INHIBITION OF ANAEROBIC DEGRADATION OF TREATED PAPER SAMPLES UNDER SIMULATED LANDFILL CONDITIONS

By

LIANG CHEN

A thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of requirements

for the degree of

Master of Science

Graduate Program in Environmental Science

written under the direction of

Professor Donna E. Fennell

and approved by

_____

_____

_____

_____

New Brunswick, New Jersey

October, 2010

ABSTRACT OF THE THESIS

INHIBITION OF ANAEROBIC DEGRADATION OF TREATED PAPER SAMPLES UNDER SIMULATED LANDFILL CONDITIONS

By Liang Chen

Thesis Director:

Donna E. Fennell, Ph.D.

An innovative idea for formulating paper products with incorporated inhibitors or competitors of methanogenesis to reduce or postpone the methane potential of paper during degradation in landfills was examined in this proof of concept study. Three types of formulae, termed BioLithe™(A), BioLithe™(B), and BioLithe™(C) consisting of various chemical compounds known to inhibit methanogenesis or serve as competitive electron acceptors, were provided and were tested for their potential for methane reduction when combined with paper.

The study utilized two research approaches. First, biogas evolution was measured and assayed for methane content and cumulative biogas and methane production were compared during decomposition of variously treated and untreated paper samples. Second, the microbial communities present during degradation of treated and untreated paper were characterized using polymerase chain reaction amplification of archaeal and bacterial 16S rRNA genes and separation of phylotypes by denaturing gradient gel electrophoresis.

These approaches were used to compare paper treated with different BioLithe formulations in four separate tests. In Test 1, BioLithe-saturated paper samples prepared in the laboratory were tested in laboratory-scale batch reactors that simulated anaerobic landfill conditions and their biogas and methane production was quantified and compared to those of untreated paper. Test 2 was designed to inspect impacts on methane generation of BioLithe solutions added to anaerobic medium. Test 3 was performed to assess methane production associated with artificially BioLithe-saturated paper prepared at different concentrations. Finally, industrially prepared BioLithe-treated paper was evaluated for methane potential in Test 4.

Results show that BioLithe™(B) could effectively control paper degradation in relatively high amounts, or decrease methane generation correspondingly even with a relatively small amount of coating on paper, while the other two formulae BioLithe™(A) and BioLithe™(C) failed to control methanogenesis. Microbial communities existing during anaerobic paper degradation also shifted in response to the presence of BioLithe™(B) and this community shift might be associated with methane production performance.

ACKNOWLEDGEMENT

First and foremost, I am heartily thankful to my research advisor, Dr. Donna E. Fennell for providing me this research opportunity and her encouragement, patient guidance and excellent advice from the initial to the final level.

The project requires a diversity of studies and knowledge, and I graciously offer thanks to Dr. Lily Young for her input and advice on various aspects of this work.

I am thankful to Dr. Peter Strom for his continuous help and guidance both on my work and study for the entire time I have been at Rutgers.

Also, I acknowledge my gratitude to Globe Die Cutting Inc. for their funding support and the original idea for this project. Dr. Brian Davis developed the BioLithe Formulae described in this thesis. We thank the staff of the Joint Meeting of Essex and Union Counties (JMEUC) Sewerage Authority in Elizabeth, NJ for providing anaerobic digester sludge.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

TABLE OF CONTENTS

LIST OF TABLES

Chapter 1

Chapter 3

Chapter 4

Chapter 5

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 4

CHAPTER 1

INTRODUCTION

1.1 Environmental Impacts of Greenhouse Gases

Global warming, defined as an increase in the average temperature of the Earth's surface caused by natural or anthropogenic climate change (Mastrandrea and Schneider, 2005), is one of the most environmentally significant challenges facing humankind. Increasing ambient temperatures are predicted to bring about a series of potentially catastrophic effects to the environment, including a probable increase in the frequency of extreme weather events, loss of plant and animal species, and rising sea levels, which directly threatens insular and coastline areas (Cooper and Alley, 2002). New Jersey is confronted with the problem of sea level rise, which will be exacerbated and greater than the global average resulting from coastline subsidence (NJ Department of Protection, 2010).

According to the 2007 Fourth Assessment Report by the Intergovernmental Panel on Climate Change (IPCC), most of the observed increase in global average temperatures since the mid-20th century has very likely been caused by the observed increase in anthropogenic greenhouse gas (GHG) concentrations in the atmosphere (IPCC, 2007). Although carbon dioxide (CO_2) is the GHG with the largest mass emission to the atmosphere (U.S. EPA, 2010), it is not the only gas that contributes to climate change. Other important GHGs include methane, nitrous oxide, ozone and chlorofluorocarbons

(U.S. EPA, 2010); in particular, methane (CH_4) is a much more powerful GHG than carbon dioxide.

Methane is second only to carbon dioxide in atmospheric abundance of anthropogenic emissions contributing to global warming; further, methane has a global warming potential (GWP)[1] which is estimated to be 25 times higher than that of carbon dioxide over a 100-year time horizon (IPCC, 2007). Notably, the global atmospheric concentration of methane has increased from a pre-industrial value of about 715 ppb to 1,732 ppb in the early 1990s, and was 1,774 ppb in 2005 (IPCC, 2007).

1.2 Methane Emissions from Landfills

Methane is thought to account for about 20% of the observed global warming (Mackie and Cooper, 2009) and two-thirds of methane emissions result from human activities such as crop cultivation, livestock production, the extraction of geologic natural gas, and emissions from landfills (U.S. EPA, 2009). Landfills are the second largest anthropogenic source of methane emissions in the U.S., accounting for 23% of all methane emissions in 2007 (U.S. EPA, 2009). Methane makes up 45 to 60% of the volume of landfill gas (Bogner et al., 1995). Methane may escape from landfills as fugitive emissions either directly to the atmosphere or by diffusion through the cover soil.

Emissions from municipal solid waste (MSW) landfills, which received about 64.5% of the total solid waste generated in the United States (Arsova et al., 2008), accounted for about 90% of the total landfill emissions, while industrial landfills

[1] Global warming potential (GWP) is a measure of how much a given mass of greenhouse gas is estimated to contribute to global warming (http://news.bbc.co.uk/2/hi/science/nature/8314501.stm).

accounted for the remainder (U.S. EPA, 2009). Landfill operators have made efforts to reduce methane emissions by capturing biogas and either flaring it or using it for energy generation, or by ensuring biofiltration of landfill gas through cover soil or waste materials where the methane is aerobically degraded to carbon dioxide by microorganisms utilizing oxygen that diffuses into the cover layer from the atmosphere (Mancinelli and McKay, 1985; Park et al., 2009; Scheutz et al., 2009). Despite these measures, the net fugitive methane emissions still account for more than half of the total methane produced in landfills, as seen as Table 1.1. In 2007, landfill methane emissions were approximately 6,327 Gg (1 Gg = 10^9 g) (U.S. EPA, 2009). This number is expected to increase because of the annually increasing amount of MSW produced as a result of the growing population in the US. The on-going biodegradation of the huge amounts of waste that are already buried can mean that methane will continue to be produced for years after landfill sites are closed (Eleazer et al., 1997; U.S. EPA, 2010).

Table 1.1 Methane Emissions from Landfills (Gg) (U.S. EPA, 2009)

	Year					
Activity	**1990**	**1995**	**2000**	**2005**	**2006**	**2007**
Methane Generation						
MSW Landfills	8,219	9,132	9,854	11,486	11,813	12,107
Industrial Landfills	554	615	687	728	730	735
Methane Recovered						
Gas-to-Energy	(635)	(1,064)	(2,348)	(2,707)	(2,819)	(3,062)
Flared	(242)	(1,048)	(1,722)	(2,743)	(2,822)	(2,750)
Oxidized [a]	(789)	(763)	(647)	(676)	(690)	(703)
Total Emission	**7,105**	**6,871**	**5,825**	**6,088**	**6,211**	**6,327**

Note: Totals may not sum due to independent rounding. Parentheses indicate negative values.

[a] Includes oxidation at both municipal and industrial landfills.

1.2.1 Construction of landfills and fugitive methane emissions

Landfills, which contain large quantities of organic material and prevailing anaerobic conditions, provide ideal conditions for methanogenesis, the final step in the anaerobic biodegradation of organic material (Eleazer et al., 1997; Barlaz M.A., 1998). The huge amounts of waste that are buried in landfills can fuel production of methane for years after the landfill is closed. It is estimated by EPA that substantial methane production typically begins one or two years after waste disposal in a landfill and continues for 10 to 60 years, or longer (U.S. EPA, 2010). In MSW landfills this process is advantageous because it leads to formation of a recoverable biofuel (methane) and it stabilizes and reduces the volume of the waste.

There are approximately 1,800 operational landfills in existence in the U.S., with the largest landfills receiving most of the waste and generating the majority of the methane (Arsova et al., 2008). During landfill operation, individual "cells" are constructed through the emplacement of MSW over time (Belevi and Baccini, 1989; GeoSyntec Consultants, 2001A). The volume of a cell varies depending upon the design and characteristics of the landfill, such as composition of waste-in-place, cell density, operational practices, size and capacity of landfill, climate, and other factors (GeoSyntec Consultants. 2001A; GeoSyntec Consultants, 2001B; Interstate Technology & Regulatory Council, 2005; U.S. EPA, 2010). The design of the cell determines the time required for cell construction which may range from months to years (Government Engineering, 2006). In most cases, the cell in a landfill may stay open without a final cap as long as possible, to take advantage of the airspace increase created by liquids recirculation (Interstate Technology & Regulatory Council, 2005). This may lead to some negative

implications, of which a major concern is control of biogas emissions. Unless captured first by a biogas recovery system, methane generated in the landfill is emitted when it migrates through the landfill cover. However, only after the cell is filled and capped are the biogas collection wells and extraction system placed online for active landfill gas collection. During the construction time, emplaced MSW begins to degrade and produce methane. While the soil oxidizes approximately 10% of the generated methane which is not recovered by landfills, the remaining 90% of it escapes as fugitive methane into the atmosphere (Mancinelli and McKay, 1985; Czepiel et al., 1996; Scharff and Jacobs, 2006; Kumar et al., 2004; Park et al., 2009).

