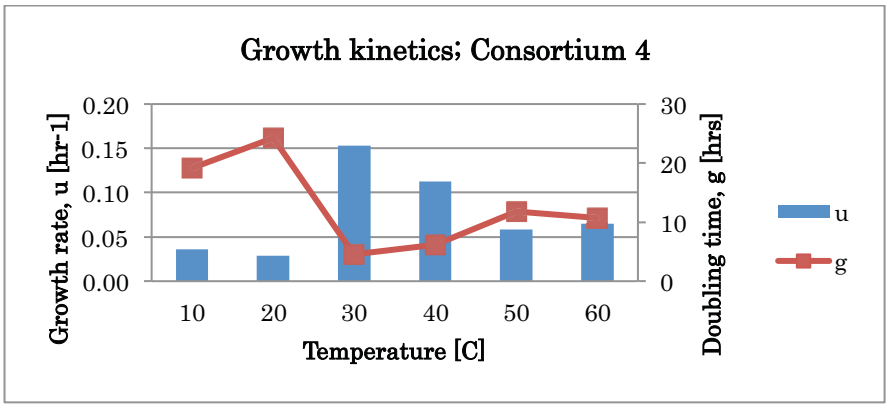

Figure E.19. Growth rate constant, μ, and doubling time, g, for consortium 4 under various temperature conditions.

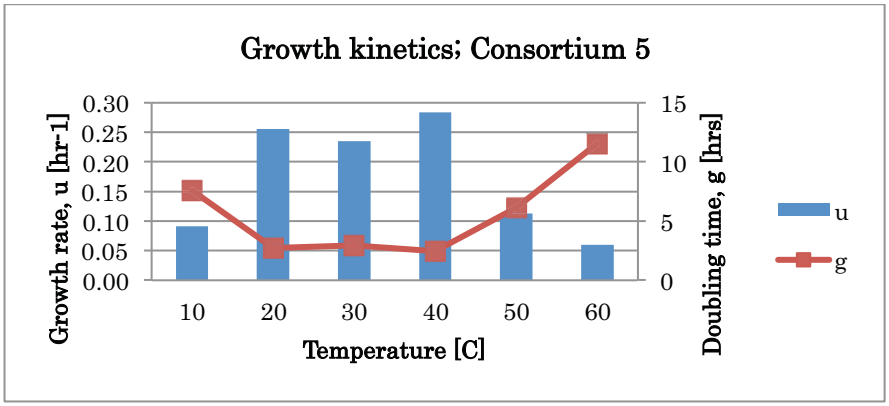

Figure E.20. Growth rate constant, μ, and doubling time, g, for consortium 5 under various temperature conditions.

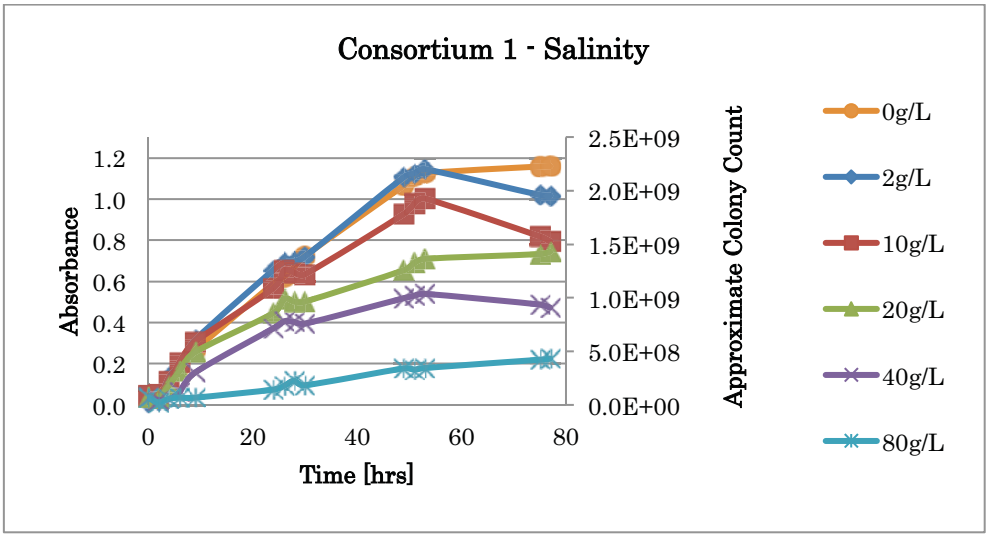

Figure E.21. Growth of consortium 1 under salinity (added as NaCl) ranging between 0-80 g/L.

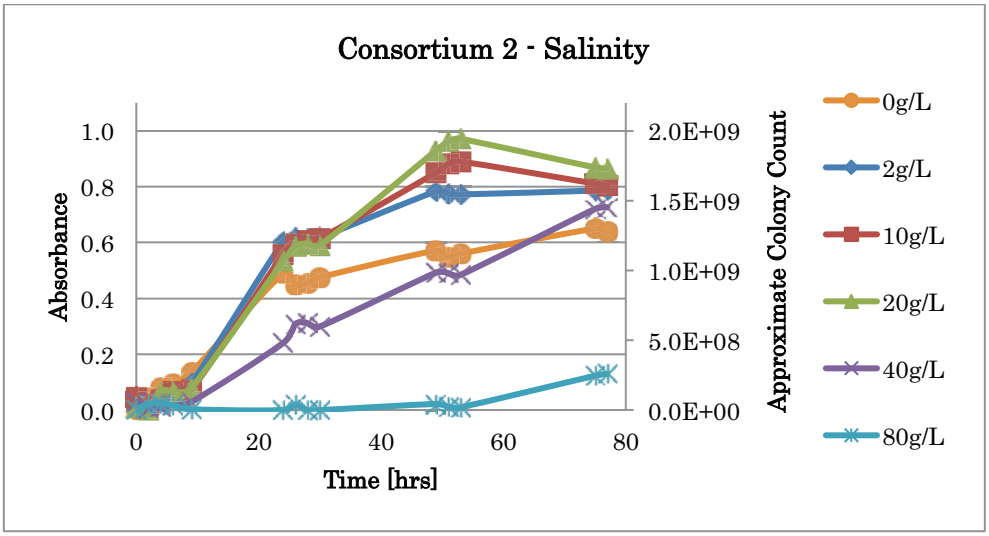

Figure E.22. Growth of consortium 2 under salinity (added as NaCl) ranging between 0-80 g/L.

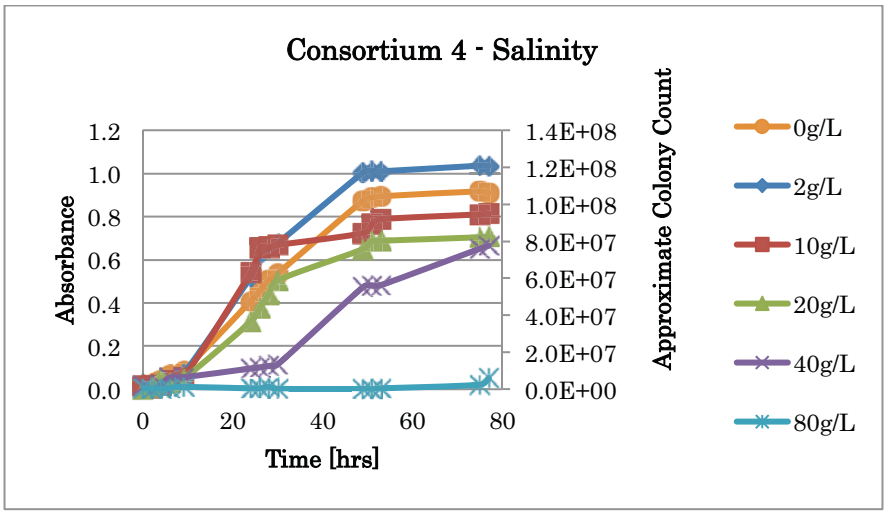

Figure E.23. Growth of consortium 4 under salinity (added as NaCl) ranging between 0-80 g/L.

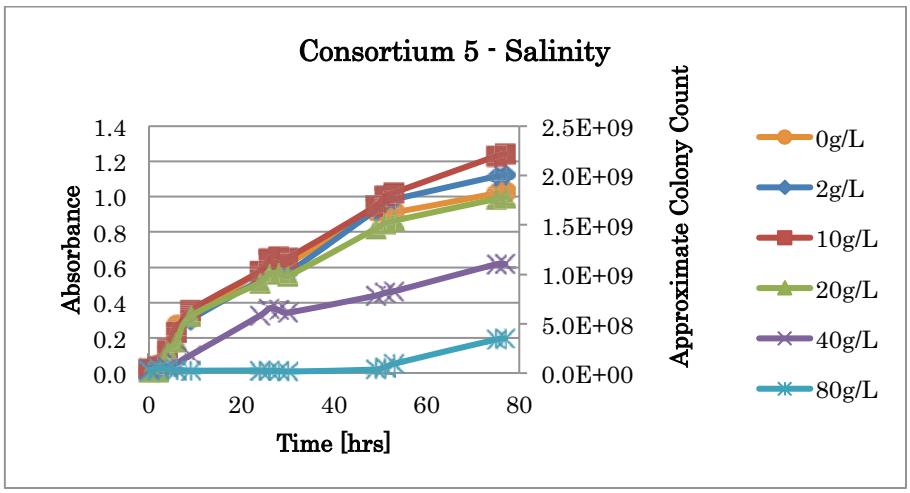

Figure E.24. Growth of consortium 5 under salinity (added as NaCl) ranging between 0-80 g/L.

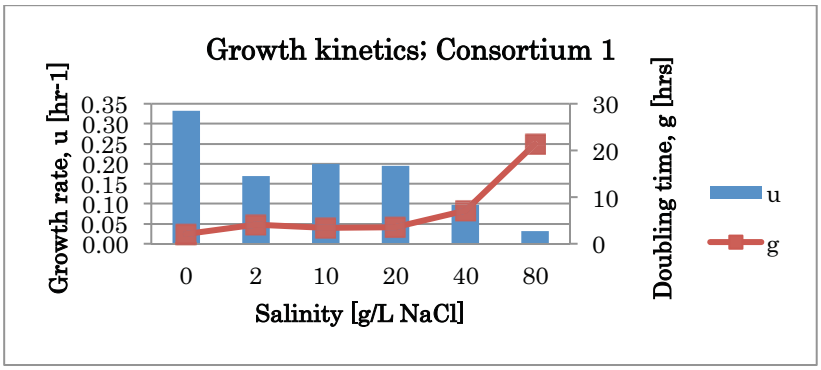

Figure E.25. Growth rate constant, μ, and doubling time, g, for consortium 1 under various salinity conditions.

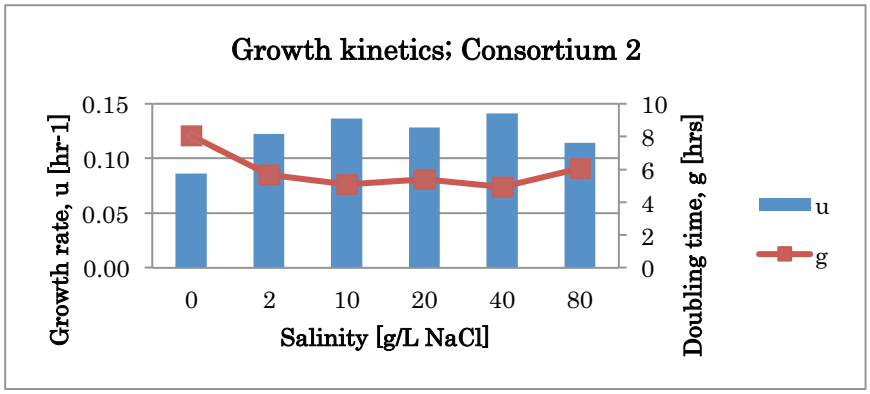

Figure E.26. Growth rate constant, μ, and doubling time, g, for consortium 2 under various salinity conditions.

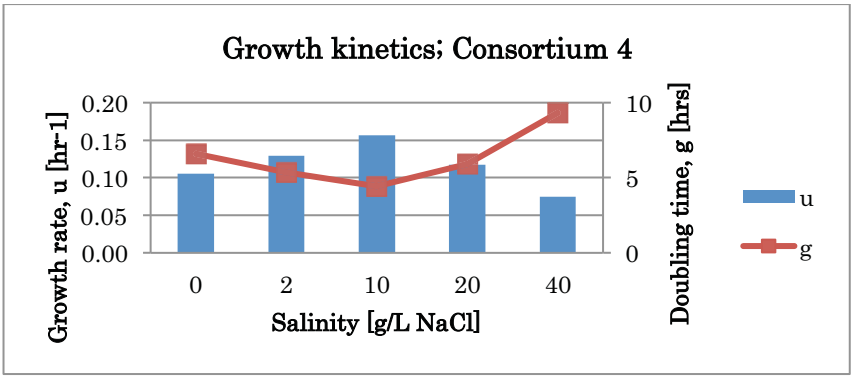

Figure E.27. Growth rate constant, μ, and doubling time, g, for consortium 4 under various salinity conditions.

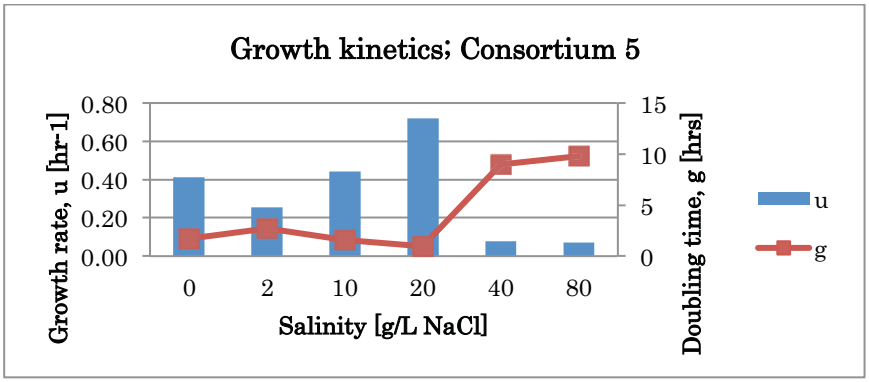

Figure E.28. Growth rate constant, μ, and doubling time, g, for consortium 5 under various salinity conditions.