1.3 Paper in Landfills

Among the diverse wastes placed in MSW landfills, more than one-third is paper (U.S. Department of Energy, 2005; Kinsella et al., 2007). The U.S. per capita paper consumption was more than 700 pounds (approximately 318 kilograms) in 2004 (Resource Information Systems Inc, 2004), making the U.S. population by far the highest per capita paper consumers in the world (Kinsella et al., 2007). Although paper recovery and recycling has increased every year over the past five years (Resource Information Systems Inc, 2007; Kinsella et al., 2007), most paper is eventually disposed of in landfills. The U.S. Environmental Protection Agency (EPA) has identified the decomposition of paper as among the most significant sources of landfill methane (U.S. EPA, 2010). To prevent further pollution from landfill methane to the atmosphere, the paper industry must formulate an environmental response and develop technologies for paper treatment to mitigate fugitive methane emissions.

1.3.1 Paper (cellulose) degradation in anaerobic environment

Paper is made from pulp which in turn is made from either wood or other lignocellulosic material (Gutleben et al., 2004). Generally, lignocellulosic material consists of roughly 60-95% holocellulosic material (a mixture of cellulose and hemicelluloses), and 5-30% lignin, as well as other inorganic material (Zou et al., 1994; Kacik et al., 2009; Gutleben et al., 2004). Office paper and newspaper are the most abundant holocellulosic material in municipal solid waste (Kim and Lee, 2008). The chemical composition of office paper was determined to be 95.8% and 2.3% holocellulose and lignin, respectively (Eleazer et al., 1997), while that of newspaper was 72.1% and 24.0% for holocellulose and lignin, respectively (Cummings and Stewart, 1994). Since cellulose is the major component of paper that is in landfills (Kosik et al., 1983; Kacik et al., 2009), the degradation of paper here is considered and analyzed as the decomposition of cellulose molecules.

After disposal in MSW landfills, paper undergoes a series of complex degradation processes, from polysaccharide hydrolysis under the effect of bacteria to methane generation by the archaeal methanogens. Figure 1.1 shows the overall process of anaerobic decomposition, in which various groups of fermentative anaerobic microorganisms cooperate in the conversion of complex organic materials ultimately to form methane and carbon dioxide. The complex polymers (here represented by cellulose) first undergo hydrolysis. Hydrolysis is a slow process mediated by hydrolytic microorganisms possessing a unique extracellular multi-enzyme complex, called cellulosome (Béguin and Lemaire, 1996； Schwarz, 2001). These multi-enzyme complexes attach both to the cell envelope and to the substrate, allowing the cells to have

proximity to the cellulose (Schwarz, 2001). The most complex and best investigated cellulosome is that of the thermophilic bacterium *Clostridium thermocellum* (Béguin et al., 1997; Bender et al., 1998). The monomer released during the hydrolysis of the polymer is then fermented by primary fermenters into a variety of fermentation products, including acetate, propionate, butyrate, succinate, alcohols, hydrogen (H_2) and carbon dioxide (Madigan and Martinko, 2006). Hydrogen produced in primary fermentation is removed by hydrogen consumers, such as methanogens and homoacetetogens. Other fermentation products are converted to hydrogen, carbon dioxide and acetate under the function of syntrophic bacteria (Zhao et al., 1996; Chakraborty et al., 2002; Rozej et al., 2008). In addition, acetate can be converted to methane by acetotrophic methanogens (Madigan and Martinko, 2006).

The entire process of cellulose degradation has the following theoretical stoichiometry (Khan et al., 1978): $nC_6H_{12}O_6 \rightarrow 3nCH_4 + 3nCO_2$. Considering paper is not 100% cellulose, the actual methane generation during paper decomposition would be less than this theoretical ratio.

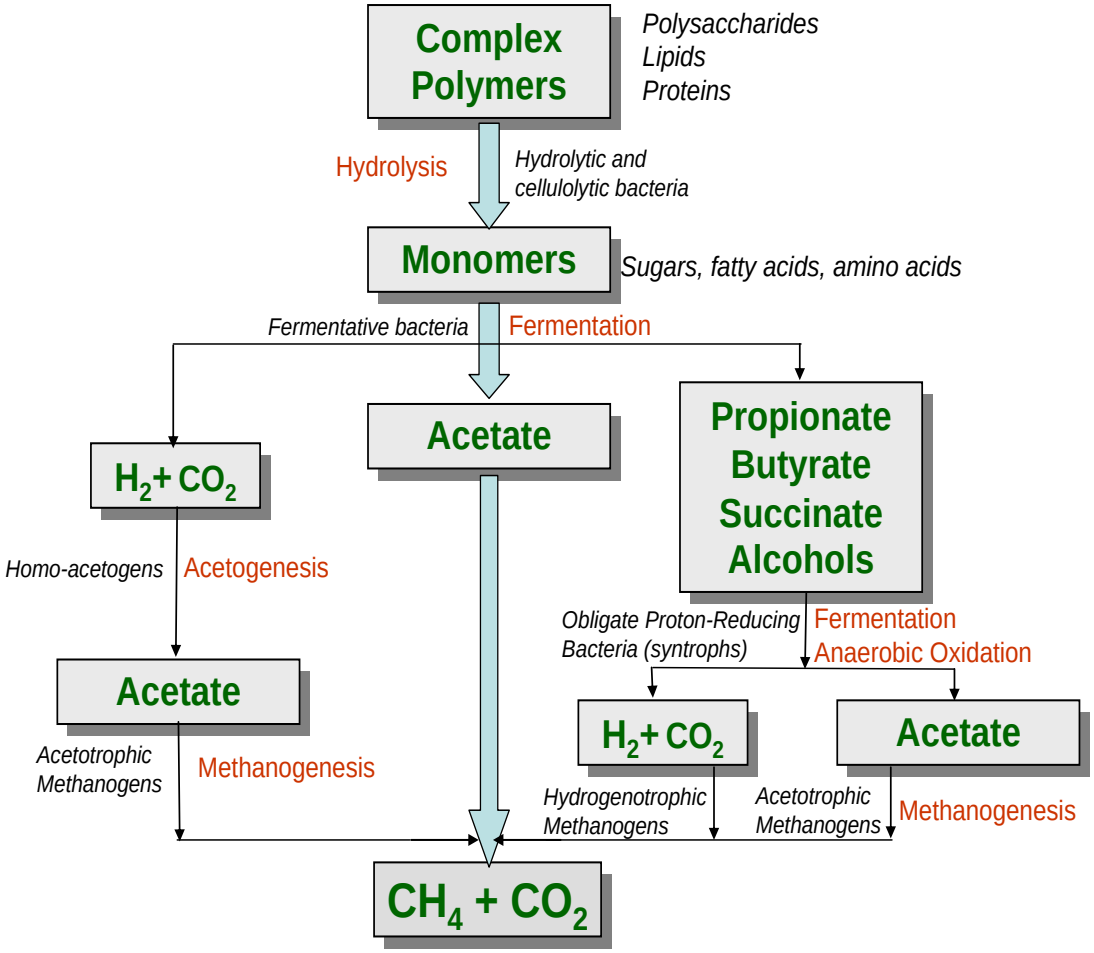


Figure 1.1 Anaerobic degradation process of converting cellulose to methane

1.4 Control of Methanogenesis using Exogenous Inhibitors

Many studies have examined additional inhibitors that are able to effect on methanogens and thus methane formation (Oremland and Taylor, 1975; In et al., 1992; Janssen and Frenzel, 1997; Dumitru et al., 2003). Inhibitors have been applied to target methanogenesis in a variety of cultures and ecosystems in order to either investigate the physiology of methanogens, investigate microbial community dynamics in anaerobic ecosystems, or to investigate technologies to expressly mitigate emissions of methane into the atmosphere.

Early on, McCarty summarized the concentrations of common cations that had an inhibitory effect on methane production in anaerobic digesters (Kugelman and McCarty, 1964). After that more chemicals were analyzed and reported for methanogen inhibition and methane reduction. A summary of methanogenic studies have concluded various chemicals that were used for methane suppression (Table 1.2).

Table 1.2 Summary of studies that investigated specific methane inhibitors

Inhibitor	System Investigated	Suppressive Concentration	Reference
Ethylene	Marine sediments	>5% v/v	Oremland and Taylor, 1975
Methyl chloride	Landfill cover soil	>0.01% v/v	Chan and Parkin, 2000
2-Bromoethane sulfonic acid	Anaerobic digested sludge + activated sludge	0.1-0.27 mM (potently inhibited)	Chae et al., 2010
		50 mM (completely inhibited)	Parameswaran et al., 2009
Thymol	Swine manure	1.5/3.0 g/L	Varel and Wells, 2007
Nickel (Ni^{2+})	Anaerobic medium	2.5 mM	Lorowitz et al., 1992
Sodium	Anaerobic digester	>3,500 mg/L	Kugelman and McCarty, 1964
Potassium	Anaerobic digester	>2,500 mg/L	Kugelman and McCarty, 1964
Calcium	Anaerobic digester	>2,500 mg/L	Kugelman and McCarty, 1964
Magnesium	Anaerobic digester	>1,000 mg/L	Kugelman and McCarty, 1964
Copper (Cu^{2+})	Anaerobic digester	10-250 mg/L	Sanchez et al., 1996
Zinc (Zn^{2+})	Anaerobic digester	10-250 mg/L	Sanchez et al., 1996
Ferric iron (Fe^{3+})	Sewage sludge	21 mg/L	Zhang et al., 2009

Several studies specifically investigated methods to inhibit methanogenesis for prevention of methane emissions to the atmosphere. Methyl chloride (CH_3Cl) was amended to landfill cover soil and found to affect methanogenesis at concentrations exceeding 0.01% (volume/volume), and a CH_3Cl concentration of 0.1% inhibited methanogenesis by 89% (Chan and Parkin, 2000). It was reported that thymol reduced methane production by 78 and 93% at applications of 1.5 and 3.0 g/L in swine manure,

respectively (Varel and Wells, 2007). Besides organic compounds, inorganic compounds especially metallic cations were shown to cause methane suppression. For example, potassium and magnesium show some inhibitory phenomenon on anaerobic digesters at concentrations of 2,500 mg/L and 1,000 mg/L, respectively, and strong inhibition at 12,000 mg/L and 3,000 mg/L, respectively (Kugelman and McCarty, 1964; Chen et al., 2007).

Besides the various ecosystems described above, inhibitors were also applied to control methanogenesis with cellulose as the carbon source. It was found that sulfide (S^{2-}) concentrations above 0.5 mM inhibited methane formation, and above 0.8 mM inhibited both cellulose degradation and methane formation (Khan et al., 1979). Other studies involving methanogenic degradation of cellulose found that inorganic sulfur compounds other than sulfate inhibited degradation of cellulose to methane, and this inhibition increased in the order thiosulfate < sulfite < sulfide < H_2S (Khan and Trottier, 1978).