APPENDIX F

RAMAN SPECTROSCOPY RESULTS

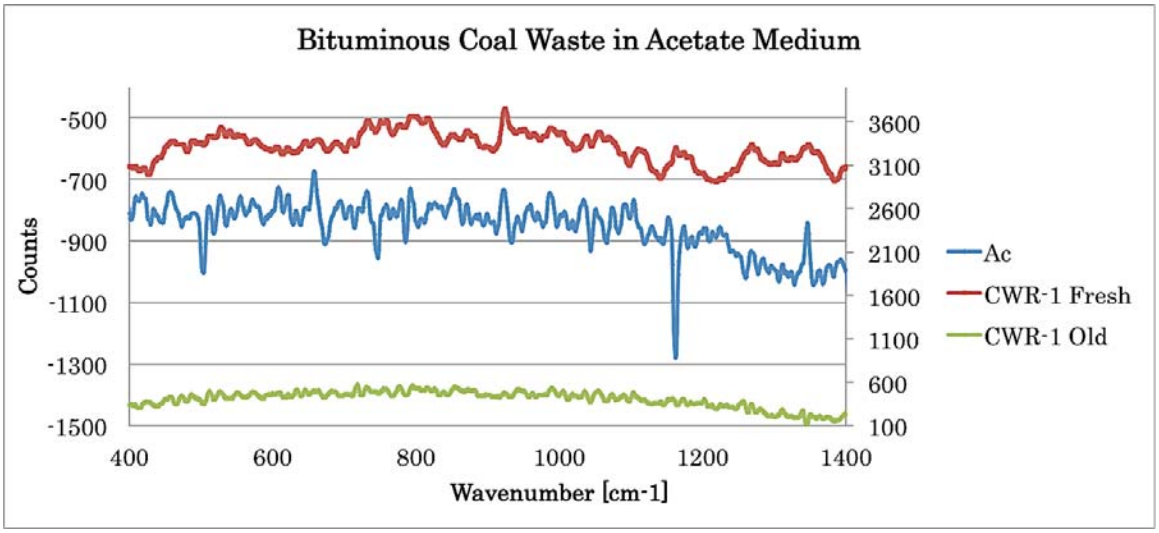

Figure F.1. Spectra of bituminous coal waste sample immersed in acetate medium for 48 hours (CWR-1 Fresh) and six months (CWR-1 Old), and of the acetate medium (Ac).

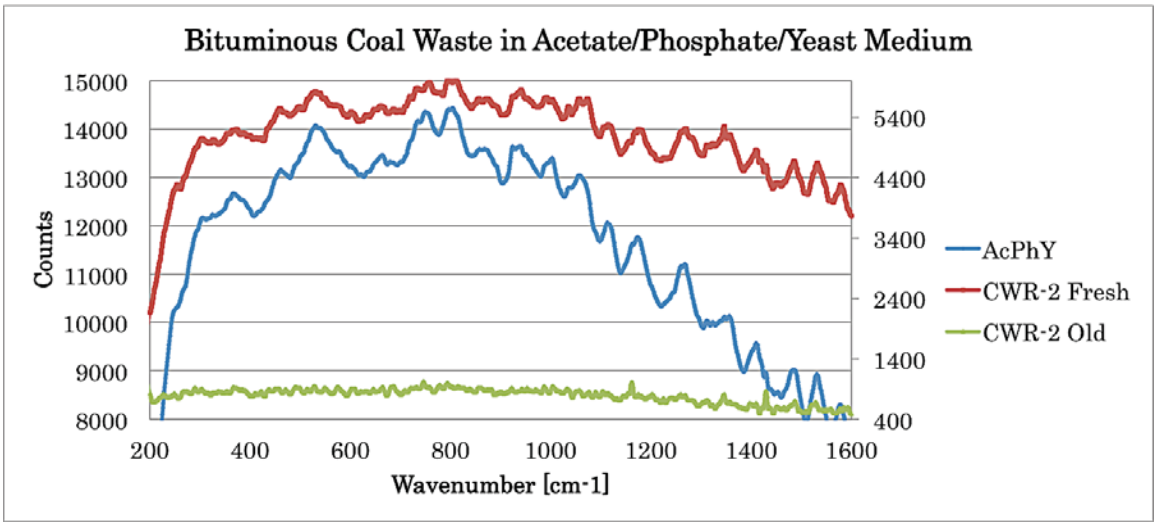

Figure F.2. Spectra of bituminous coal waste sample immersed in acetate, phosphate, yeast medium for 48 hours (CWR-2 Fresh) and six months (CWR-2 Old), and of the acetate, phosphate, yeast medium (AcPhY).

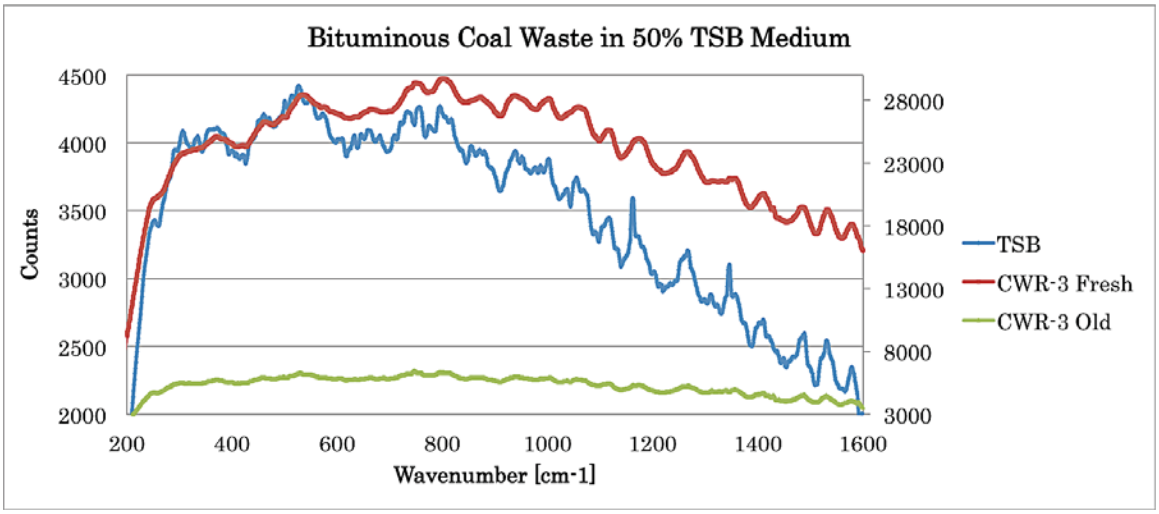

Figure F.3. Spectra of bituminous coal waste sample immersed in TSB medium for 48 hours (CWR-3 Fresh) and six months (CWR-3 Old), and of the TSB medium (TSB).

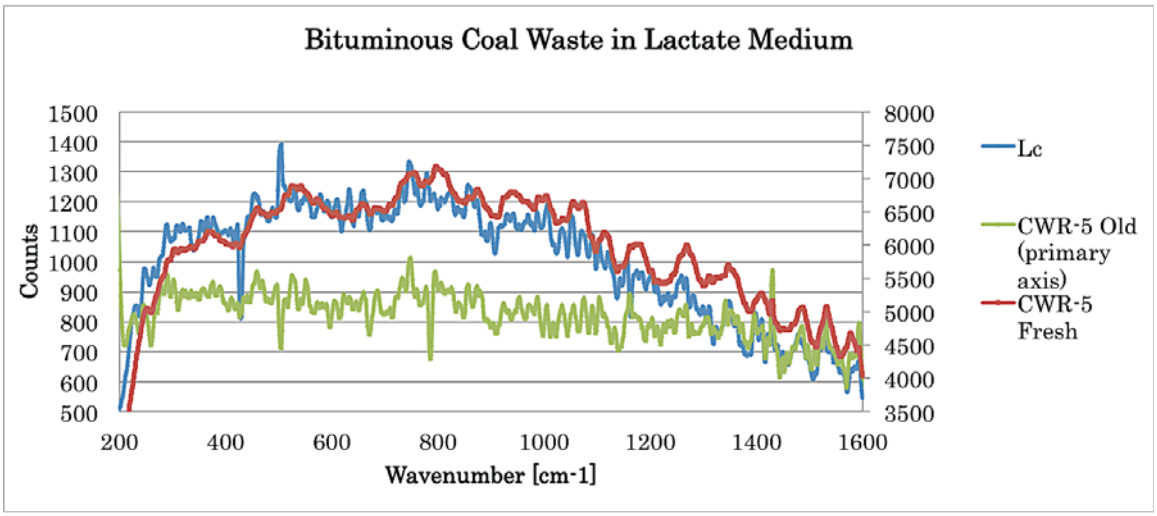

Figure F.4. Spectra of bituminous coal waste sample immersed in lactate medium for 48 hours (CWR-5 Fresh) and six months (CWR-5 Old), and of the lactate medium (Lc).

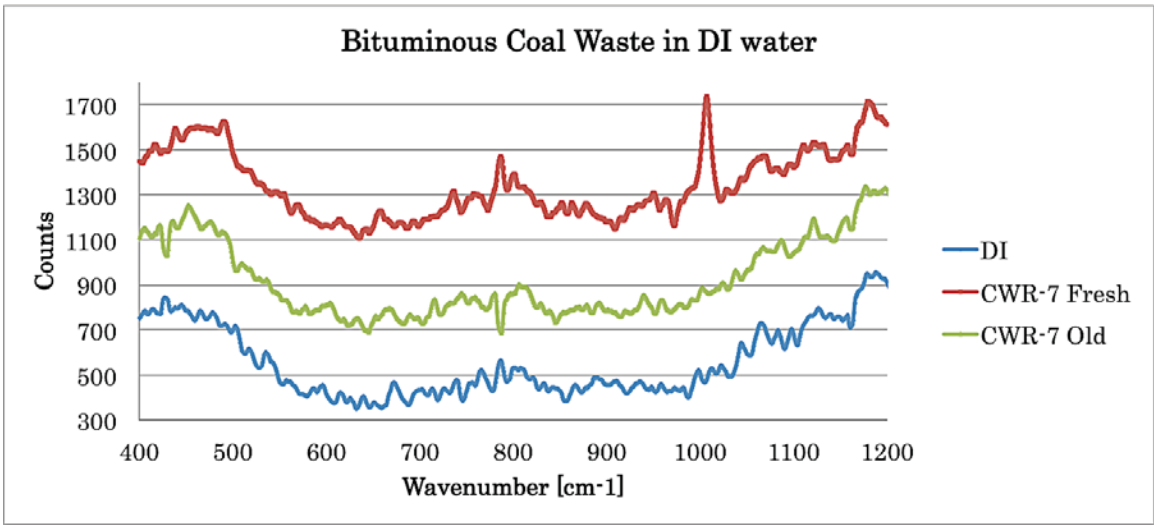

Figure F.5. Spectra of bituminous coal waste sample immersed in DI water for 48 hours (CWR-7 Fresh) and six months (CWR-& Old), and of the DI water (DI).

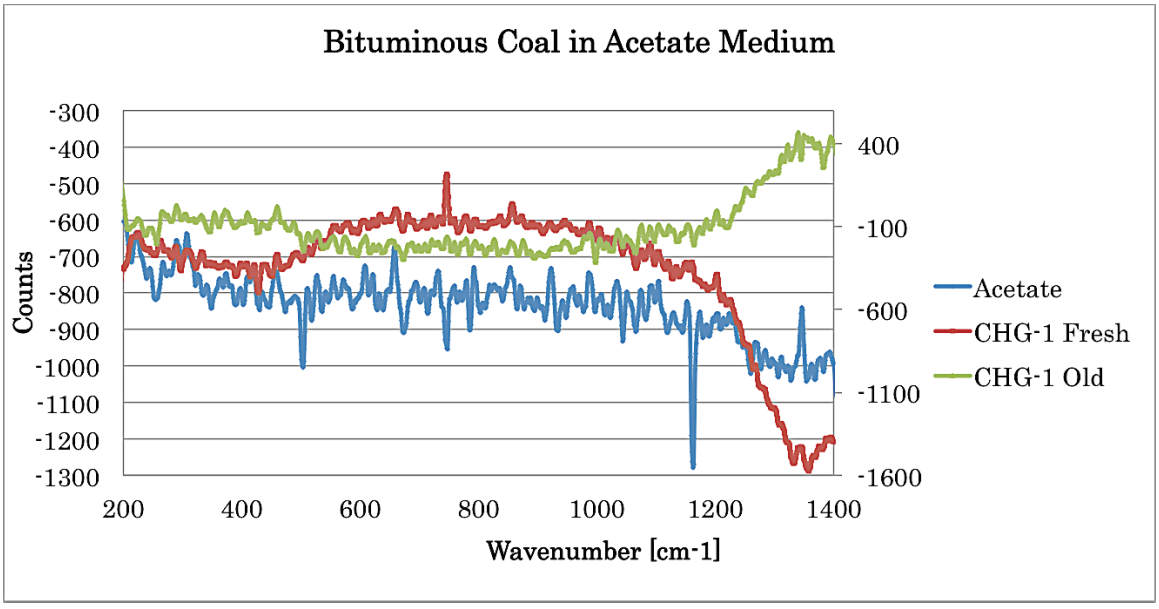

Figure F.6. Spectra of bituminous coal sample immersed in acetate medium for 48 hours (CHG-1 Fresh) and six months (CHG-1 Old), and of the acetate medium (Ac).

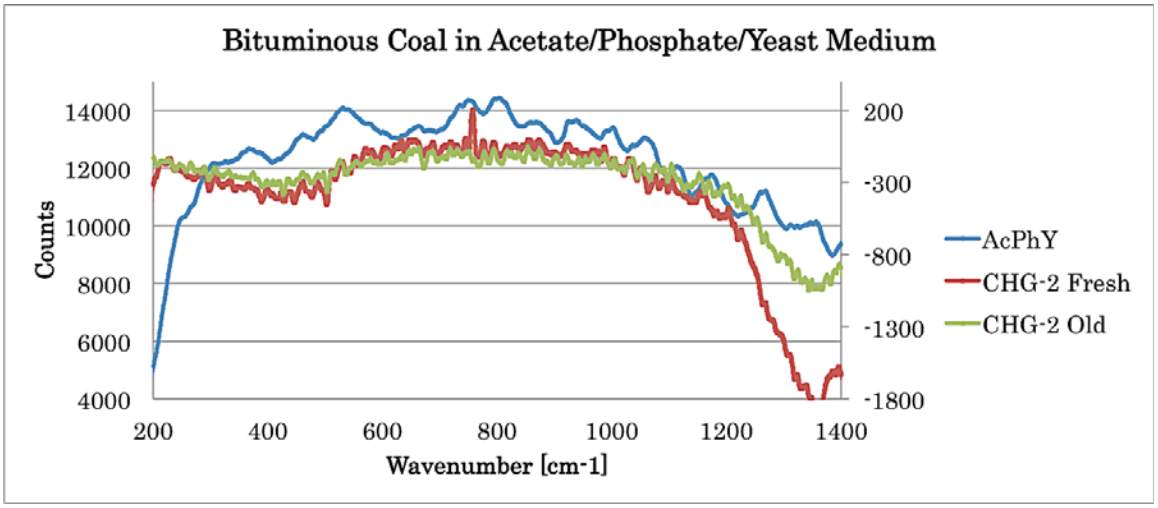

Figure F.7. Spectra of bituminous coal sample immersed in acetate, phosphate, yeast medium for 48 hours (CHG-2 Fresh) and six months (CHG-2 Old), and of the acetate, phosphate, yeast medium (AcPhY).

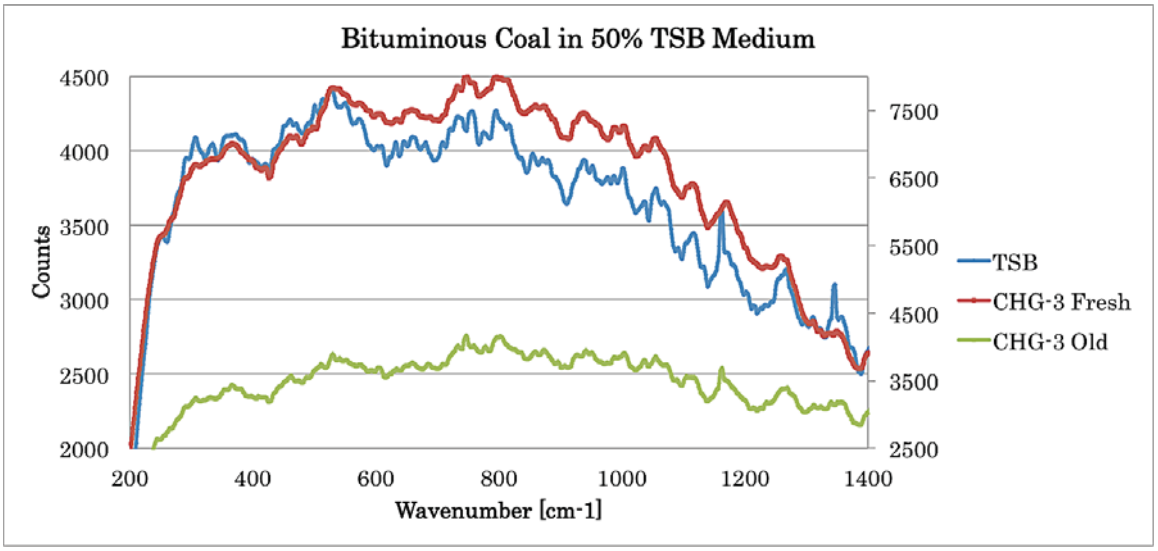

Figure F.8. Spectra of bituminous coal sample immersed in TSB medium for 48 hours (CHG-3 Fresh) and six months (CHG-3 Old), and of the TSB medium (TSB).

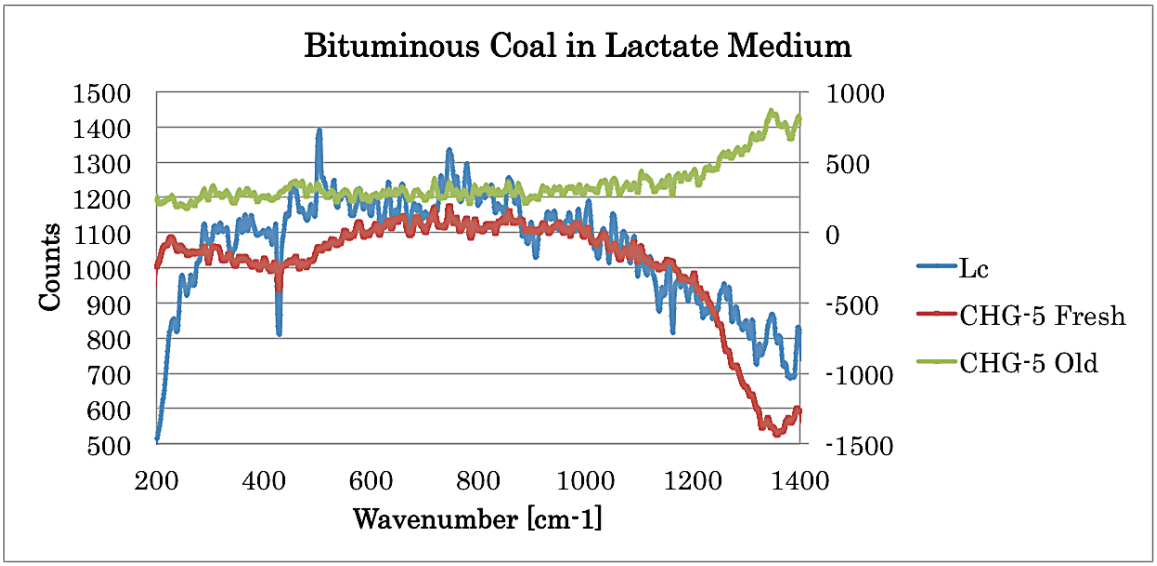

Figure F.9. Spectra of bituminous coal sample immersed in lactate medium for 48 hours (CHG-4 Fresh) and six months (CHG-4 Old), and of the lactate medium (Lc).

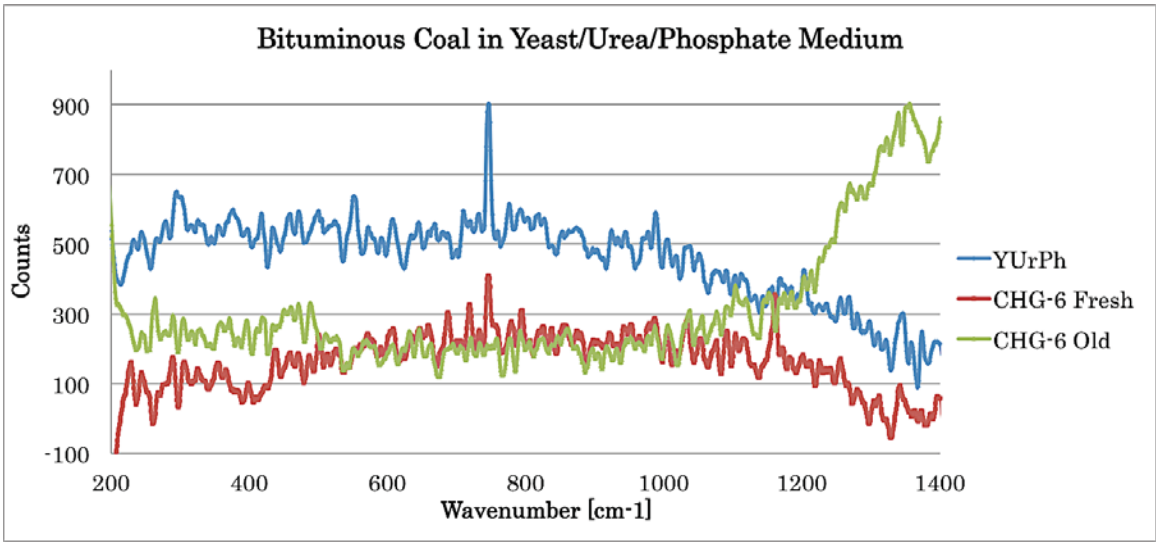

Figure F.10. Spectra of bituminous coal sample immersed in yeast, ura, phosphate medium for 48 hours (CHG-6 Fresh) and six months (CHG-6 Old), and of the yeast, urea, phosphate medium (YUrPh).

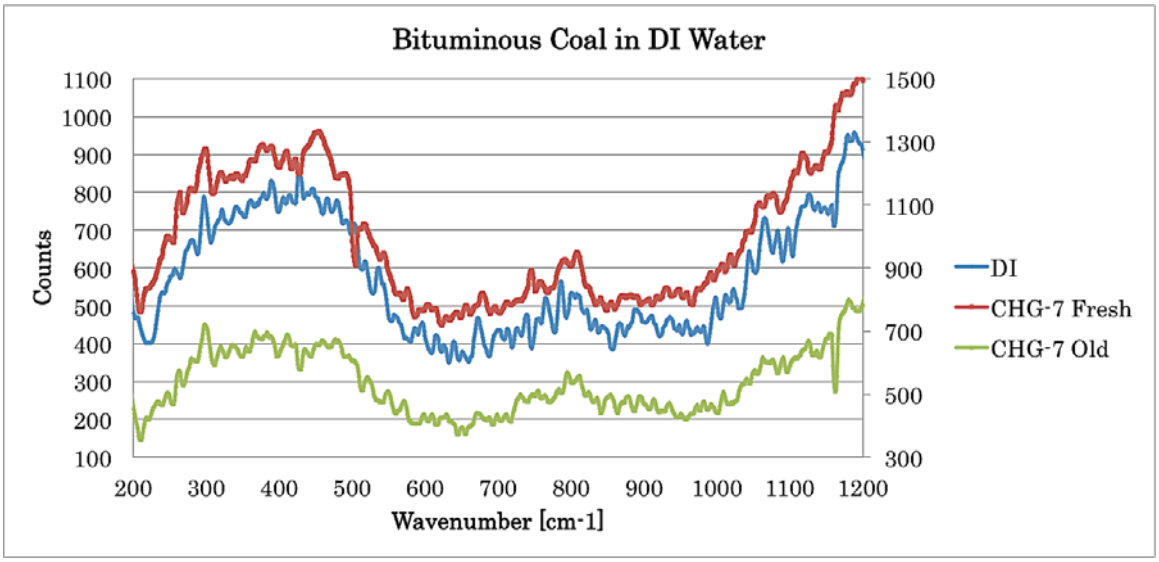

Figure F.11. Spectra of bituminous coal sample immersed in DI water for 48 hours (CHG-7 Fresh) and six months (CHG-7 Old), and of the DI water (DI).

APPENDIX G

MICROBES IN LIQUID MEDIA RESULTS

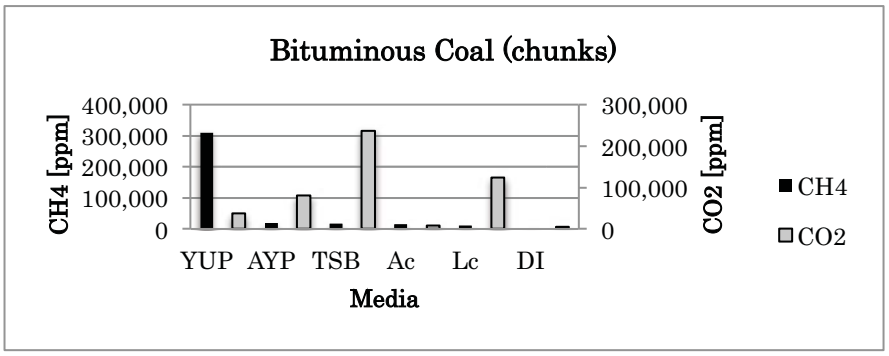

Figure G.1. Methane (black bars) and carbon dioxide (gray bars) generation from the coarse bituminous coal in various media.

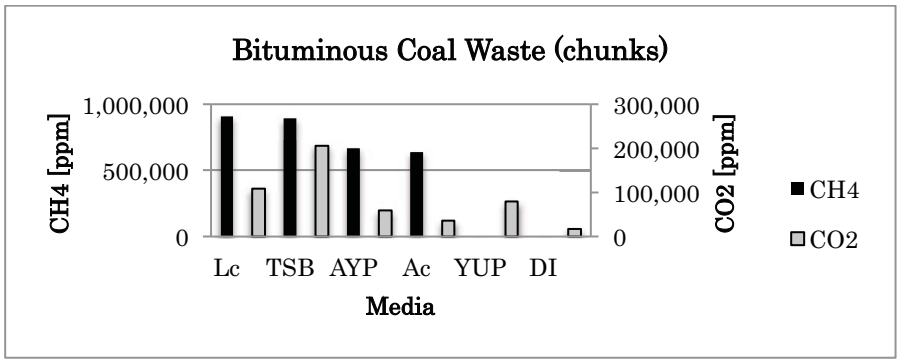

Figure G.2. Methane (black bars) and carbon dioxide (gray bars) generation from the coarse bituminous coal waste in various media.

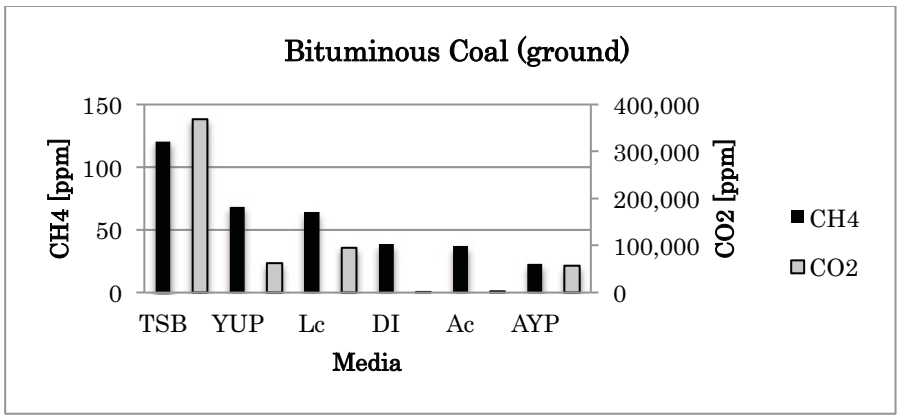

Figure G.3. Methane (black bars) and carbon dioxide (gray bars) generation from the ground bituminous coal in various media.

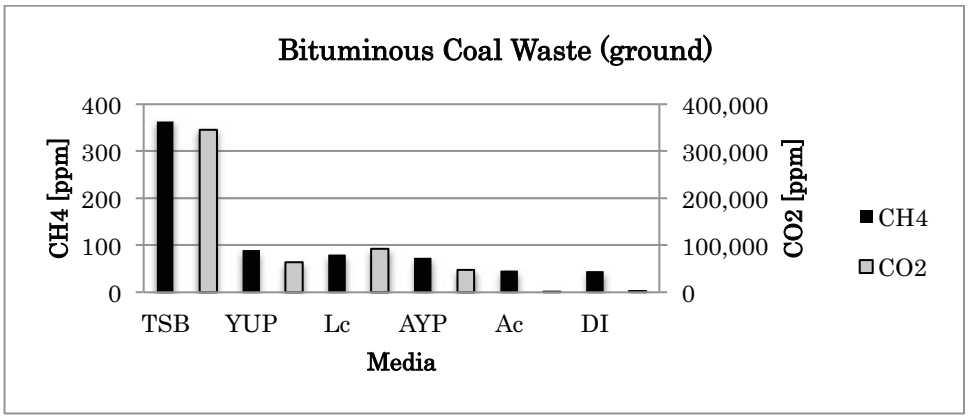

Figure G.4. Methane (black bars) and carbon dioxide (gray bars) generation from the ground bituminous coal waste in various media.

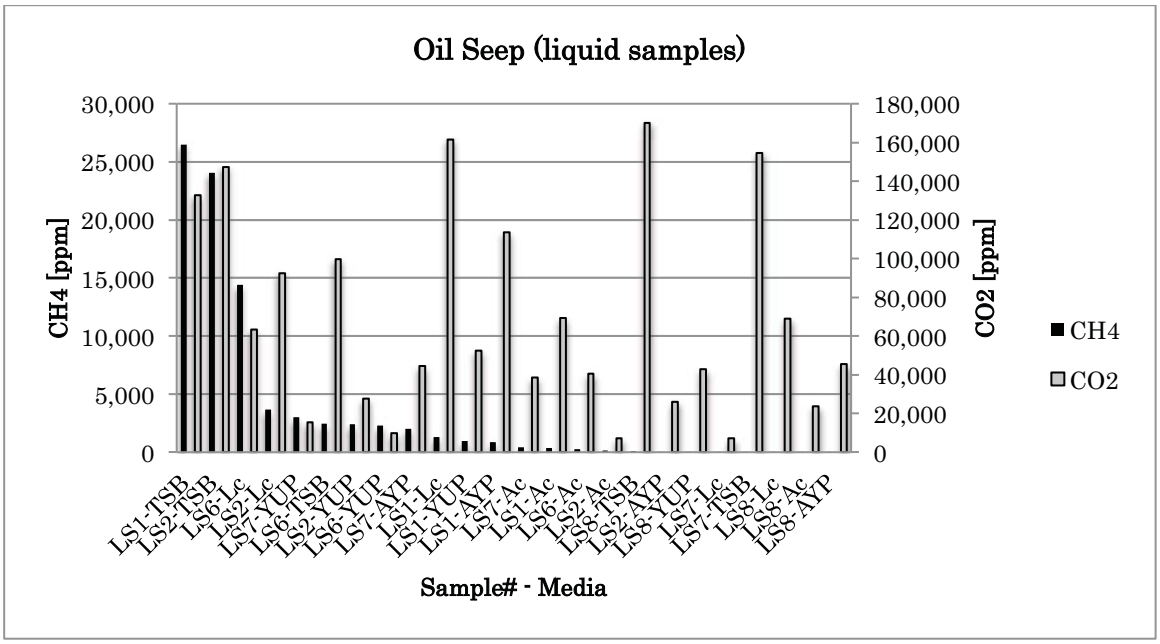

Figure G.5. Methane (black bars) and carbon dioxide (gray bars) generation from the liquid oil seep samples in various media.