All these studies indicate that methanogenesis during cellulose degradation could be affected by a variety of inhibitors. At the same time this implies that a new approach for reducing methane emissions during paper degradation could be accomplished by treating paper with some of these compounds so as to inhibit methanogenic or bacterial activity (either completely or partially) during the landfilling of paper, and thus decrease methane emissions from landfills to the atmosphere.

1.5 Detection and Identification of Methanogens using PCR-DGGE

Many methods have been applied to analyze archaeal methanogens in various environments. For example, methanogenic communities have been intensively investigated in rice paddy soil microcosms by using clone library and terminal RFLP (restriction fragment length polymorphism) techniques (Grobkopf et al., 1998; Chin et al., 1999; Fey et al., 2001; Webber et al., 2001). However the use of traditional microbiological techniques in determining population structures and characteristics is limited as it has been shown that many organisms are not readily cultured on selective media (Briones and Raskin, 2003). Denaturing gradient gel electrophoresis (DGGE) of small subunit rRNA genes amplified by polymerase chain reaction (PCR) has been broadly employed in methanogenic community studies since the method was established, and has rapidly developed (Muyzer et al., 1993, Achenbach and Woese, 1995; Cahyani et al., 2003; Watanabe et al. 2004; Keyser et al., 2006). The PCR-DGGE method has the particular advantage of allowing phylogenetic information about the microorganisms present to be obtained by excising and sequencing DNA from bands that migrate separately on DGGE gels. Further, examination of DGGE banding patterns through time-course sampling allows community structure changes to be followed.

The PCR-based DGGE method has been used for analysis of methanogens in various and complex environments, for example for a petroleum hydrocarbon-contaminated aquifer (Kleikemper et al., 2005) and for rumen liquid, solid and epithelium fraction of cattle (Pei et al., 2009), among others. Archaeal species were identified in different types of UASB (upflow anaerobic sludge blanket) granules using a PCR-based DGGE approach (Keyser et al., 2006). It was reported that methanogenic granule

populations depended mainly on the composition of the substrate (Lévesque and Guiot, 2004) and the methanogenic populations were an indicator of changes in temperature and pH stability, as well as the solids retention time (Casserly and Erijman, 2003).

Most methanogens detected by 16S rRNA gene analysis from environmental samples belonged to the phyla Euryarchaeota and Crenarchaeota while for some environments only members of the Crenarchaeota were detected (Watanabe et al. 2004). Different primers and PCR conditions were developed to identify diverse archaeal methanogenic species (Amann et al., 1995; Øvreås et al., 1997; Bundt et al., 2001). In some studies, primers for amplifying 16S rRNA genes of methanogenic archaea were modified based on existing archaeal primers for the purpose of being more specific to a target microbial community (Casamayor et al., 2002; Watanabe et al., 2004).

CHAPTER 2

RATIOANLE AND OBJECTIVES

2.1 The Origin and Relevance of the Research

Paper, as introduced in Chapter 1, is the most frequent item encountered in MSW landfills (Kinsella et al., 2007), making paper decomposition one of the most important sources of landfill methane production (U.S. EPA, 2010). On average, paper accounts for more than 40% of the composition by mass in landfills (Baird and Colin, 2004). This proportion has held steady for decades and in some landfills has actually increased (Baird and Colin, 2004; Kinsella et al., 2007). To prevent further environmental deterioration from landfill methane emissions, scientists, governments and the paper industry have investigated more environmentally and socially responsible alternatives to control and reduce the negative influence from successive treatment of paper, which is ultimately primarily disposed of in landfills.

Based on the severe environmental problems resulting from methane emissions from paper degradation in landfills, BioLithe LLC, a company founded with a focus on producing environmentally friendly products, has proposed a technological approach for producing paper with reduced methane potential. As indicated in Figure 2.1, the idea proposed by BioLithe LLC is that paper could be treated prior to use to prevent, lessen, or delay methanogenesis by some BioLithe formulae, to reduce the release of the potent greenhouse gas, methane upon the ultimate disposal of paper in a landfill.

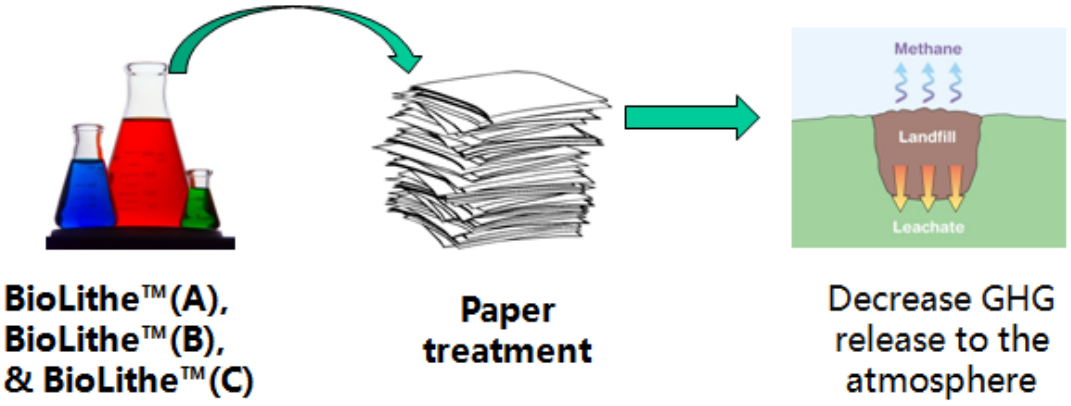


Figure 2.1 The rational of the BioLithe Study

Figure 2.2 shows a conceptual model of the hypothetical methane emissions from "Ordinary Paper" versus "BioLithe Paper" during the degradation process in landfills. Assume the decomposition process of ordinary paper leading to methane formation is represented by the solid line, and the shaded area under the curve labeled ① represents the methane emission to the atmosphere before cell closure when active methane recovery starts. In the case of BioLithe treated paper in landfills, for which methane production would be initially inhibited, the methane emission is represented by the dashed line, the volume of escaped methane would be decreased to the shaded area under the curve labeled ③, and the shaded area under the curve labeled ② represents the potentially lost methane that now could be captured before cell closure. Accordingly, by formulating paper with BioLithe that intrinsically delays or inhibits methanogenesis, less methane (shaded area ③) would escape into the environment during internment, and more gas production (shadow part ②) could be captured via landfill gas wells and later supplied as energy to factories and homes.

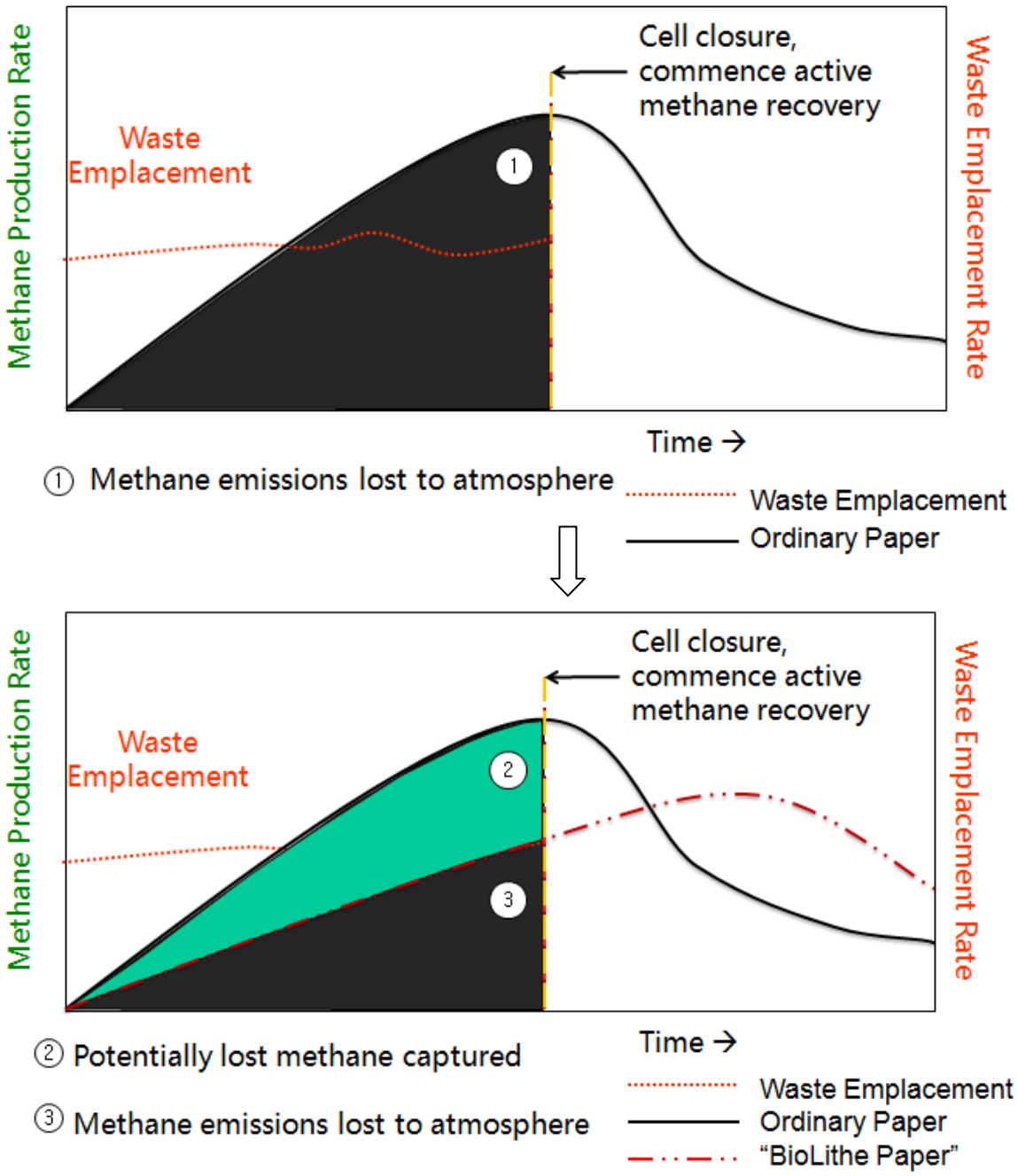


Figure 2.2 Conceptual model of hypothetical methane emission during paper degradation

An alternate mode of effectiveness would be if the degradation of the paper were channeled to carbon dioxide production while methanogens were competitively excluded through the presence of an alternate electron acceptor (e.g., Lovley and Klug, 1983). This model would mean that overall less of the potent GHG methane would be produced.