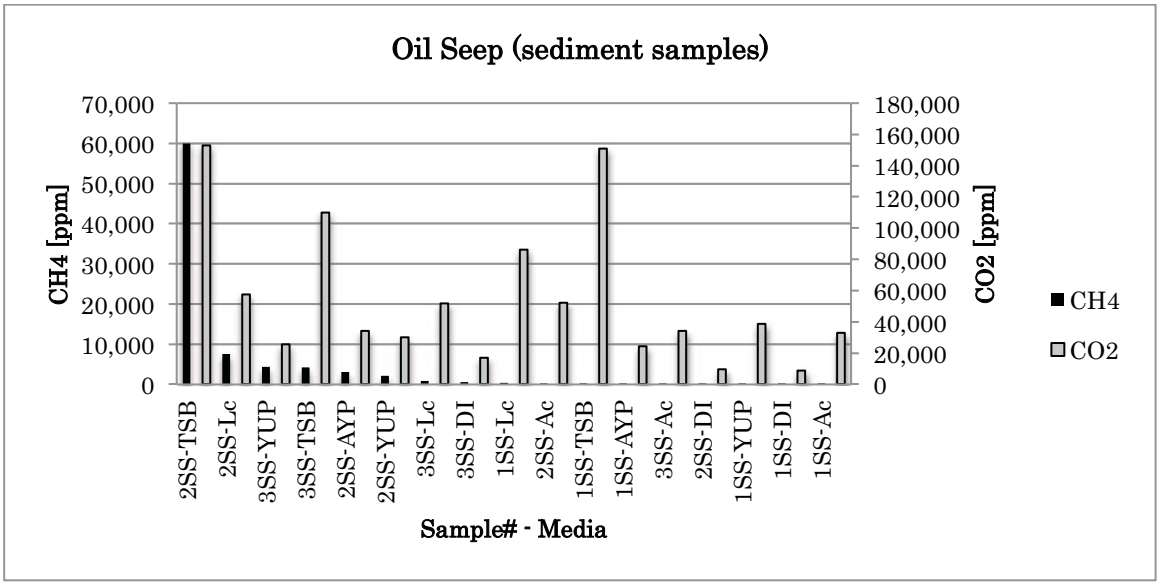

Figure G.6. Methane (black bars) and carbon dioxide (gray bars) generation from the oil seep sediments in various media.

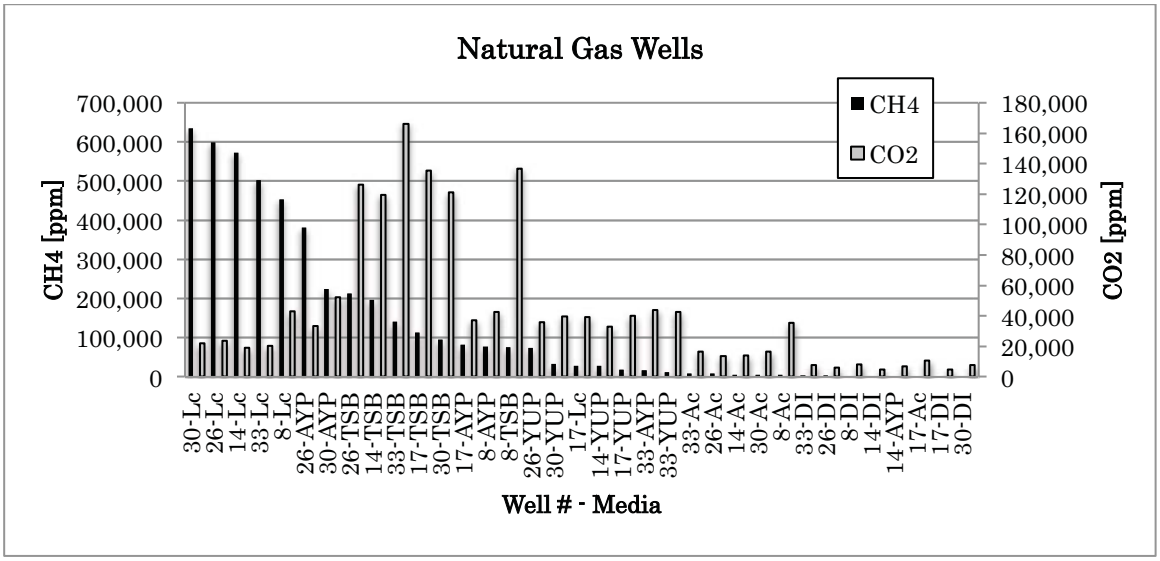

Figure G.7. Methane (black bars) and carbon dioxide (gray bars) generation from the natural gas wells production water in various media.

APPENDIX H

MICROBES IN COAL SAMPLES RESULTS

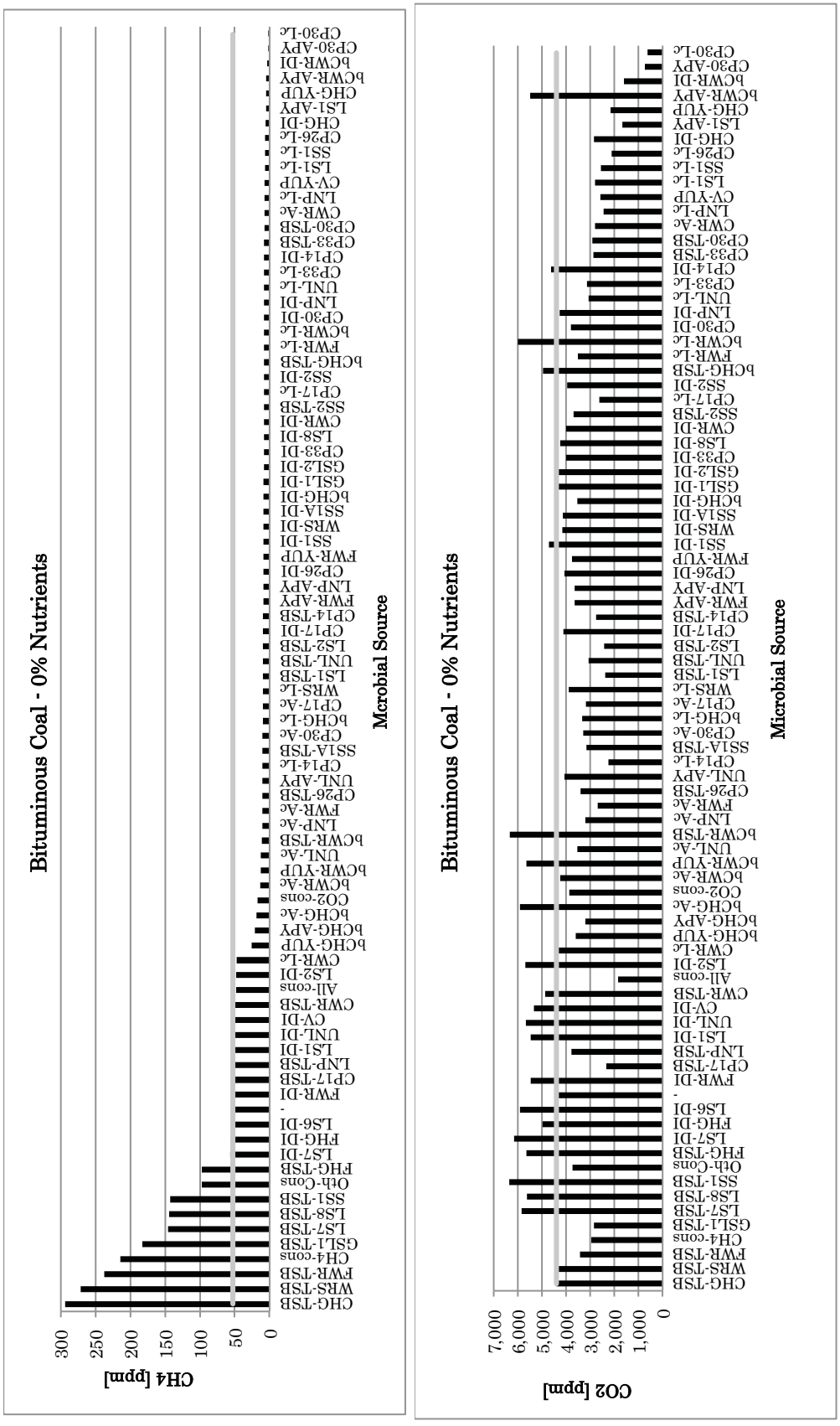

Figure H.1. Methane (upper graph) and carbon dioxide (lower graph) generated from bituminous coal samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with no nutrient amendment. Solid gray line represent the DI control samples.

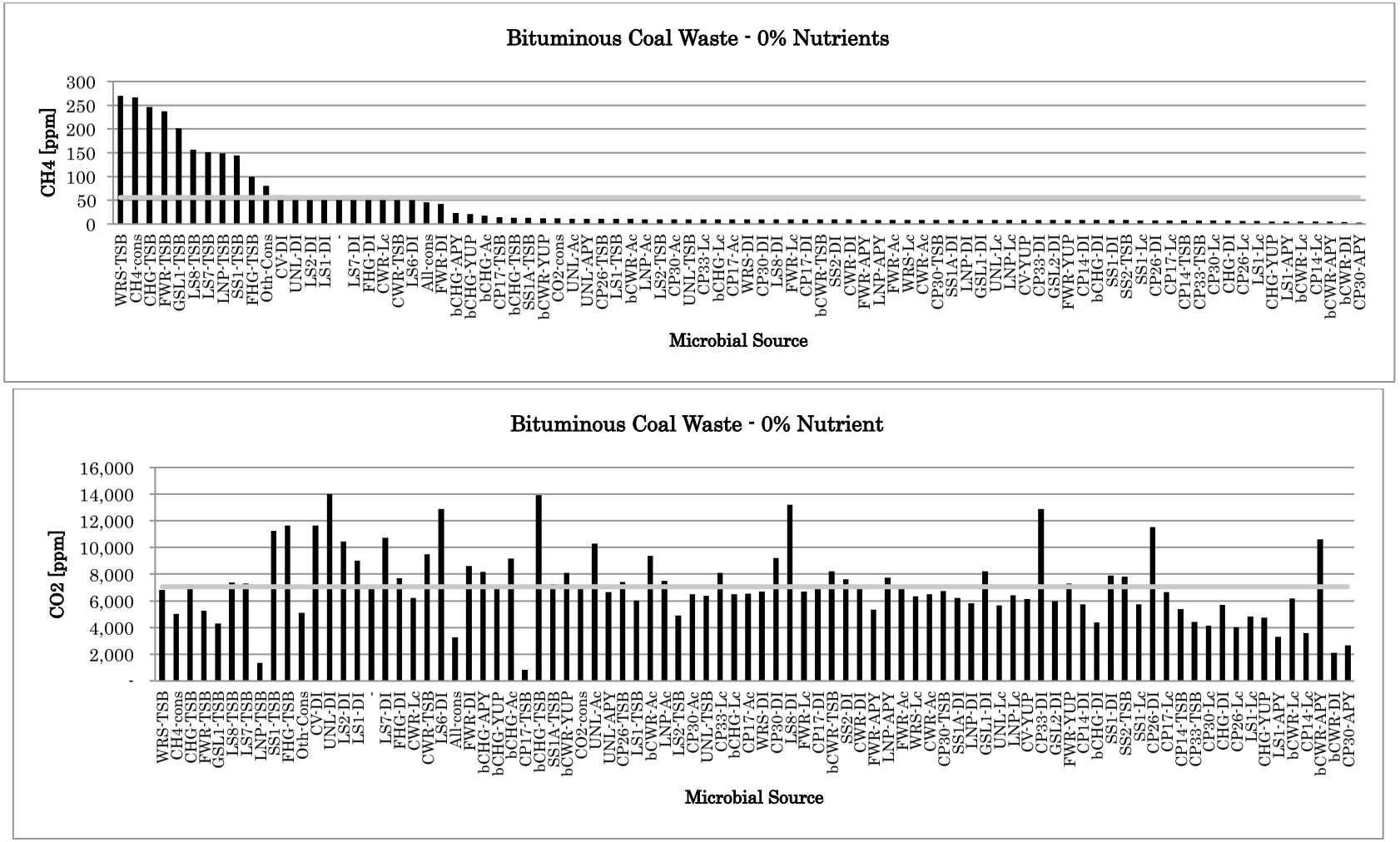

Figure H.2. Methane (upper graph) and carbon dioxide (lower graph) generated from bituminous coal waste samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with no nutrient amendment. Solid gray line represent the DI control samples.

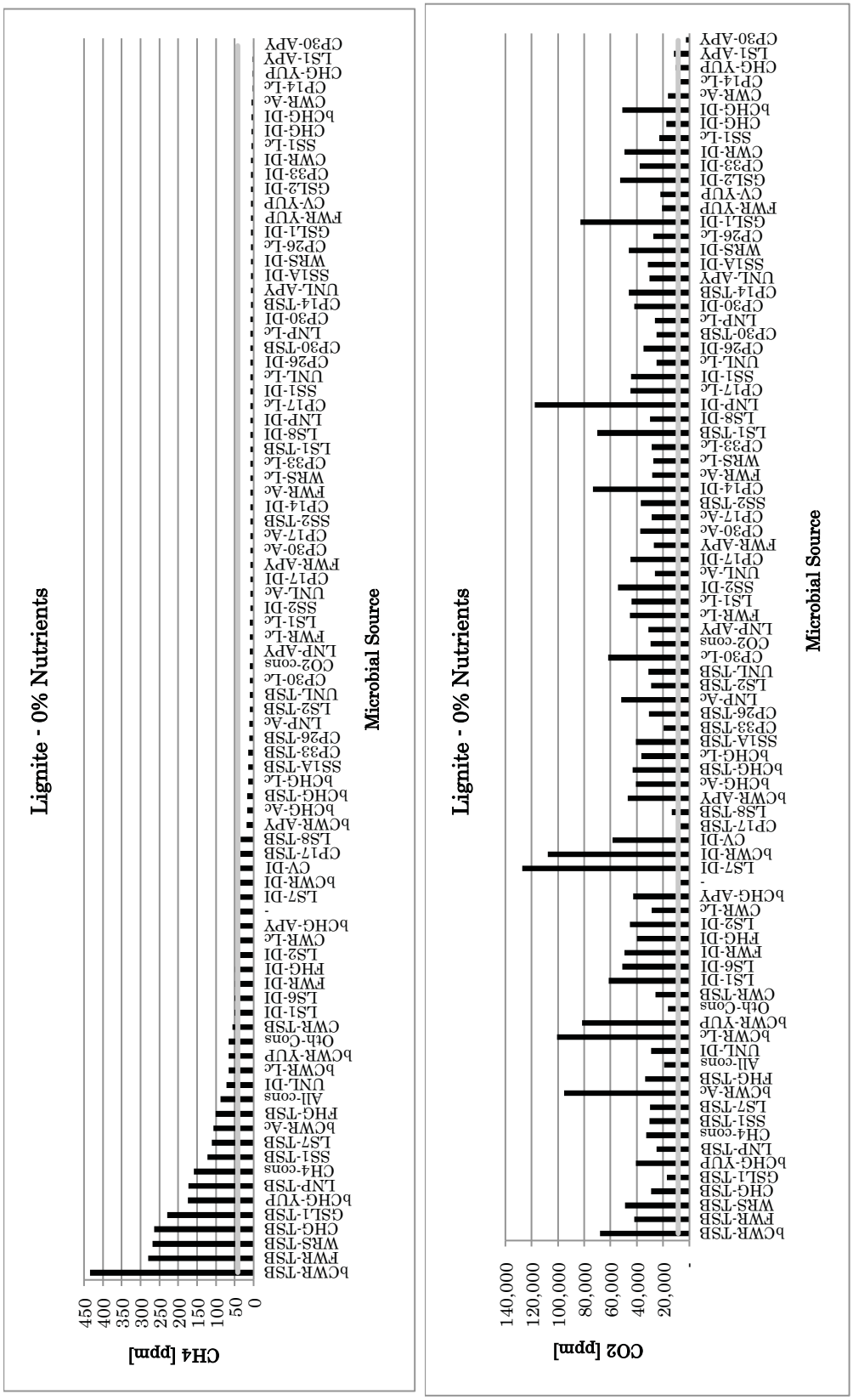

Figure H.3. Methane (upper graph) and carbon dioxide (lower graph) generated from lignite samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with no nutrient amendment. Solid gray line represent the DI control samples.

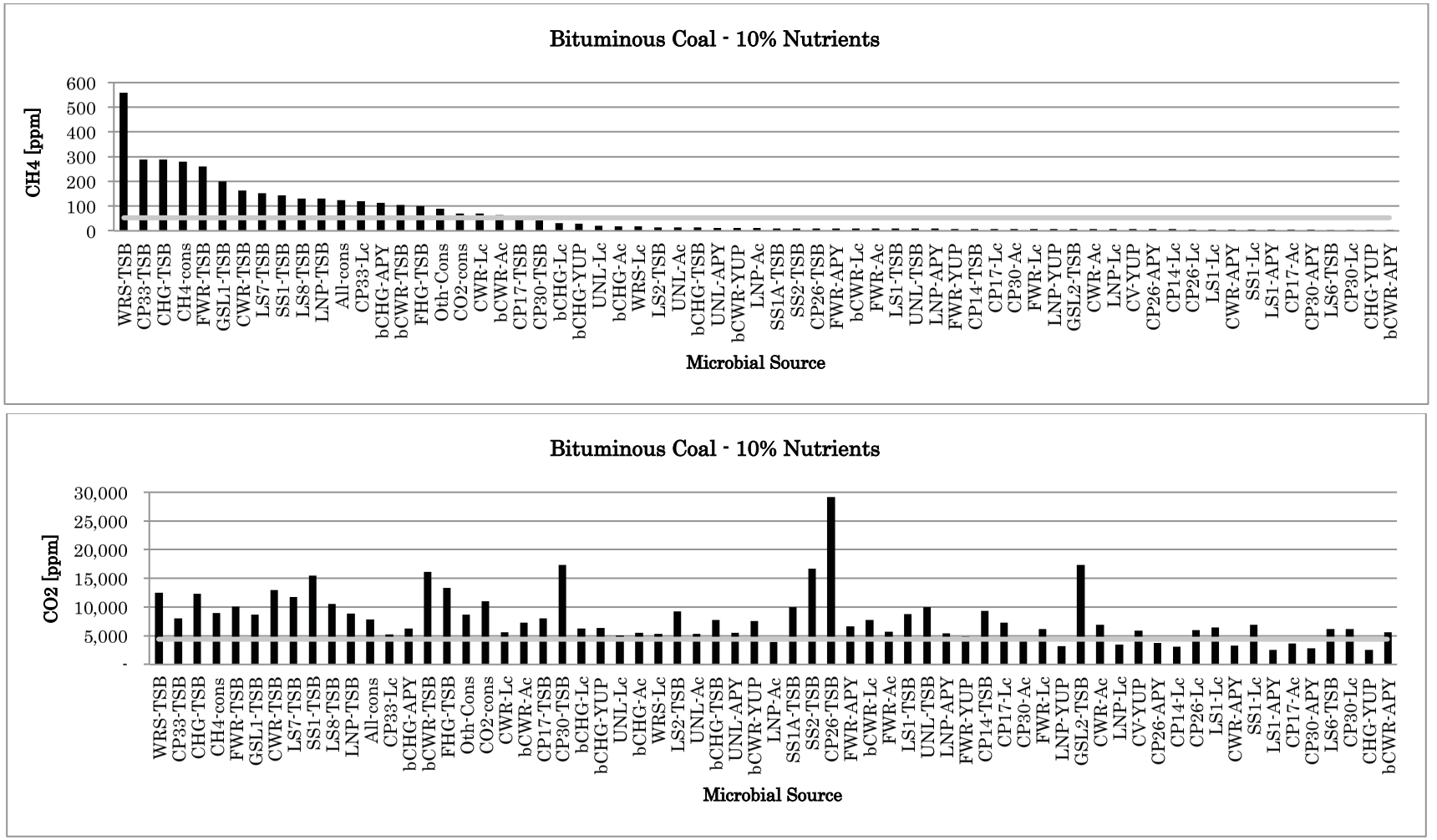

Figure H.4. Methane (upper graph) and carbon dioxide (lower graph) generated from bituminous coal samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with 10% nutrient amendment. Solid gray line represent the DI control samples.

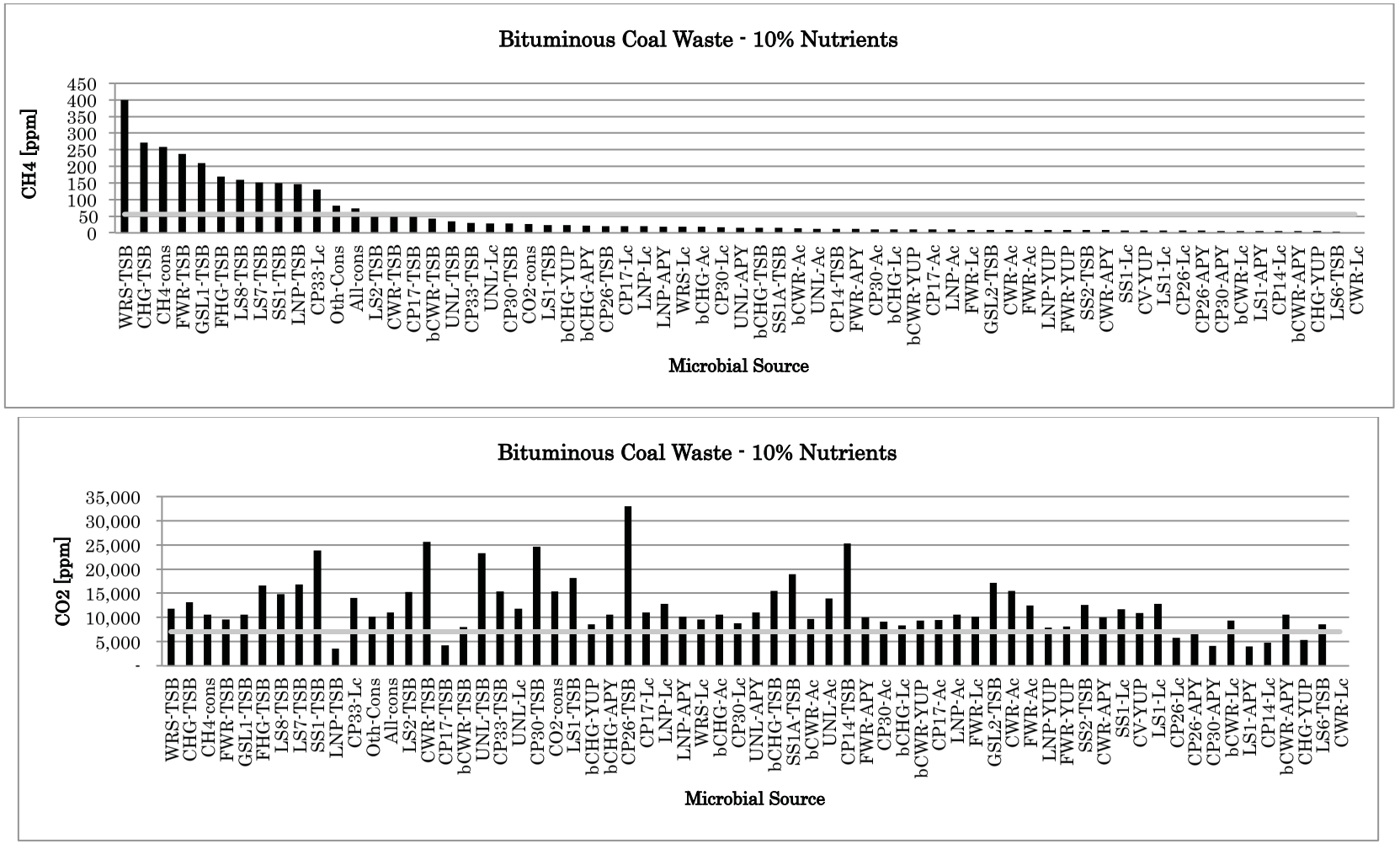

Figure H.5. Methane (upper graph) and carbon dioxide (lower graph) generated from bituminous coal waste samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with 10% nutrient amendment. Solid gray line represent the DI control samples.

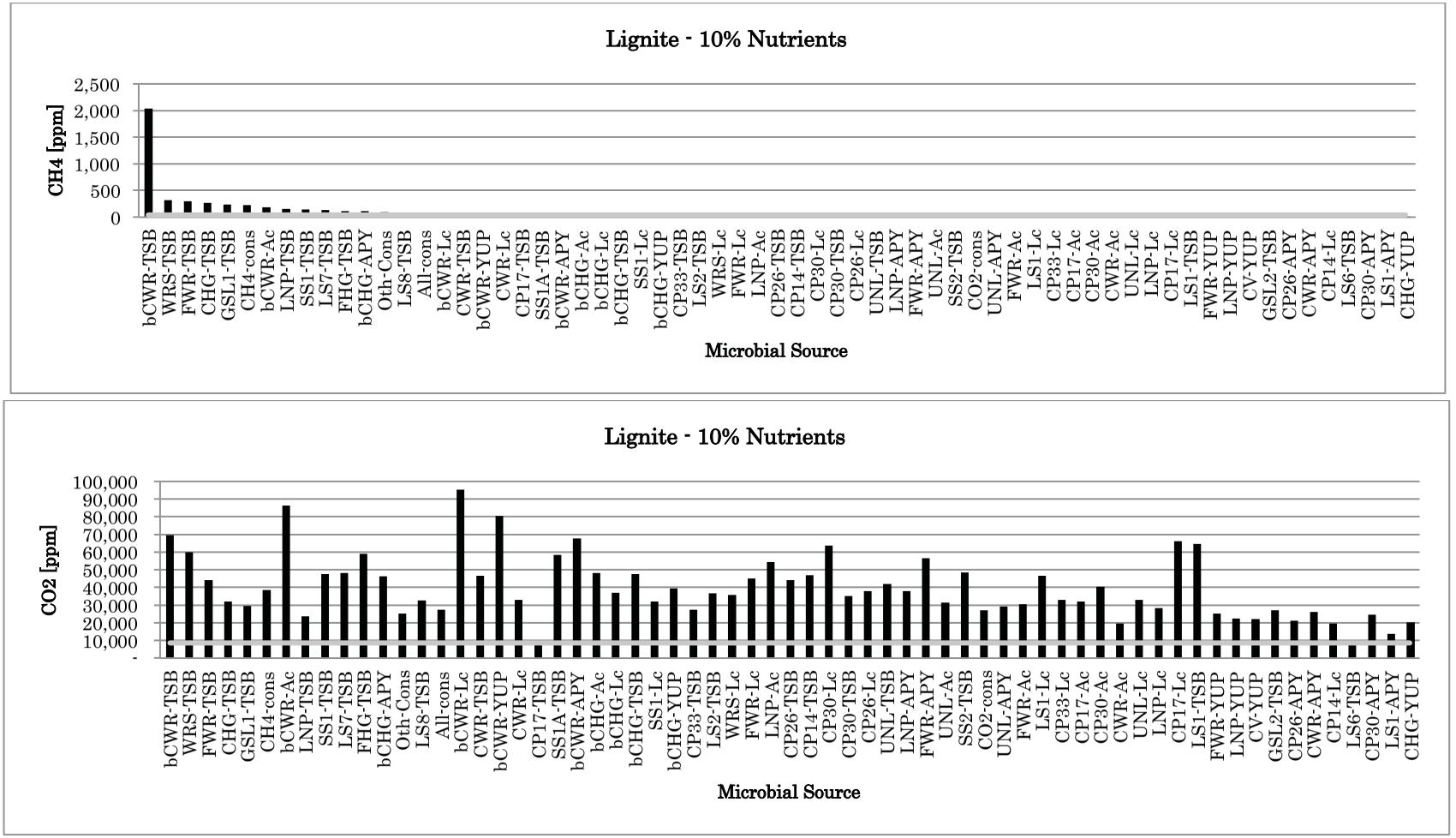

Figure H.6. Methane (upper graph) and carbon dioxide (lower graph) generated from lignite samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with 10% nutrient amendment. Solid gray line represent the DI control samples.