This thesis describes a study contracted by and conducted in support of technology development for BioLithe. The experiments described were intended to test methane inhibition and/or altered rates of biogas and methane production from simulated or prototype paper products amended with inhibitors of methanogenesis. Further, the research utilized molecular biology tools to assess the impact of different paper treatment formulations on the microbial community present during paper degradation.

2.2 Research Approach and Hypotheses

The project was designed and implemented in a series of successive test phases as follows: TEST 1, Degradation of Paper Saturated with Inhibitors I; TEST 2, Degradation of Paper in the Presence of Dissolved Inhibitors; TEST 3, Degradation of Paper Saturated with Inhibitors II; and TEST 4, Degradation of Paper Coated with Inhibitors in an Industrial Process, as shown in Figure 2.3. Each experiment was designed to test a specific hypothesis and to accomplish specific goals, as described in the subsequent sections of this chapter. Materials and methods related to practical operation were revised as needed during the broader study. The specific details of each experiment are presented in Chapter 3, Materials and Methods.

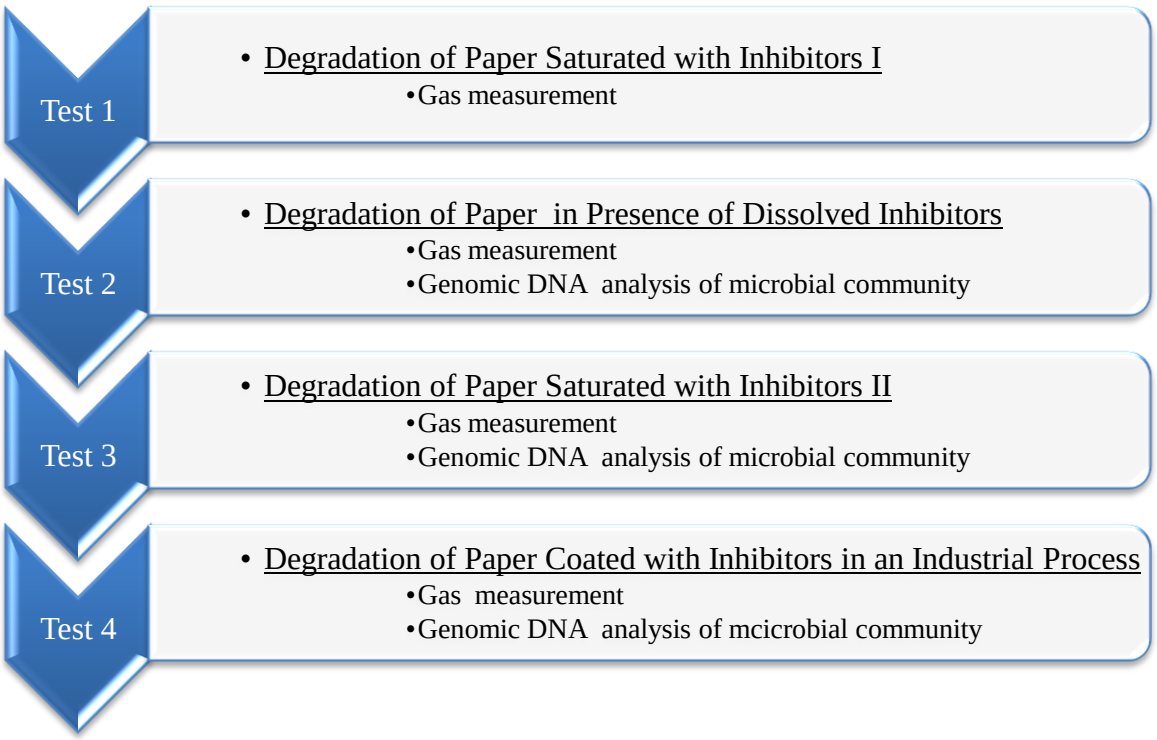


Figure 2.3 Chronological Flow Chart of the BioLithe Study

2.2.1 Research hypotheses

This project was developed stepwise, starting from simple tests of the effectiveness of different BioLithe formulae for inhibiting production or channeling the degradation towards carbon dioxide by addition of a competitive electron acceptor, sulfate (e.g., Lovley and Klug, 1983), and proceeding to determination of the methane production potential of paper coated with BioLithe formulae in an industrially applied operation. All tests were carried out with the following hypotheses to be examined:

1) During anaerobic degradation, paper treated with BioLithe solutions would exhibit inhibited or delayed methanogenesis or a lower methane production potential compared to ordinary (untreated) paper.

2) The different BioLithe solutions and aqueous coating material would have different effects on either the archaeal methanogenic community, or the bacteria community responsible for fermentation and production of substrates for methanogenesis.

3) The magnitude of additional BioLithe chemicals on paper might affect the degree of paper decomposition, implying that the inhibitor application concentration correlates to a corresponding amount of methane production.

4) By virtue of an industrial paper treatment process, BioLithe solutions could be mechanically coated on the surface of paper and play a role in methane control.

2.2.2 Chronological order of tests during the BioLithe study and corresponding rationales

TEST 1. <u>Degradation of Paper Saturated with Inhibitors I</u>: Two paper treatment formulae BioLithe™(A) and BioLithe™(B) were provided by BioLithe LLC. Each formula was to be applied to paper (chipboard) and the resulting treated paper product was tested for methane emission control during degradation in an anaerobic environment intended to mimic landfill conditions. Paper was saturated in the two BioLithe solutions, and as a result, the paper fiber was assumed to be relatively evenly coated with the BioLithe chemicals. The inoculum used in this test was a mixture of materials from three different origins intended to provide a complex anaerobic microbial environment similar to that in landfills. The purpose of this experiment was to observe the effect of BioLithe chemicals on paper degradation, and the difference in biogas and methane production between the treated paper and untreated paper.

TEST 2. <u>Degradation of Paper in the Presence of Dissolved Inhibitors</u>: This test had three major objectives. The paper employed in this experiment was printer paper, broadly used in the packaging and advertising industries.

Printer paper is usually coated with a layer of aqueous coating to protect the paper from cracking or scuffing after printing. This coating material would act as a medium in which to dissolve the BioLithe formulae to add it to paper through an industrial coating process. Thus BioLithe chemicals would likely always be applied to paper along with the aqueous coating material. Therefore, during this test, the first objective was to test the BioLithe formulae for methane inhibition in combination with the aqueous coating. (Note that the individual effect of the aqueous coating material on paper degradation was not determined during this test).

The second objective of this test was to evaluate the methane inhibition effect of BioLithe™ (C), which was substituted for BioLithe™ (A) (which was found not to be effective during the test, <u>Degradation of Paper Saturated with Inhibitors I</u>).

A third objective of this experiment was to test the impact of BioLithe solutions on the microbial communities involved in paper degradation. The BioLithe chemicals were not soaked into the paper or coated on the paper during this test, but were added to the bulk medium in which the microbial inoculum and paper were also added. Therefore, BioLithe inhibitors were dissolved and acting on microorganisms in the presence of the paper samples.

TEST 3. <u>Degradation of Paper Saturated with Inhibitors II</u>: The purpose of all the previous tests carried out was to examine the methane inhibition potential of the BioLithe formulae. After the validation of BioLithe chemicals, they would be

transitioned to an industrial operation associated with paper coating. Before proceeding to an applied operation, however, a formula concentration test was run to determine the relationship between the concentration of BioLithe formulae applied to paper (g/g) and the corresponding methane production. Therefore, paper treated with BioLithe solutions at different concentrations was prepared for this methane inhibition experiment. During this test an additional objective was to test the impact of the BioLithe solutions on the microbial communities involved in paper degradation.

TEST 4. Degradation of Paper Coated with Inhibitors in an Industrial Process: This experiment addressed an applied coating test with different combinations of paper and BioLithe formulae. The paper samples were coated with BioLithe chemicals dissolved in aqueous coating via a mechanized industrial coating technique. The treated paper then underwent testing to monitor methane generation. During this test an additional objective was to test the impact of the BioLithe solutions on the microbial communities involved in paper degradation.

2.2.3 Research approach

As shown in Figure 2.3, the entire BioLithe study mainly employed two research approaches - biogas measurement and DNA-based analysis of the microbial community. Gas analysis would be applied to all BioLithe tests, to measure cumulative gas production and methane content of the gas during paper decomposition, and thus directly reflect differences in methane production potential of various paper samples. Tests 2, 3 and 4 further characterized the microbial communities that developed in the presence of the different BioLithe formulae using the genomic DNA of the total microbial population

during anaerobic degradation. This was done to analyze the influence of BioLithe chemicals on the microorganisms carrying out paper decomposition

Overall the importance of this research is in developing methods to reduce the greenhouse effect by taking steps to help reduce the amount of methane gas emitted during paper degradation in landfills. In all, this series of tests conducted for the BioLithe project with new types of formulated paper can prove to be a benefit, since it can encourage the public to adopt paper which is more favorable to our environment and establish a market for eco-friendly paper products.

CHAPTER 3

MATERIALS AND METHODS

3.1Preparation for Bioreactor Set Up

3.1.1 BioLithe formulae for testing

Three types of BioLithe formulae were tested—BioLithe™(A), BioLithe™(B) and BioLithe™(C) (see Tables 3.1.1, 3.1.2, and 3.1.3). These formulae were developed by Dr. Brian Davis and provided to us by Globe Die Cutting, Inc. (Metuchen, NJ). The formulae are under patent and were prepared in the laboratory at Rutgers University using chemicals provided by Globe Die Cutting, Inc. (Metuchen, NJ).

Table 3.1.1 Composition of BioLithe™(A)

Chemical[a] Component	**Concentration (g/L)**	**Mass Percentage of Component (%)**
Ferric ammonium citrate	47.9	40.6
Ferrous sulfate heptahydrate	50.0	42.4
Manganese gluconate	4.8	4.1
Copper sulfate anhydrate	2.5	2.1
Zinc gluconate	12.7	10.8
Total	117.9	100

[a] Chemicals provided by Globe Die Cutting, Inc. (Metuchen, NJ)

Table 3.1.2 Composition of BioLithe™(B)

Chemical[a] Component	**Concentration[b] (g/L)**	**Concentration[c] (g/L)**		**Mass Percentage of Component (%)**
		High Conc.[d]	**Low Conc.**	
Ferric ammonium citrate	47.9	71.9	7.2	34.8
Magnesium sulfate heptahydrate	44.4	66.6	6.7	32.3
Manganese gluconate	9.6	14.4	1.4	7.0
Copper sulfate anhydrate	5.0	7.5	0.8	3.6
Zinc gluconate	30.6	45.9	4.6	22.3
Total	137.5	206.3	20.6	100

[a]Chemicals provided by Globe Die Cutting, Inc. (Metuchen, NJ)
[b]Concentration applied in Test 1 and Test 4.
[c]Concentration applied in Test 3.
[d]Concentration applied in Test 2

Table 3.1.3 Composition of BioLithe™(C)

Chemical[a] Component	**Concentration[b] (g/L)**	**Concentration[c] (g/L)**		**Mass Percentage of Component (%)**
		High Conc.[d]	**Low Conc.**	
Sodium Chloride	30.0	66.6	6.7	32.3
Potassium chloride	30.0	66.6	6.7	32.3
Magnesium chloride	18.0	39.9	4.0	19.4
Ammonium chloride	15.0	33.3	3.3	16.1
Total	93	206.4	20.6	100

[a]Chemicals provided by Globe Die Cutting, Inc. (Metuchen, NJ)
[b]Concentration applied in Test 4
[c]Concentration applied in Test 3
[d]Concentration applied in Test 2

3.1.2 Paper preparation

For testing the three types of formulae "BioLithe™(A), BioLithe™(B) and BioLithe™(C)" for methane inhibition during paper degradation, several paper types and preparation techniques were utilized.

TEST 1, Degradation of Paper Saturated with Inhibitors I: Chipboard was provided by Globe Die Cutting, Inc. (Metuchen, NJ). The paper was first weighed to prepare a 5.00 g portion to be placed in each serum bottle. Second, as required for treated paper, the 5.00 g paper sample was completely saturated with either BioLithe™(A) or BioLithe™(B), (Table 3.1.3 and Table 3.1.2) respectively. After saturation in the solution for 6 hours, the paper was removed from the solution, hung and air dried overnight. Finally, treated and untreated paper samples were reduced in particle size by cutting with scissors to less than 5 mm (one dimension) so that the paper could fit through the

bioreactor (160 mL serum bottle) opening. The resulting paper pieces were irregular in shape and size, but one dimension was <5 mm.

TEST 2, Degradation of Paper in Presence of Dissolved Inhibitors: Printer paper provided by Globe Die Cutting Inc. was punched into circular 5 mm pieces using a punch (Swingline, Lincolnshire, IL) and weighed to obtain 2.00 g portions for each bioreactor to be tested.

TEST 3, Degradation of Paper Saturated with Inhibitors II: Printer paper from Globe Die Cutting Inc. was first punched into 5 mm circular pieces using a punch (Swingline, Lincolnshire, IL) and 2.00 g was weighed out for each bioreactor. Paper was grouped and then accordingly saturated with BioLithe™(B) and BioLithe™(C) (Table 3.1.2 and Table 3.1.3) respectively in different concentrations. Saturated samples were air dried in a chemical fume hood overnight and punched into pieces prior to placement into serum bottles.

TEST 4, Degradation of Paper Coated with Inhibitors in an Industrial Process: BioLithe™(B) and BioLithe™(C), respectively, were mixed with aqueous coating at a 10% (v/v) ratio. Aqueous coating is a water-based coating applied after printing for protecting the paper surface from dirt, smudges, fingerprints and scratching. Printer paper provided by Globe Die Cutting Inc. was then coated with the mixed solutions as a very thin layer on one side of the surface. The paper coating was processed and applied to paper by an industrial partner of Globe Die Cutting Inc. at a paper printing factory. Treated and untreated paper were grouped (as shown in Table 3.1.7), weighed (2.00 g for each bottle), and reduced to an average diameter of 5 mm using a punch (Swingline, Lincolnshire, IL) so that the pieces could fit into the bioreactors.

3.1.3 BioLithe formulae added in each Test

TEST 1, Degradation of Paper Saturated with Inhibitors I: Paper was weighed before and after saturation with the BioLithe formulae made as described in Table 3.1.1 and Table 3.1.2. The compositions and weight of the solution remaining on the paper was used to determine the amount of BioLithe chemicals loaded on paper as shown in Table 3.1.4.

Table 3.1.4 Experimental Protocol for the Degradation of Paper Saturated with Inhibitors I

Description (Saturated)	**Anaerobic Mineral Medium (mL)**	**Inoculum (mL)**	**Paper[a] Mass (g)**		**BioLithe Per Paper Mass[b] (g/g)**
			Before treatment	**After treatment[b]**	
BioLithe™(A)	80	20	5.00	5.60 ±0.03	0.12 ±0.006
BioLithe™(B)	80	20	5.00	5.74 ±0.05	0.15 ±0.01
Untreated Control	80	20	5.00	5.00	na
Inoculum Control	80	20	na	na	na

[a]Paper type-Chipboard (Globe Die Cutting, Inc., Metuchen, NJ)
[b]Based on triplicates
na = not applicable

TEST 2, Degradation of Paper in Presence of Dissolved Inhibitors: In this test BioLithe formulae were added to the bottles in liquid form rather than saturating or coating the paper. The concentration of BioLithe in the bulk liquid was calculated by multiplying the volume of the BioLithe formula injected into the bioreactor by the concentration of the chemicals in the BioLithe solutions as seen in Table 3.1.5.

Table 3.1.5 Experimental Protocol for the Degradation of Paper in Presence of Dissolved Inhibitors

Description (Dissolved in Inoculum)	**Anaerobic Mineral Medium (mL)**	**Inoculum (mL)**	**Paper[a] Mass (g)**	**Aqueous Coating in Mixed Inoculum (mL/100 mL)**	**BioLithe in Mixed Inoculum (g/100 mL)**
Aqueous Coating with BioLithe™(B)	80	20	2.00	0.50	0.10
Aqueous Coating with BioLithe™(C)	80	20	2.00	0.50	0.10
Untreated Control	80	20	2.00	na	na
Inoculum Control	80	20	na	na	na

[a]Paper type-Chipboard (Globe Die Cutting, Inc., Metuchen, NJ)
na = not applicable

TEST 3, Degradation of Paper Saturated with Inhibitors II: The mass of BioLithe formula on paper was determined from the weights of the paper before saturation and after saturation and air drying (Table 3.1.6).

Table 3.1.6 Experimental Protocol for the Degradation of Paper Saturated with Inhibitors II

Name	**Anaerobic Mineral Medium (mL)**	**Inoculum (mL)**	**Paper[a] Mass (g)**		**BioLithe Per Paper Mass[b] (g/g)**
			Before treatment	**After treatment[b]**	
BioLithe™ (B) in High Concentration	80	20	2.00	2.88 ±0.006	0.44 ±0.003
BioLithe™(B) in Low Concentration	80	20	2.00	2.13 ±0.005	0.065 ±0.003
BioLithe™(C) in High Concentration	80	20	2.00	3.01 ±0.02	0.51 ±0.01
BioLithe™(C) in Low Concentration	80	20	2.00	2.12 ±0.004	0.06 ±0.002
Untreated Control	80	20	na	2.00	na
Inoculum	80	20	na	na	na

[a]Paper type- printer paper (Globe Die Cutting, Inc., Metuchen, NJ)
[b]Based on triplicates
na = not applicable

TEST 4, Degradation of Paper Coated with Inhibitors in an Industrial Process:

Paper with and without coatings in bulk were weighed to assess amounts of composite coating consisting of BioLithe chemicals and aqueous coating material on the paper. BioLithe chemicals and aqueous coating material consisted of the BioLithe solutions mixed with aqueous coating at a 10% v/v ratio. To determine the dry weight of BioLithe chemicals coated on paper, mixed samples of 20 mL were weighed in ceramic dishes and evaporated to dryness in an oven (Arieve Corporation, Round Lake, IL) at 105 ℃, for more than 12 h. The dry residue was weighed afterwards to determine the mass percentage of BioLithe chemicals in the mixed dry weight. Finally, the mass of BioLithe

chemicals and aqueous coating load on paper was computed by the respective weight percentage and total mass of the composite coating.

Table 3.1.7 Experimental Protocol for the Degradation of Paper Coated with Inhibitors in an Industrial Process

Description (Treatment)	**Anaerobic Mineral Medium (mL)**	**Inoculum (mL)**	**Paper[a] Mass (after coating)[b] (g)**	**Coating Per Paper Mass (mg/g)**	**BioLithe Per Paper Mass (mg/g)**
Aqueous Coating[c]	80	20	2.00	16.40	na
Aqueous Coating with BioLithe™(B)	80	20	2.00	14.94	0.46
Aqueous Coating with BioLithe™(C)	80	20	2.00	18.88	0.62
Untreated Control	80	20	2.00	na	na
Inoculum	80	20	na	na	na

[a]Paper type- printer paper (Globe Die Cutting, Inc., Metuchen, NJ)
[b]Mass based on paper weight after coating. Mass of coating on paper is so little that it could be neglected.
[c]Aqueous coated paper without BioLithe treatment
na = not applicable

3.1.4 Anaerobic minimal salts medium

Anaerobic mineral salts medium to support methanogenic growth was prepared according to Table 3.1.8 (Shelton and Tiedje, 1984).

Table 3.1.8 Anaerobic minimal medium composition

Component	Amount per L medium
KCl	1.3 g
KH_2PO_4	0.2 g
NaCl	1.17 g
NH_4Cl	0.5 g
$CaCl_2$-$2H_2O$	0.1 g
$MgCl_2$-$6H_2O$	0.18 g
$NaHCO_3$	2.5 g
Resazurin	1 mL
Vitamin Solution[a]	5 mL
Trace Salts I[b]	1 mL
Trace Salts II[c]	0.1 mL

a Vitamin solution contained g/L: 0.02, d-biotin; 0.02, folic acid; 0.1, pyridoxine hydrochloride; 0.05, thiamin hydrochloride; 0.05, riboflavin; 0.05, nicotinic acid; 0.05, DL-calcium pantothenate; 0.01, vitamin B12; 0.05, *p*-aminobenzoic acid; 0.05, lipoic acid; 0.04, 1,4-naphthaquinone; 0.1, nicotinamide; and 0.01, hemin.

b Trace Salts I stock solution contained g/L: 5, $MnCl_2$-$6H_2O$; 0.5, H_3BO_4; 0.5, $ZnCl_2$; 0.5, $CoCl_2$-$6H_2O$; 0.46, $NiCl_2$-$6H_2O$; 0.3, CuCl-$2H_2O$; 0.1, $NaMoO_4$-$2H_2O$; and 1.49, $FeCl_2$-$4H_2O$.

c Trace Salts II stock solution contained g/L: 0.03, $NaSeO_3$; and 0.08, Na_2WO_4.