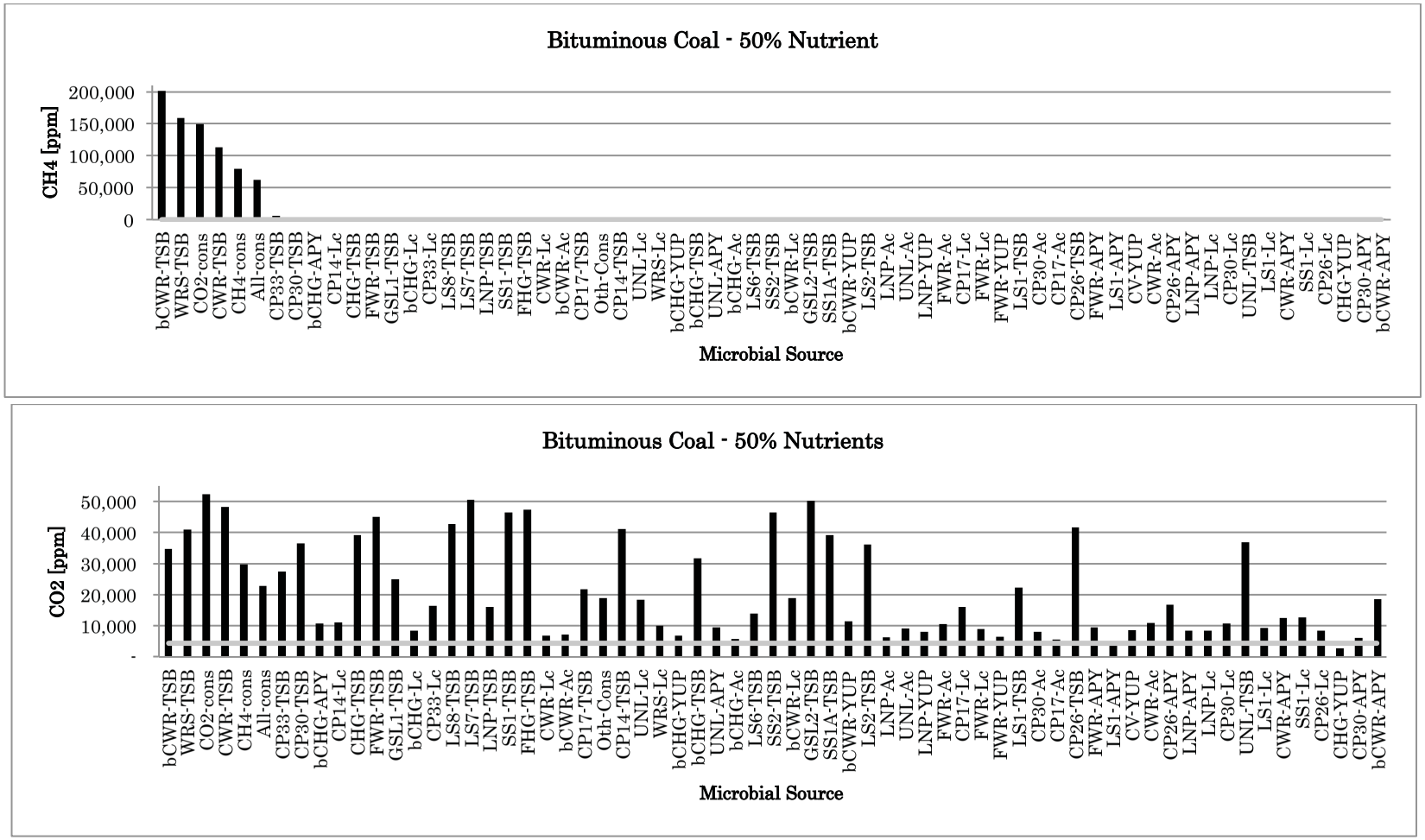

Figure H.7. Methane (upper graph) and carbon dioxide (lower graph) generated from bituminous coal samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with 50% nutrient amendment. Solid gray line represent the DI control samples.

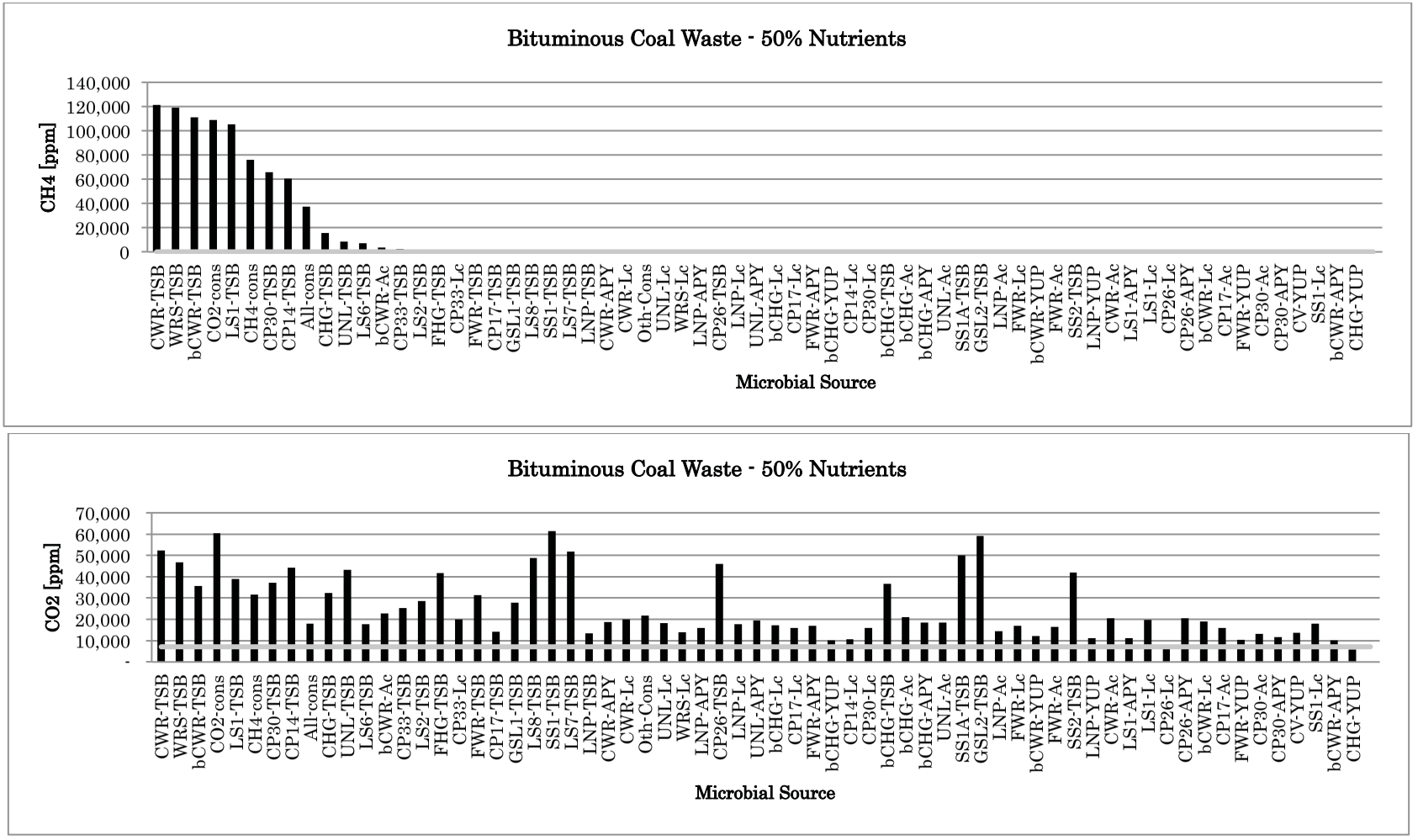

Figure H.8. Methane (upper graph) and carbon dioxide (lower graph) generated from bituminous coal waste samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with 50% nutrient amendment. Solid gray line represent the DI control samples.

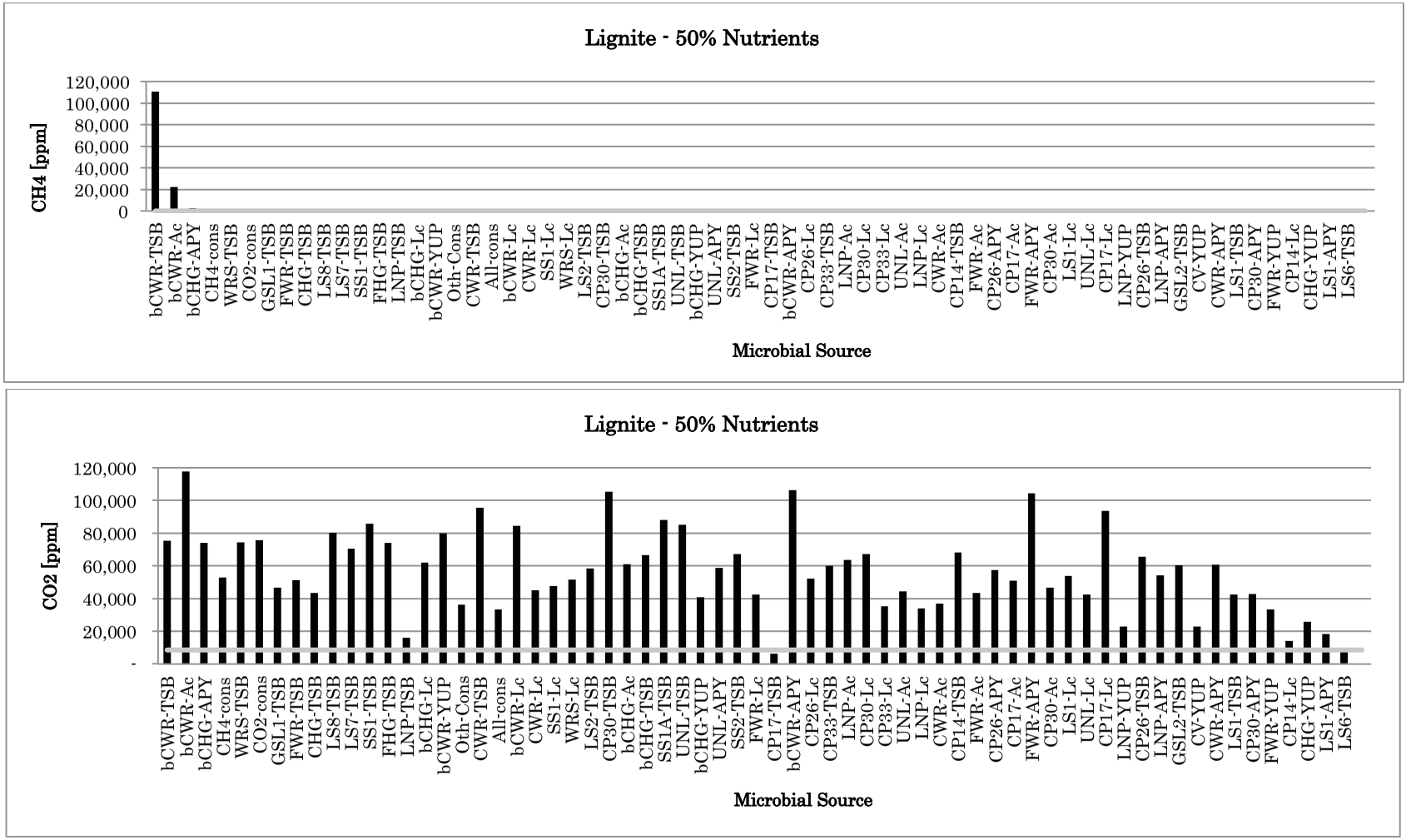

Figure H.9. Methane (upper graph) and carbon dioxide (lower graph) generated from lignite samples inoculated with various microbial consortia (represented by the x-axis) after 30 days incubation period with 50% nutrient amendment. Solid gray line represent the DI control samples.

APPENDIX I

MICROBIAL CONSORTIA IN PRETREATED COAL SAMPLES

Table I.1. Methane and carbon dioxide generation from bituminous coal waste samples immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), hydrogen peroxide (HP) pretreatments and normal saline solution (DI) for 14 and 28 days.

	Chemical – 14 days		Chemical – 28 days	
Pretreatment	CH_4 [ppm]	CO_2 [ppm]	CH_4 [ppm]	CO_2 [ppm]
Ni	8.84	13,927.6	17.70	12,199.9
LA	249.66	111,289.2	155.70	16,449.9
HP	10.99	13,781.2	12.75	8,097.3
DI	7.52	13,981.2	7.32	5,436.4

Table I.2. Methane and carbon dioxide generation from bituminous coal waste samples immersed in normal saline solution and inoculated with four microbial consortia for 14 and 28 days.

	Microbial – 14 days		Microbial – 28 days	
Consortium	CH_4 [ppm]	CO_2 [ppm]	CH_4 [ppm]	CO_2 [ppm]
1	13.15	21,943.2	12.96	17,840.2
3	15.00	20,282.7	14.34	16,080.1
4	12.71	13,488.3	13.77	15,058.7
5	11.96	14,877.4	12.12	15,538.1

Table I.3. Methane and carbon dioxide generation from bituminous coal waste samples immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), hydrogen peroxide (HP) pretreatments and inoculated with four microbial consortia for 14 and 44 days.

	Chemical+Micro – 14 days		Chemical+Micro – 44 days	
Pretreatment	CH_4 [ppm]	CO_2 [ppm]	CH_4 [ppm]	CO_2 [ppm]
Ni-1	11.68	23,143.3	11.85	14,820.0
Ni-3	9.33	29,421.0	12.59	17,474.1
Ni-4	9.92	22,090.9	11.05	14,579.9
Ni-5	12.67	22,689.7	10.18	14,446.9
LA-1	117.29	23,866.6	88.35	53,561.1
LA-3	120.33	39,723.9	298.56	69,635.4
LA-4	93.20	30,943.8	39.03	33,934.4
LA-5	47.28	32,759.8	24.38	17,445.3
HP-1	11.33	20,553.5	10.53	12,048.0
HP-3	12.23	21,119.6	13.76	14,576.3
HP-4	10.62	14,317.9	9.34	11,953.9
HP-5	12.99	19,156.6	12.99	12,477.3

Table I.4. Methane and carbon dioxide generation from bituminous coal waste samples immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), hydrogen peroxide (HP) pretreatments, inoculated with microbial consortium 3, centrifuged and re-suspended in normal saline solution for 14 and 44 days.

	Solids – 14 days		Solids – 44 days	
Pretreatment	**CH_4 [ppm]**	**CO_2 [ppm]**	**CH_4 [ppm]**	**CO_2 [ppm]**
Ni+3	5.24	9,504.9	6.27	9,211.0
LA+3	18.63	21,141.4	93.59	30,306.0
HP+3	6.20	7,927.1	5.44	9,465.9

Table I.5. Methane and carbon dioxide generation from centrifuged bituminous coal waste supernatant immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), and hydrogen peroxide (HP) pretreatments, and inoculated with microbial consortium 3 for 14 and 44 days.

	Liquids – 14 days		Liquids – 44 days	
Pretreatment	**CH_4 [ppm]**	**CO_2 [ppm]**	**CH_4 [ppm]**	**CO_2 [ppm]**
Ni+3	3.35	8,435.8	2.94	6,410.6
LA+3	9.49	15,825.0	116.26	7,676.9
HP+3	4.20	9,049.2	4.03	7,138.1

Table I.6. Methane and carbon dioxide generation from bituminous coal samples immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), hydrogen peroxide (HP) pretreatments and normal saline solution (DI) for 14 and 28 days.

	Chemical – 14 days		Chemical – 28 days	
Pretreatment	**CH_4 [ppm]**	**CO_2 [ppm]**	**CH_4 [ppm]**	**CO_2 [ppm]**
Ni	13.64	11,404.0	17.85	8,961.8
LA	171.08	35,711.0	202.47	14,355.5
HP	11.84	10,555.6	12.98	10,005.1
DI	7.01	3,974.7	10.15	5,757.4

Table I.7. Methane and carbon dioxide generation from bituminous coal samples immersed in normal saline solution and inoculated with four microbial consortia for 14 and 28 days.

	Microbial – 14 days		Microbial – 28 days	
Consortium	CH_4 [ppm]	CO_2 [ppm]	CH_4 [ppm]	CO_2 [ppm]
1	10.02	8,110.9	8.27	10,053.3
3	10.32	8,572.2	10.43	12,141.3
4	10.00	4,383.1	11.49	7,634.6
5	9.47	5,085.9	12.08	10,933.9

Table I.8. Methane and carbon dioxide generation from bituminous coal samples immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), hydrogen peroxide (HP) pretreatments and inoculated with four microbial consortia for 14 and 44 days.