3.1.5 Inocula

For TEST 1, Paper Saturated with Inhibitors I, inoculum was prepared under anoxic conditions by mixing on a volume:volume basis: one third mesophilic anaerobic digester sludge from the Joint Meeting of Essex and Union Counties (JMEUC) Sewerage Authority in Elizabeth, NJ; one third pond sediment from Boyd Ponds, North Brunswick, NJ; and one third material from aged landfill microcosms (MSW Landfill, Burlington,

NJ). After mixing these inoculum sources, 20 mL was added to each test bottle under an anaerobic 70% N_2/30% CO_2 purge gas.

For the remaining experiments, TEST 2, Paper in Presence of Dissolved Inhibitors, TEST 3, Paper Saturated with Inhibitors II, and TEST 4, Paper Coated with Inhibitors in an Industrial Process, mesophilic anaerobic digester sludge from JMEUC was exclusively employed as inoculum to maximize the degradation extent and efficiency, since it was thought that the methanogen concentration in this material was greater than the other inoculum sources (pond sediment and landfill microcosms), and to maintain a more unified /constant microorganism community for the different experiments.

3.1.6 Experimental setup of bioreactors

Methane inhibition tests were conducted to compare methane production from paper with different inhibitory treatments of BioLithe™(A), BioLithe™(B) or BioLithe™(C), to methane production from untreated (control) paper. Each experiment was conducted using bioreactors consisting of 160 mL serum bottles (Wheaton Science Products, Millville, NJ). For each test, the appropriate paper was placed into the serum bottle and a purge gas of 70% N_2 and 30% CO_2 was introduced and maintained during the remaining steps. Except for Test 3, anaerobic inoculum (described in section 3.1.5) was added and each bottle was filled to the 100 mL mark with anaerobic mineral medium (described in Table 3.1.8). The serum bottles were sealed with butyl rubber stoppers (Wheaton Science Products, Millville, NJ) and crimped with aluminum seals (Agilent, South Plainfield, NJ). In Test 3, 20 mL anaerobic digester sludge was added via a 50 mL disposable syringe (Becton, Dickinson and Company, Franklin Lakes, NJ) which had

been flushed with nitrogen gas after the bottles were sealed. Bioreactors were treated and grouped as shown in Table 3.1.6.

For Test 2, after all bottles were sealed with butyl rubber stoppers, BioLithe solutions were purged with oxygen-free 70%N_2/30%CO_2 for 30 min and then injected into bottles using a 1 mL disposable syringe (Becton, Dickinson and Company, Franklin Lakes, NJ) that had been flushed with nitrogen, in accordance with Table 3.1.5.

Triplicate bioreactors were set up for each treatment. The serum bottle reactors were operated as shaken (150 rpm) batch systems at 37 ℃. Triplicate control bottles amended with inoculum only, to correct for biogas production by endogenous decay of the inoculum, were also run. There were 18 groups (Test 2 and Test 4 shared the same Inoculum group) of triplicate bottles or 54 bottles in total.

3.2 Analytical Methods

3.2.1 Biogas volume measurement

Biogas was released from reactors once or twice per week depending on gas production and at the same time the volume was measured at atmospheric pressure using a water displacement system constructed from a 100 mL or 500 mL burette and a water tank open to the atmosphere.

3.2.2 Methane measurement

Biogas was analyzed for methane content (%), determined by removing an 0.25 mL headspace sample from each reactor using a gas-tight syringe (Valco® Precision Sampling, Baton Rouge, LA), and injecting the sample into a Shimadzu GC-8AI gas chromatograph (GC) equipped with a thermal conductivity detector and a 15 ft x 1/8 in.

stainless steel column packed with Carboxen-1000 (60/80 mesh) (Sigma-Aldrich, Columbia, MD). Standards for methane over a concentration range from 0 to 100% by volume in air were prepared at Day 0, 45 and 90 days using certified gas standards for methane (99% purity; Matheson Tri-Gas, Inc., Montgomeryville, PA).

3.2.3 pH test

Some bioreactors were tested for pH in order to ascertain the environmental conditions for the microorganisms. The pH value was tested by saturating Hydrion Insta-Chek pH Paper 0-13 (Micro Essential Laboratory, Brooklyn, NY) with a small amount of liquid which had been removed from the serum bottle under anoxic conditions.

3.3 Statistical Analyses

Biogas and methane measurement was a direct indicator to analyze the effects of the three BioLithe formulae on methane inhibition during paper degradation under anaerobic conditions. One-way Analysis of Variance (one-way ANOVA) in conjunction with Tukey's Studentized Range Test (Tukey's HSD (Honestly Significant Difference)) was then employed to statistically analyze the significance of numerical data.

In every test, treatment groups were compared with the untreated group together by ANOVA, and each treated group was then separately compared to the "Untreated Control" by the Tukey's HSD test to find which mean was significantly different from the control group. The significant level was set to $\alpha = 0.05$. The statistical analysis was conducted using SAS® 9.1.3 (SAS Institute Inc., Cary, NC, USA). The bottle set "Inoculum Control", which was set up for to determine the methane production potential from inoculum alone, was excluded from the statistical analysis.

3.4 Molecular Biology Analysis

3.4.1 DNA extraction and Polymerase Chain Reaction (PCR)

DNA extraction from the microcosm samples was performed using the PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) from 1 mL slurry samples according to the manufacturer's instructions. The DNA extracts were analyzed by 1.5% agarose gel electrophoresis. The DNA was electrophorized in the gel by staining in 0.1% ethidium bromide (EtBr) (Bio-Rad Laboratories, Hercules, CA, USA) solution for 20 min and visualized using UV on a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA, USA).

Different primers sets were used separately to amplify 16S ribosomal RNA (16S rRNA) genes of members of the archaea and bacteria in DNA extracts by means of polymerase chain reaction (PCR). The PCR amplification of partial archaeal 16S rRNA genes was performed by using primers 0348aF (with GC-clamp), which has been used for sequencing of methanogenic archaea 16S rRNA genes (Achenbach et al., 1995), and 0691R, which was modified as reported previously (Watanabe et al., 2004) to improve the specificity (see Table 3.4.1). The PCR mixture (50 µL) contained 0.5 µL of forward and reverse primers (50 pmol μL^{-1} each) (USB Corporation, Cleveland, OH), 0.5 µL of *Taq* polymerase (USB), 5 µL of 10x buffer (USB), 1.25 µL of dNTP mixture (10 mM each) (USB), 4 µL of Mg^{2+} mixture (25 mM Mg^{2+} each) (USB), 2 µL of DNA template, and balance autoclaved MilliQ water. PCR amplification was performed using a Bio-Rad Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: 94 ℃ for 3 min (initial denaturation), 40 cycles of denaturation at 94 ℃ for 1

min, annealing at 49 ℃ for 1 min and extension at 72 ℃ for 2 min, and a final extension at 72 ℃ for 8 min.

The PCR amplification of partial bacterial 16S rRNA genes was performed with forward (338-GC F/ 27 F) and reverse primers (519 R), as shown as Table 3.4.1. The amplification program was performed as follows (Watanabe et al., 2004): denaturation at 94 ℃ for 5 minutes (Achenbach et al., 1995); 30 cycles (or more depending upon the concentration of DNA in the template) of denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s and extension/elongation at 72 ℃ for 30 s. The final elongation step was occasionally performed at a temperature of 72 ℃ for 7 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA was fully extended. The final hold was at 4 ℃ for an indefinite time. PCR products from the initial and diluted genomic DNA extracts were analyzed by 1.5% agarose gel electrophoresis and the resulting amplicons were visualized in the gel by staining in 0.1% ethidium bromide (EtBr) (Bio-Rad Laboratories, Hercules, CA, USA) solution for 20 min and then imaging using a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA, USA).

Table 3.4.1 Primer sets for PCR analysis

Primer		Sequence	Reference
Archaea (Methanogenic)	0348aF	TCC AGG CCC TAC GGG	Achenbach et al., 1995
	0691R	GGA TTA CAR GAT TTC AC	Watanabe et al., 2004
	GC-Clamp	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G	Muyzer et al., 1993
Bacteria	27F	AGA GTT TGA TCM TGG CTC AG	Lane, D. J. 1991
	338F	TCC TAC GGG AGG CAG CAG	Nakatsu et al., 2000
	519R	ATT ACC GCG GCT GCT GG	Nakatsu et al., 2000
	GC-Clamp	CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CC	Nakatsu et al., 2000

Denaturing gradient gel electrophoresis (DGGE) (Asakawa et al., 1993) of PCR amplified community DNA was carried out and dominant bands were excised and sequenced to examine the community diversity by comparing sequences with those available in the 16S rRNA gene libraries of Genbank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The PCR-DGGE product was applied onto an 8% (w/v) polyacrylamide gel in 1×TAE. The denaturant gradient range of the gel, in which 100% denaturant contained 7 M urea and 40% (v/v) formamide, was from 25-65% for archaea analysis, and 20-60% for bacteria analysis. Electrophoresis was run at 60 ℃ for 14 h at 100 V for the archaeal product and 16 h at 60 V for the bacterial product. The timing varied depending upon the gradient of the DGGE gel.

Nucleotide sequences of DNA fragments recovered from bands on DGGE gels were determined by the following method. The gel strip of a band was excised from a DGGE gel, and the DNA was eluted in 20 μL MilliQ water at 4 ℃ overnight. The DNA

fragment was amplified from the eluted DNA by PCR, and the PCR products were further purified with UltraClean PCR Clean-up kit (Mo-bio, Solana Beach, CA) according to the manufacturer's instructions. Clean gene fragments were then sent for sequencing to Genewiz, Inc (Plainfield, NJ).

Sequencing results of amplified 16S rRNA gene fragments were analyzed and compared to the sequences in GenBank database by BlastN (National Center for Biotechnology Information database) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 TEST 1, Degradation of Paper Saturated with Inhibitors I

4.1.1 Biogas and methane measurements

Paper samples treated by two different solutions – BioLithe™(A) and BioLithe™(B) (Experimental protocol see Table 3.1.4) – were tested for biogas and methane production and compared to untreated paper. After setup, the medium in the bottles was clear, indicating reduced conditions (note that the resazurin indicator included in the medium produces a pink color under oxidizing conditions), however, the color of the contents was light brown (Figure 4.1.1). Cumulative biogas production for each treatment over 119 days is shown in Figure 4.1.2. The untreated control produced the highest amount of biogas, and the color of the material turned darker within a few days (indicating a more reduced environment) (see Figure 4.1.1). The BioLithe™(A) treatment produced low amounts of biogas until 21 days of operation, when material in two replicates turned darker, and biogas production substantially increased. Color change in the third BioLithe™(A) replicate occurred around day 35 and its biogas yield thereafter increased substantially. BioLithe™(B) exhibited low biogas production throughout the test and the color of the materials in the reactors remained light brown. BioLithe™(A) and the Untreated Control produced the largest volumes of methane, as shown in Figure 4.1.3.

Biogas production by the BioLithe™(A) and Untreated Control increased rapidly in the first few weeks, but stabilized in BioLithe™(B) and Inoculum after 14 days of

operation. With respect to the effectiveness of the paper treatments, BioLithe™(B) had the greatest impact and achieved nearly total suppression of all biological activity, including methanogenesis. Thus it was apparent that paper degradation in BioLithe™(B) was almost completely inhibited.

Figure 4.1.1 Photograph of different treatments during TEST 1, Degradation of Paper Saturated with Inhibitors I. Note color differences where darker bottles produce greater amounts of biogas and methane than lighter colored bottles.

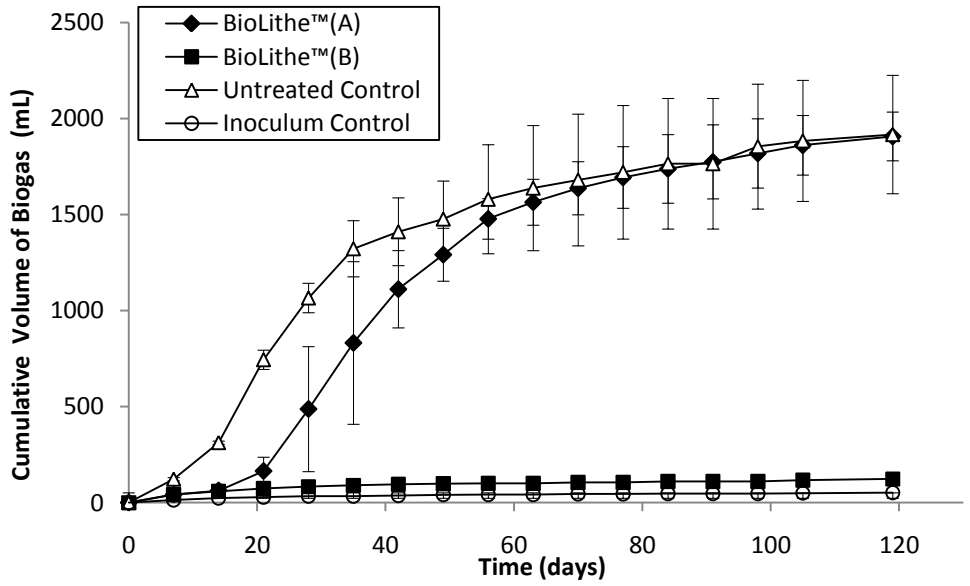


Figure 4.1.2 Cumulative volume of biogas produced by different treatments during TEST 1, Degradation of Paper Saturated with Inhibitors I. Symbols are averages of triplicate bottles and error bars are one standard deviation.

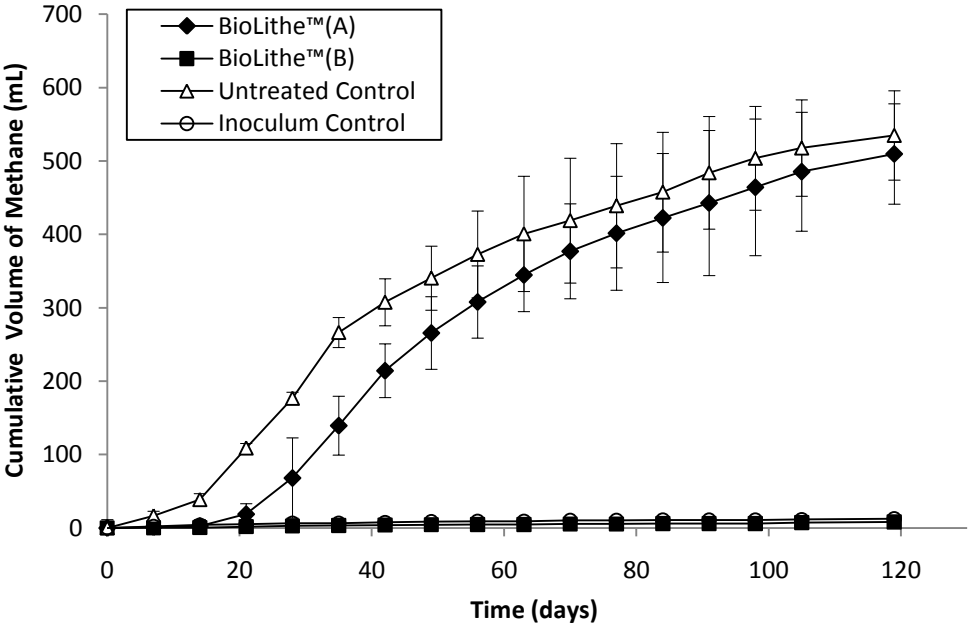


Figure 4.1.3 Cumulative volume of methane produced by different treatments during TEST 1, Degradation of Paper Saturated with Inhibitors I. Symbols are averages of triplicate bottles and error bars are one standard deviation.

With comparison of biogas and methane generation, it was noted that the cumulative methane production of Untreated Control was about one fourth of that of cumulative biogas production, less than the theoretically stoichiometrical equation (section 3.1.1). Paper samples used in this test were chipboard which had a lower cellulose component than printer paper, which therefore resulted in less methane production. Also, the methane content (%) in the biogas during the first few weeks was rather low, about 20-30%, and this number had gradually increased as the paper degradation proceeded. The methane content (%) increased to approximately 50% in the latter phase of the test, and this higher methane content was maintained as the test continued.

4.1.2 Statistical Analyses

TEST 1, Degradation of Paper Saturated with Inhibitors I: Analysis of Variance (ANOVA) showed that numerical data from both cumulative biogas and cumulative methane production were significantly different between treatments and the untreated control, with a P-value < 0.0001 (Table 4.1.1). According to Tukey's Studentized Range (HSD) Test, cumulative biogas and methane generation from the BioLithe™(B) treatment was significantly lower than those of the Untreated Control. However there was no statistical difference between BioLithe™(A) and the Untreated Control for biogas and methane production.

With results from ANOVA analysis, it was apparent that BioLithe™(B) had a significant effect on inhibition of biogas and methane, by reducing biogas approximately 93.56% and methane 98.48%, while BioLithe™(A) had no statistically significant reduction in gas production compared to the Untreated Control. Thus, testing of

BioLithe™(A) was discontinued for the subsequent BioLithe tests, but BioLithe™(B) was carried into next step of the testing and analysis. Note that complete and permanent inhibition of methane production is not the ultimate goal for the BioLithe treatments. Rather, delay of methanogenesis or channeling the carbon released during degradation to carbon dioxide, instead of methane, is the desired effect. In a landfill environment it is likely that the inhibitors present on the paper would be eventually be diluted or washed out during leachate movement, allowing biological activity to recover. In this case however, the significant effect of BioLithe™(B) indicated that it could be useful with respect to delaying methane production, and was thus tested further.

Table 4.1.1 Results of TEST 1, Degradation of Paper Saturated with Inhibitors I

Comparison Group	Biogas			Methane		
	ANOVA	HSD[b]	Biogas Reduction[c] (%)	ANOVA	HSD[b]	Methane Reduction[c] (%)
Treated groups[a] and Untreated Control	**P-value <0.0001**			**P-value <0.0001**		
BioLithe™(A) and Untreated Control		NSD	0.51		NSD	4.72
BioLithe™(B) and Untreated Control		**SD**	93.56		**SD**	98.48

[a] BioLithe™(A) and BioLithe™(B)
[b] NSD means not significantly different; SD means significantly different
[c] Based on average gas production of each group

4.2 TEST 2, Paper Degradation in Presence of Dissolved Inhibitors

4.2.1 Biogas and methane measurements

Biogas and methane production from the degradation of paper in the presence of dissolved inhibitors in the form of BioLithe™(B) or BioLithe™(C) (prepared in the aqueous coating) was compared to biogas and methane production from plain paper (as an Untreated Control). For experimental protocol see Table 3.1.5.

Biogas production is shown in Figure 4.2.1. The test was operated for 99 days, and in the last two weeks, all bioreactors yielded very little gas. Thus it could be assumed that the paper degradation was close to the later stages, and that microorganisms in each bottle were in the stationary phase. One bioreactor in the Aqueous Coating with BioLithe™(B) treatment was lost on day 78, thus data of this triplicate was eliminated. Until day 99, the Untreated Control had cumulatively produced the most biogas. Aqueous Coating with BioLithe™(B) and Aqueous Coating with BioLithe™(C) produced similar amounts of biogas which were substantially less than that of the Untreated Control (see Figure 4.2.1). Aqueous Coating with BioLithe™(B) produced 225.34 ± 42.76 mL (based on duplicates) biogas and Aqueous Coating with BioLithe™(C) produced 236.32 ± 71.22 mL biogas less than the Untreated Control. Standard deviations were relatively large in the middle portion of the test, resulting from uneven performance in the triplicates in each bottle set. In the later phase, the bioreactors that had exhibited a lag in biogas production, began to catch up and subsequently accumulated additional biogas, which led to smaller error bars at the end of the test. Note that the Inoculum Control was run with slightly different time points of analysis.

The results of methane production corresponded to biogas production where the Untreated Control had exhibited the most methane potential at the beginning of the test and maintained that trend until the end of the test. Aqueous Coating with BioLithe™(B) and Aqueous Coating with BioLithe™(C) accumulated lower methane amounts as indicated in Figure 4.2.2, and until day 99, the latter accumulated the least volume of methane. Aqueous Coating with BioLithe™(B) had 75.65 ± 1.23 mL (based on duplicates) less methane, while Aqueous Coating with BioLithe™(C) had 78.52 ± 20.82 mL less methane than that of Untreated Control. The cumulative gas production curves climbed rapidly at the beginning of the test, but began to level off at the end of it, indicating declining rates of gas generation.