	Chemical+Micro – 14 days		Chemical+Micro – 44 days	
Pretreatment	CH_4 [ppm]	CO_2 [ppm]	CH_4 [ppm]	CO_2 [ppm]
Ni-1	13.87	21,206.3	9.39	9,177.2
Ni-3	9.72	18,305.1	9.04	8,313.9
Ni-4	16.81	17,397.8	9.14	8,716.8
Ni-5	20.11	20,768.2	11.90	11,008.6
LA-1	147.90	20,024.7	53.67	45,160.2
LA-3	203.14	20,103.6	112.16	6,918.9
LA-4	171.63	13,696.4	124.87	6,041.7
LA-5	86.77	53,729.2	32.03	26,837.5
HP-1	18.87	20,109.7	12.20	8,102.8
HP-3	19.46	22,307.1	14.11	9,756.3
HP-4	18.51	14,297.6	11.77	7,759.5
HP-5	16.63	14,112.5	12.51	7,587.7

Table I.9. Methane and carbon dioxide generation from bituminous coal samples immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), hydrogen peroxide (HP) pretreatments, inoculated with microbial consortium 3, centrifuged and re-suspended in normal saline solution for 14 and 44 days.

	Solids – 14 days		Solids – 44 days	
Pretreatment	**CH_4 [ppm]**	**CO_2 [ppm]**	**CH_4 [ppm]**	**CO_2 [ppm]**
Ni+3	6.76	8,710.2	5.81	6,922.1
LA+3	100.60	34,583.8	714.30	26,815.3
HP+3	7.35	11,554.3	6.84	10,312.1

Table I.10. Methane and carbon dioxide generation from centrifuged bituminous coal supernatant immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), and hydrogen peroxide (HP) pretreatments, and inoculated with microbial consortium 3 for 14 and 44 days.

	Liquids – 14 days		Liquids – 44 days	
Pretreatment	**CH_4 [ppm]**	**CO_2 [ppm]**	**CH_4 [ppm]**	**CO_2 [ppm]**
Ni+3	3.08	9,547.7	3.50	7,409.5
LA+3	69.76	37,106.8	5,367.6	44,877.7
HP+3	3.91	15,770.2	4.25	10,626.3

Table I.11. Methane and carbon dioxide generation from lignite samples immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), hydrogen peroxide (HP) pretreatments and normal saline solution (DI) for 14 and 28 days.

	Chemical – 14 days		Chemical – 28 days	
Pretreatment	**CH_4 [ppm]**	**CO_2 [ppm]**	**CH_4 [ppm]**	**CO_2 [ppm]**
Ni	236.74	233,479	26.18	33,708
LA	505.26	109,806	82.84	30,163
HP	194.75	223,991	17.45	25,765
DI	5.33	145,503	6.74	47,939

Table I.12. Methane and carbon dioxide generation from lignite samples immersed in normal saline solution and inoculated with four microbial consortia for 14 and 28 days.

	Microbial – 14 days		Microbial – 28 days	
Consortium	CH_4 [ppm]	CO_2 [ppm]	CH_4 [ppm]	CO_2 [ppm]
1	17.54	102,290	10.43	102,760
3	16.74	99,026	11.63	37,664
4	14.95	81,749	7.99	39,539
5	17.17	95,058	22.07	66,642

Table I.13. Methane and carbon dioxide generation from lignite samples immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), hydrogen peroxide (HP) pretreatments and inoculated with four microbial consortia for 14 and 44 days.

	Chemical+Micro – 14 days		Chemical+Micro – 44 days	
Pretreatment	CH_4 [ppm]	CO_2 [ppm]	CH_4 [ppm]	CO_2 [ppm]
Ni-1	37.43	33,701.3	6.41	19,482.0
Ni-3	27.79	26,600.5	5.91	20,219.9
Ni-4	26.29	31,434.5	4.40	14,654.9
Ni-5	24.54	39,890.7	5.32	17,453.3
LA-1	80.06	40,034.6	8.68	20,414.7
LA-3	26.56	25,070.2	6.68	20,349.7
LA-4	81.94	31,736.7	10.18	23,323.9
LA-5	68.44	39,020.9	8.77	23,102.4
HP-1	3.07	4,901.3	4.81	17,014.2
HP-3	14.80	35,854.5	5.61	20,885.8
HP-4	14.29	28,342.3	4.35	16,522.9
HP-5	15.19	46,048.4	4.56	17,528.4

Table I.14. Methane and carbon dioxide generation from lignite samples immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), hydrogen peroxide (HP) pretreatments, inoculated with microbial consortium 3, centrifuged and re-suspended in normal saline solution for 14 and 44 days.

	Solids – 14 days		Solids – 44 days	
Pretreatment	CH_4 [ppm]	CO_2 [ppm]	CH_4 [ppm]	CO_2 [ppm]
Ni+3	3.93	9,505.0	5.25	18,446.3
LA+3	4.77	20,043.0	5.01	18,969.6
HP+3	4.09	12,952.9	3.85	11,242.3

Table I.15. Methane and carbon dioxide generation from centrifuged lignite supernatant immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), and hydrogen peroxide (HP) pretreatments, and inoculated with microbial consortium 3 for 14 and 44 days.

	Liquids – 14 days		Liquids – 44 days	
Pretreatment	CH_4 [ppm]	CO_2 [ppm]	CH_4 [ppm]	CO_2 [ppm]
Ni+3	2.11	9,663.6	4.11	10,720.9
LA+3	2.03	68,192.5	5.66	38,943.9
HP+3	2.00	10,618.4	2.13	8,084.7

Table I.16. Methane and carbon dioxide generation from corn samples immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), hydrogen peroxide (HP) pretreatments and normal saline solution (DI) for 14 and 28 days.

	Chemical – 14 days		Chemical – 28 days	
Pretreatment	CH_4 [ppm]	CO_2 [ppm]	CH_4 [ppm]	CO_2 [ppm]
Ni	8.77	28,692.5	15.54	44,757.3
LA	18.68	41,934.2	50.94	49,504.9
HP	7.89	30,314.3	10.19	27,802.6
DI	4.90	16,237.8	7.47	14,141.6

Table I.17. Methane and carbon dioxide generation from corn samples immersed in normal saline solution and inoculated with four microbial consortia for 14 and 28 days.

	Microbial – 14 days		Microbial – 28 days	
Consortium	CH_4 [ppm]	CO_2 [ppm]	CH_4 [ppm]	CO_2 [ppm]
1	3.95	97,789.9	2.93	72,772.2
3	3.04	115,045.8	3.37	107,638.2
4	3.81	196,173.8	3.37	70,401.2
5	3.90	216,749.3	2.98	19,643.7

Table I.18. Methane and carbon dioxide generation from corn samples immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), hydrogen peroxide (HP) pretreatments and inoculated with four microbial consortia for 14 and 44 days.

	Chemical+Micro – 14 days		Chemical+Micro – 44 days	
Pretreatment	CH_4 [ppm]	CO_2 [ppm]	CH_4 [ppm]	CO_2 [ppm]
Ni-1	7.72	16,348.6	10.02	22,219.7
Ni-3	5.00	9,377.0	9.73	18,546.7
Ni-4	8.97	23,538.2	7.79	18,978.8
Ni-5	77.39	34,055.0	8.77	19,156.0
LA-1	24.46	30,172.9	35.87	30,983.2
LA-3	49.96	44,616.1	36.61	32,177.5
LA-4	25.55	32,654.7	36.23	31,757.1
LA-5	46.05	39,161.4	45.54	32,718.7
HP-1	11.22	29,578.5	10.69	26,081.3
HP-3	8.79	25,862.9	9.07	21,811.6
HP-4	26.07	15,563.5	8.93	23,878.9
HP-5	10.53	38,449.7	13.16	30,094.0

Table I.19. Methane and carbon dioxide generation from corn samples immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), hydrogen peroxide (HP) pretreatments, inoculated with microbial consortium 3, centrifuged and re-suspended in normal saline solution for 14 and 44 days.

	Solids – 14 days		Solids – 44 days	
Pretreatment	**CH_4 [ppm]**	**CO_2 [ppm]**	**CH_4 [ppm]**	**CO_2 [ppm]**
Ni+3	3.91	6,449.6	5.73	8,919.5
LA+3	14.16	15,343.8	20.09	18,574.5
HP+3	7.46	20,498.4	8.39	21,781.8

Table I.20. Methane and carbon dioxide generation from centrifuged corn supernatant immersed in $NiCl_2/Al_2O_3/SiO_2$ (Ni), lactic acid (LA), and hydrogen peroxide (HP) pretreatments, and inoculated with microbial consortium 3 for 14 and 44 days.

	Liquids – 14 days		Liquids – 44 days	
Pretreatment	**CH_4 [ppm]**	**CO_2 [ppm]**	**CH_4 [ppm]**	**CO_2 [ppm]**
Ni+3	3.67	5,172.4	3.91	4,462.1
LA+3	3.22	1,843.7	2.90	1,482.8
HP+3	3.34	4,514.3	5.10	6,913.5

APPENDIX J

BENCH SCALE RESULTS

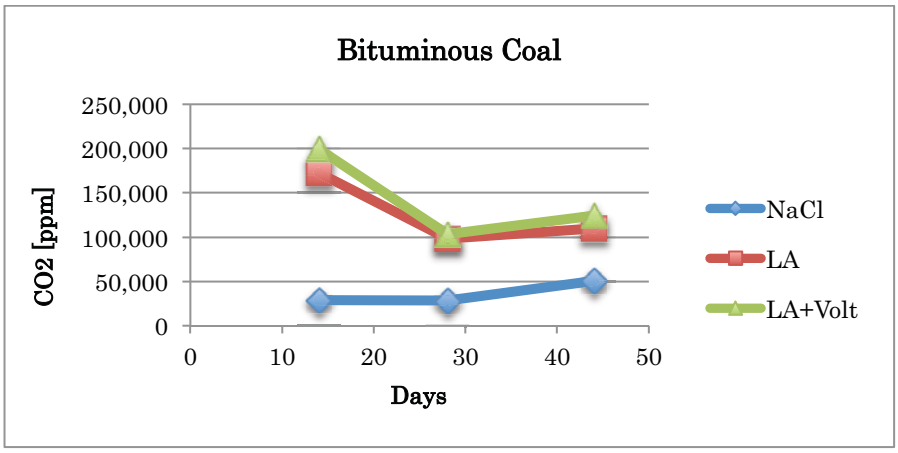

Figure J.1. Carbon dioxide generated from microbiochemically pretreated bituminous coal samples after 14, 28, and 44 days. Blue line shows samples immersed in normal saline solution, red lines shows samples immersed in lactic acid treatment, and green lines show samples immersed in lactic acid treatment and placed in electro-biochemical reactors.

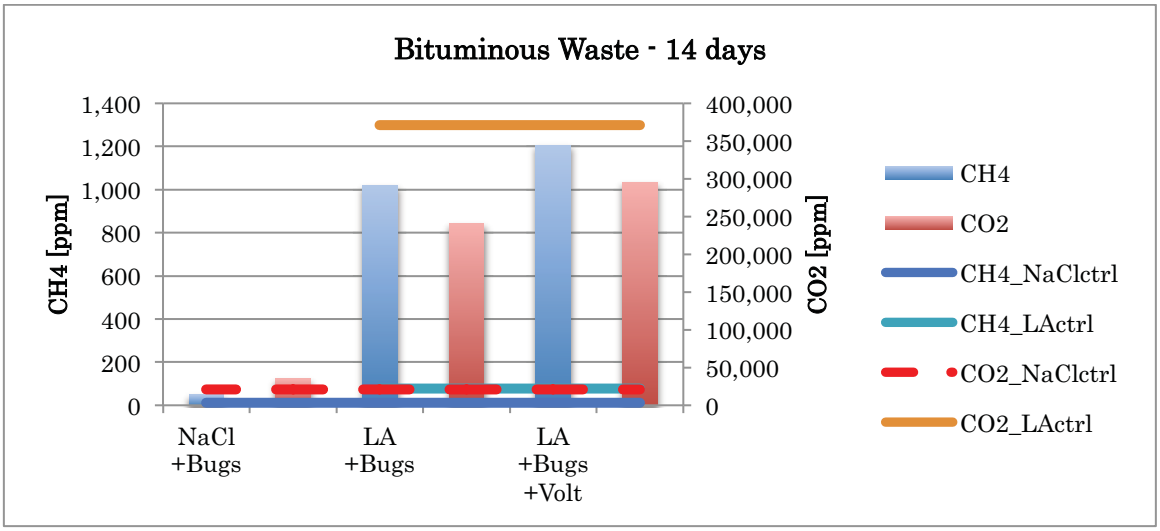

Figure J.2. Methane (blue bars) and carbon dioxide (red bars) generated from microbiochemically treated bituminous coal waste samples after 14 days. Blue and red lines represent "no microbes" saline controls of methane and CO_2, respectively. Light blue and orange lines represent "no coal" microbial controls of methane and CO_2, respectively.

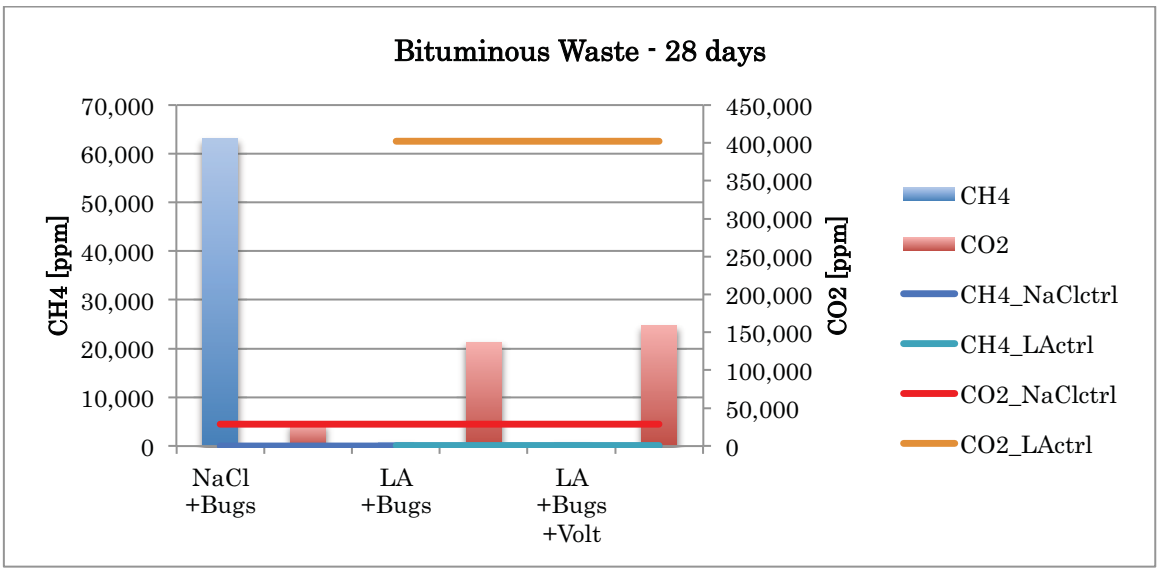

Figure J.3. Methane (blue bars) and carbon dioxide (red bars) generated from microbiochemically treated bituminous coal waste samples after 28 days. Blue and red lines represent "no microbes" saline controls of methane and CO_2, respectively. Light blue and orange lines represent "no coal" microbial controls of methane and CO_2, respectively.

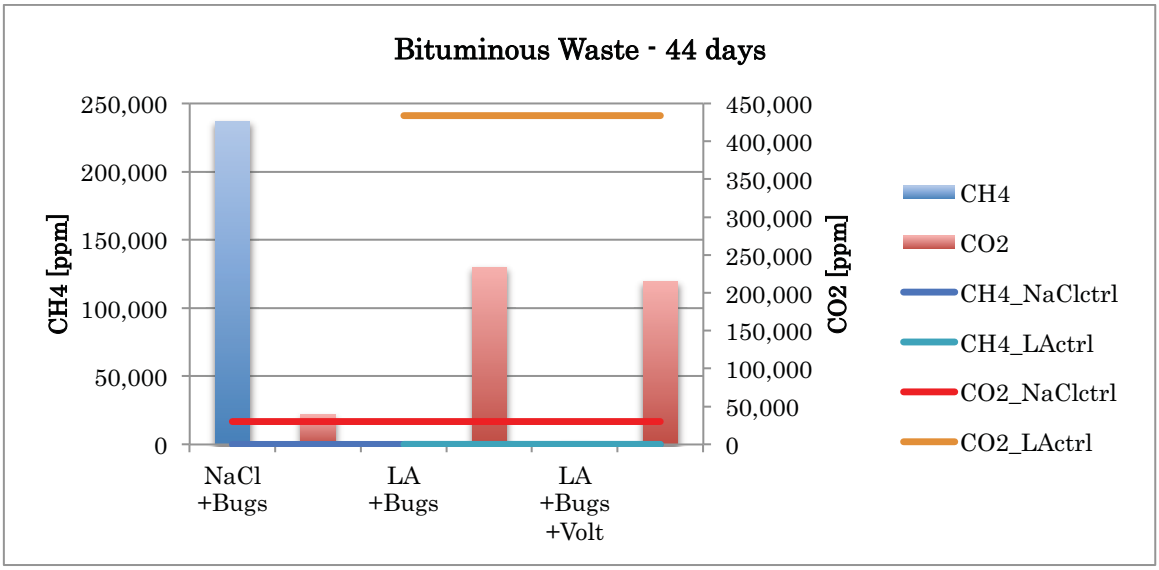

Figure J.4. Methane (blue bars) and carbon dioxide (red bars) generated from microbiochemically treated bituminous coal waste samples after 44 days. Blue and red lines represent "no microbes" saline controls of methane and CO_2, respectively. Light blue and orange lines represent "no coal" microbial controls of methane and CO_2, respectively.

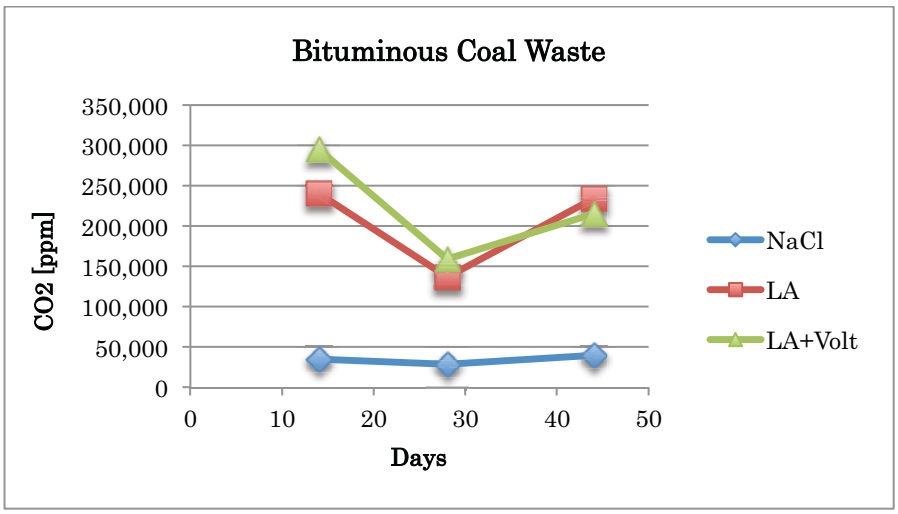

Figure J.5. Carbon dioxide generated from microbiochemically pretreated bituminous coal waste samples after 14, 28, and 44 days. Blue line shows samples immersed in normal saline solution, red lines shows samples immersed in lactic acid treatment, and green lines show samples immersed in lactic acid treatment and placed in electro-biochemical reactors.

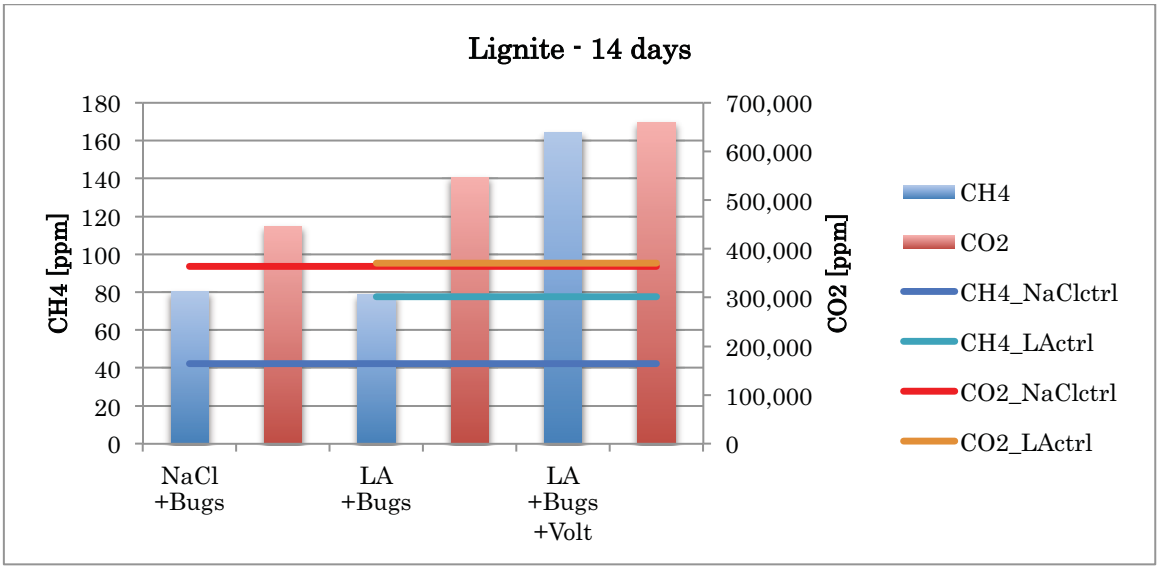

Figure J.6. Methane (blue bars) and carbon dioxide (red bars) generated from microbiochemically treated lignite samples after 14 days. Blue and red lines represent "no microbes" saline controls of methane and CO_2, respectively. Light blue and orange lines represent "no coal" microbial controls of methane and CO_2, respectively.

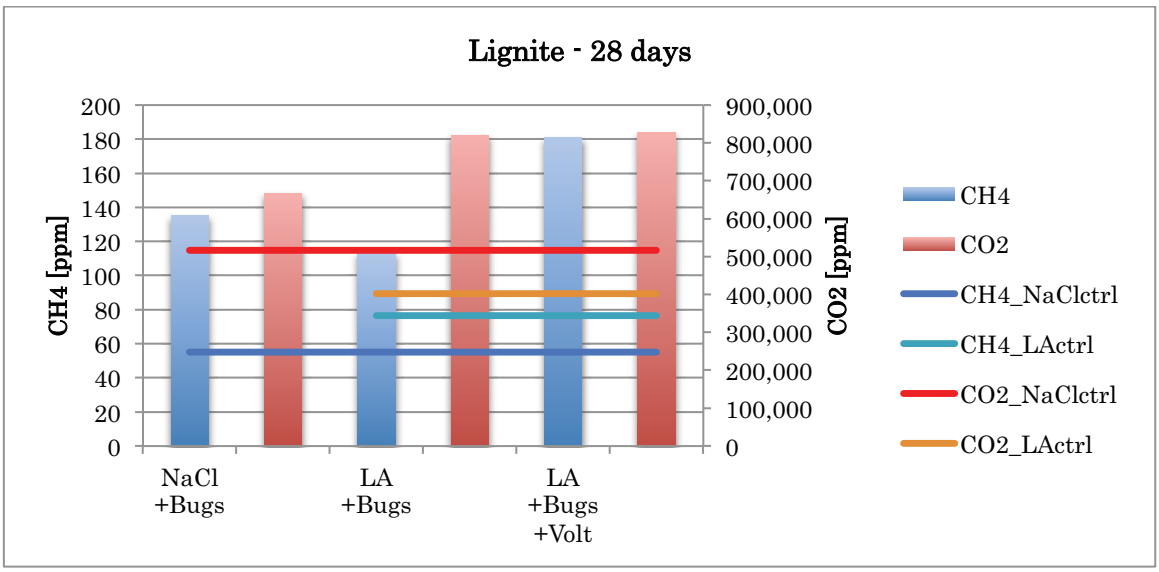

Figure J.7. Methane (blue bars) and carbon dioxide (red bars) generated from microbiochemically treated lignite samples after 28 days. Blue and red lines represent "no microbes" saline controls of methane and CO_2, respectively. Light blue and orange lines represent "no coal" microbial controls of methane and CO_2, respectively.

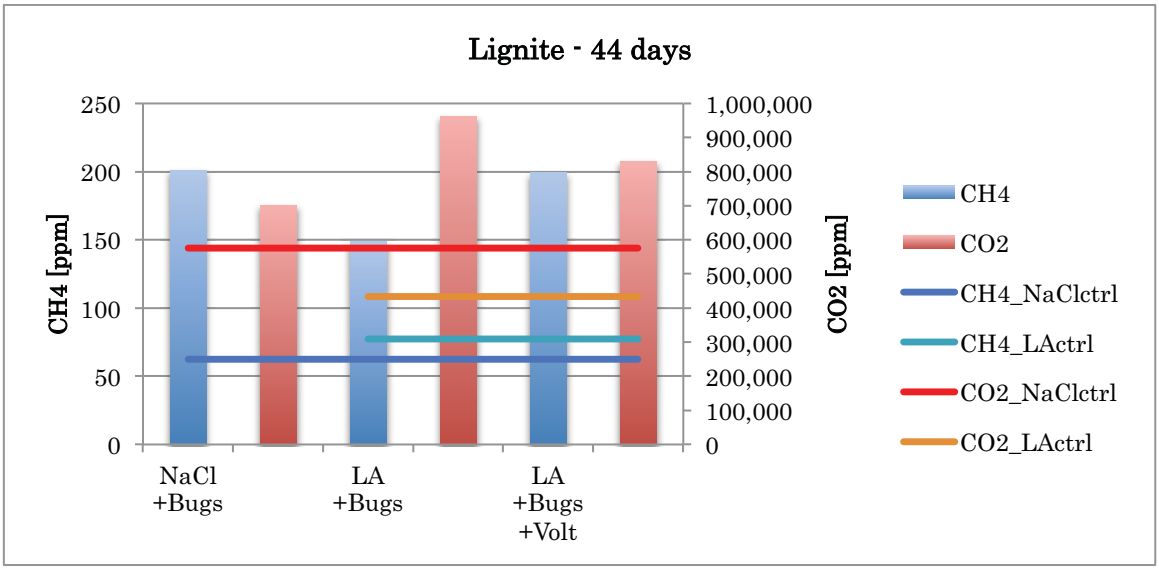

Figure J.8. Methane (blue bars) and carbon dioxide (red bars) generated from microbiochemically treated lignite samples after 44 days. Blue and red lines represent "no microbes" saline controls of methane and CO_2, respectively. Light blue and orange lines represent "no coal" microbial controls of methane and CO_2, respectively.

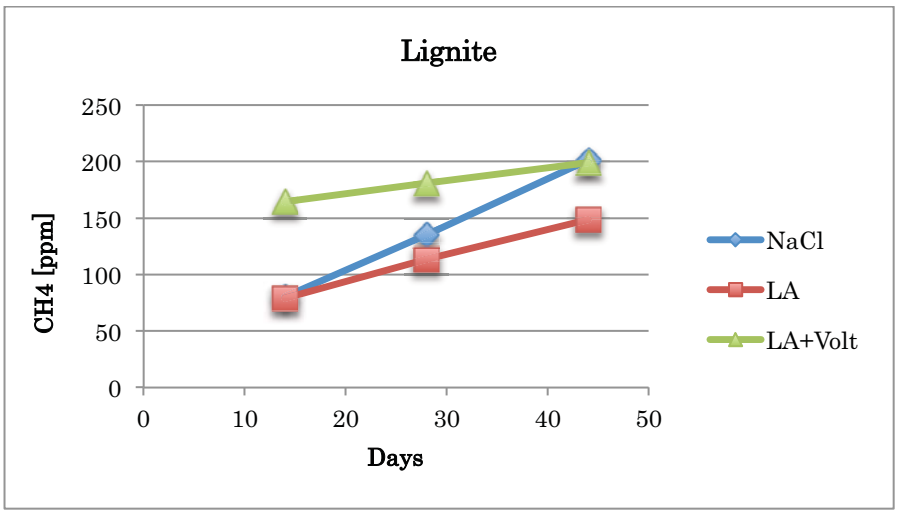

Figure J.9. Methane generated from microbiochemically pretreated lignite samples after 14, 28, and 44 days. Blue line shows samples immersed in normal saline solution, red lines shows samples immersed in lactic acid treatment, and green lines show samples immersed in lactic acid treatment and placed in electro-biochemical reactors.

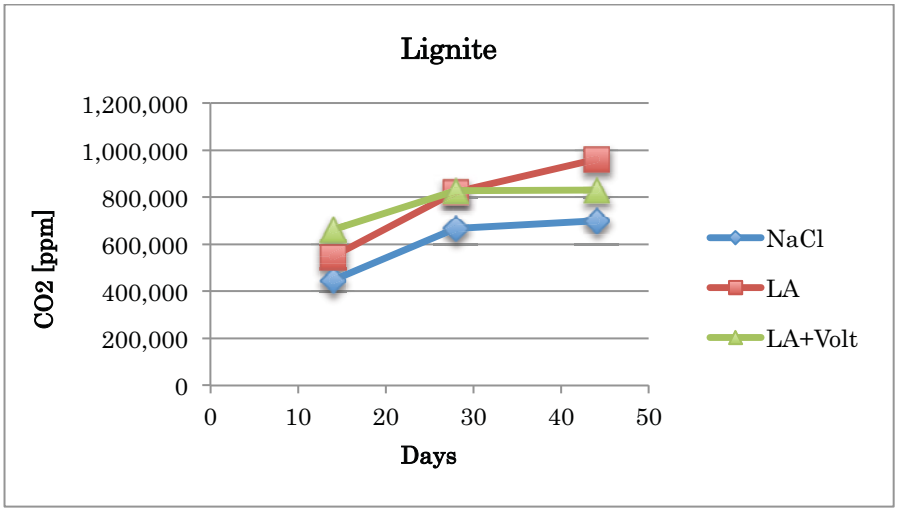

Figure J.10. Carbon dioxide generated from microbiochemically pretreated lignite samples after 14, 28, and 44 days. Blue line shows samples immersed in normal saline solution, red lines shows samples immersed in lactic acid treatment, and green lines show samples immersed in lactic acid treatment and placed in electro-biochemical reactors.

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