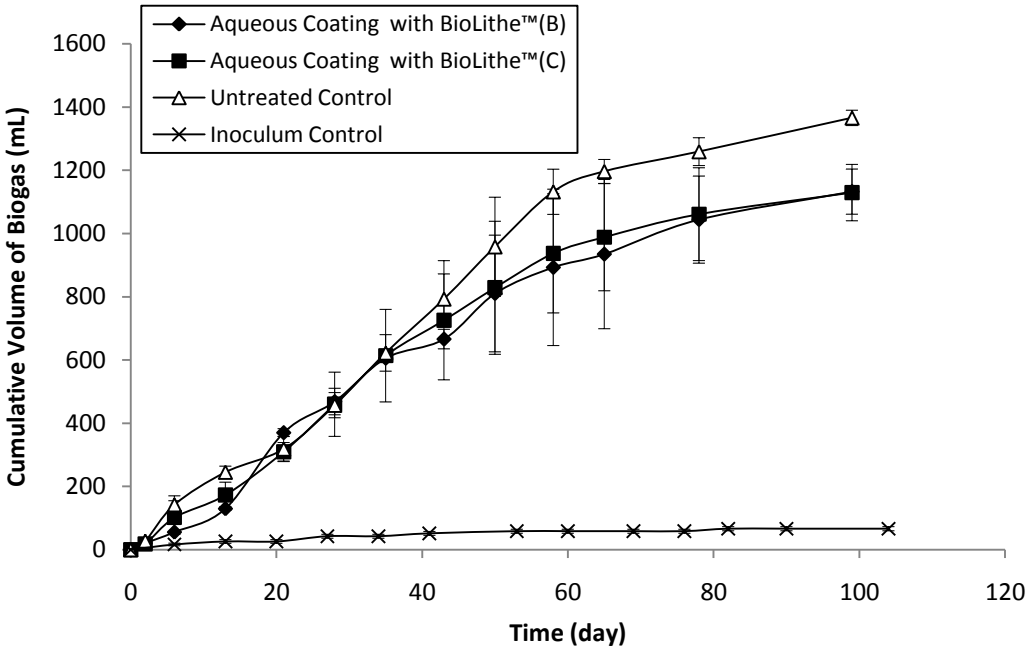


Figure 4.2.1 Cumulative volume of biogas produced by different treatments during TEST 2, Paper Degradation with in Presence of Dissolved Inhibitors. Symbols are averages of triplicate bottles and error bars are one standard deviation.

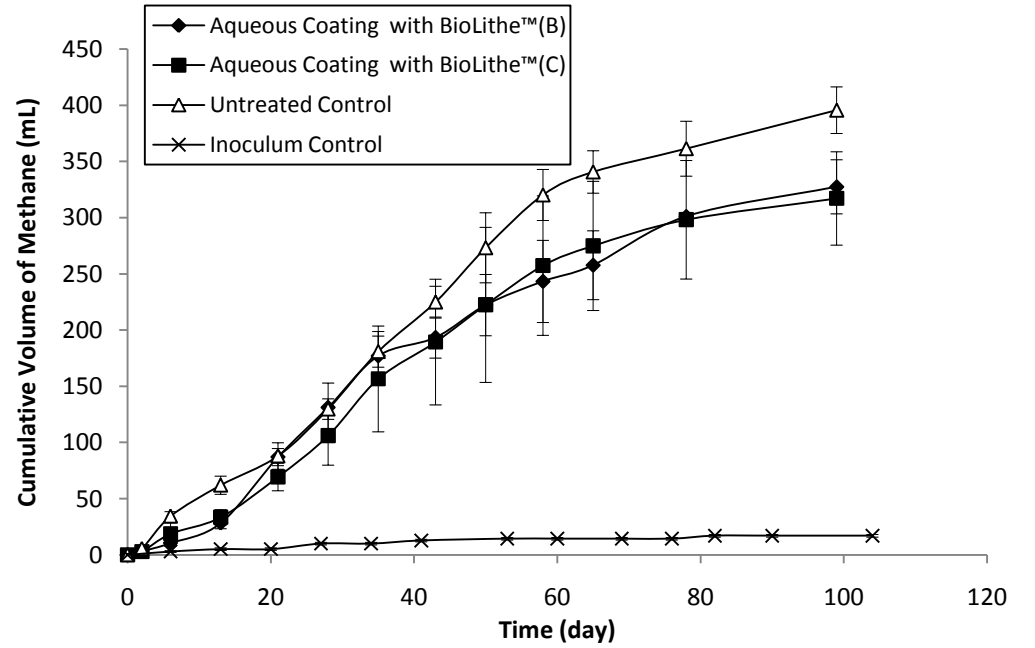


Figure 4.2.2 Cumulative volume of methane produced by different treatments during TEST 2, Paper Degradation with in Presence of Dissolved Inhibitors. Symbols are averages of triplicate bottles and error bars are one standard deviation.

4.2.2 Statistical analyses

TEST 2, Paper Degradation with in Presence of Dissolved Inhibitors: A new BioLithe formula BioLithe™(C) was substituted for BioLithe™(A) in the continuing testing and analysis. The test was designed to analyze the direct effects of dissolved BioLithe solutions (BioLithe™(B) and BioLithe™(C)) during paper degradation. It was observed that cumulative biogas production was statistically different as indicated by a P-value < 0.05, while the cumulative methane production was not different, showing a P-value > 0.05 (ANOVA). When individually compared to the Untreated Control, Aqueous Coating with BioLithe™(B) and Aqueous Coating with BioLithe™(C) had significant difference in biogas production, however had no significant difference in methane production Tukey's Studentized Range (HSD) Test.

Tukey's Studentized Range (HSD) Test concluded that biogas production of Aqueous Coating with BioLithe™(B) and Aqueous Coating with BioLithe™(C) were significantly lower than the Untreated Control by approximately 17% as shown in Table 4.2.1. Methane production in the treated groups was about 17-20% lower than the untreated group, however this difference was not statistically significant. It was thus difficult to make the conclusion that BioLithe™(B) and BioLithe™(C) had an influence on biogas and methane reduction because the results were also affected from the addition of Aqueous Coating, which was initially added in both treatment groups, but was unfortunately lacking in the control group. It was thus necessary to eliminate the effect and interference from the Aqueous Coating, and the study progressed into next phase.

Table 4.2.1 Results of TEST 2, Paper Degradation with in Presence of Dissolved Inhibitors

Comparison Group	Biogas			Methane		
	ANOVA	HSD[b]	Biogas Reduction[c] (%)	ANOVA	HSD[b]	Methane Reduction[c] (%)
Treated groups[a] and Untreated Control	**P-value < 0.05**			P-value > 0.05		
Aqueous Coating with BioLithe™(B) and Untreated Control		**SD**	17.06		NSD	17.23
Aqueous Coating with BioLithe™(C) and Untreated Control		**SD**	17.3		NSD	19.84

[a] Aqueous Coating with BioLithe™(B) and Aqueous Coating with BioLithe™(C)
[b]SD means significantly different; NSD means not significantly different
[c]Based on average gas production of each group.

4.2.3 Analysis of genomic DNA of methanogenic archaea and bacteria by DGGE

Bioreactors were sampled on day 69 for DNA extraction and DGGE analysis. Primer pairs 0348aF-GC and 0691R; 338F-GC and 519R (Table 3.4.1) were used to amplify 16 S rRNA genes from methanogenic archaea and bacteria by PCR from genomic DNA as described in section 3.3.1. Ratios of template to product varied among different bottle sets, as shown in Figure 4.2.3 and Figure 4.2.4. For example, products of Untreated Control display dark and thick bands while those of A and B were relatively light.

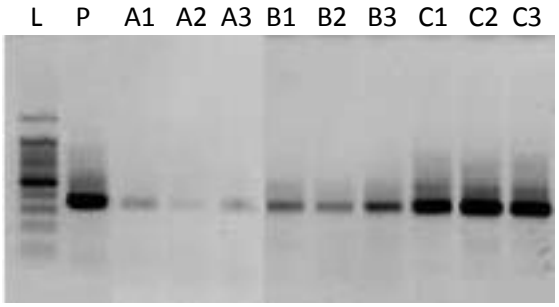


Figure 4.2.2 PCR products of methanogenic archaea in TEST 2, Paper Degradation with Presence of Dissolved Inhibitors. A represents Aqueous Coating with BioLithe™(B); B, Aqueous Coating with BioLithe™(C); C, Untreated Control (in triplicates); L, ladder; P, digester sludge as positive control.

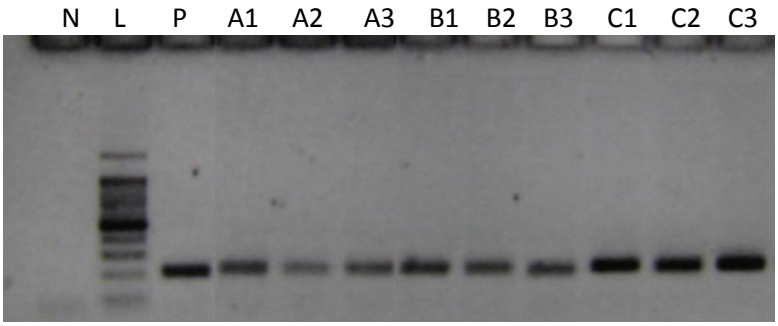


Figure 4.2.3 PCR products of bacteria in TEST 2, Paper Degradation with Presence of Dissolved Inhibitors. A represents Aqueous Coating with BioLithe™(B); B, Aqueous Coating with BioLithe™(C); C, Untreated Control (in triplicates); N, negative control (PCR products without DNA template); L, ladder; P, digester sludge as positive control.

Methanogenic archaeal and bacterial PCR products were then analyzed by DGGE and results are shown in Figure 4.2.5 and Figure 4.2.6, respectively. For the archaeal analysis, samples from different bioreactors had different banding patterns. All bottle sets had differing banding patterns from the digester sludge that was the source of original inoculum and used here as a positive control. This indicates that the microbial community was affected by the presence of paper with/without treatments, and had therefore shifted away from the original inoculum profile by 69 days of operation. While columns

corresponding to C (Untreated Control) demonstrate clear and dark bands, suggesting the presence of methanogens in the Untreated Control, columns corresponding to A (Aqueous Coating with BioLithe™(B)) and B (Aqueous Coating with BioLithe™(C)) exhibited bands that were relatively lighter, weaker and more highly variable between triplicates. This difference might result from the inhibitory/competitive effects of the BioLithe chemicals. For bacterial analysis, columns C had stronger banding patterns than those of A and B, indicating a possibility that bacteria were also affected by BioLithe solutions.

It was observed that triplicate bioreactors in a group actually did not result in the same band patterns in DGGE gels. For example, the triplicate samples from Aqueous Coating with BioLithe™(C) indicated in columns B of Figure 4.2.5 show quite different banding patterns. However, treated groups did have relatively distinct differences when compared with the control group. These differences in triplicates could also have formed during the initiation of the bioreactors, or gradually shifted during the period of the paper decomposition process. Therefore, it was important to trace the structural changes in the microbial community during the time progression of anaerobic degradation. This was carried out for Test 3 as described in Section 4.3.

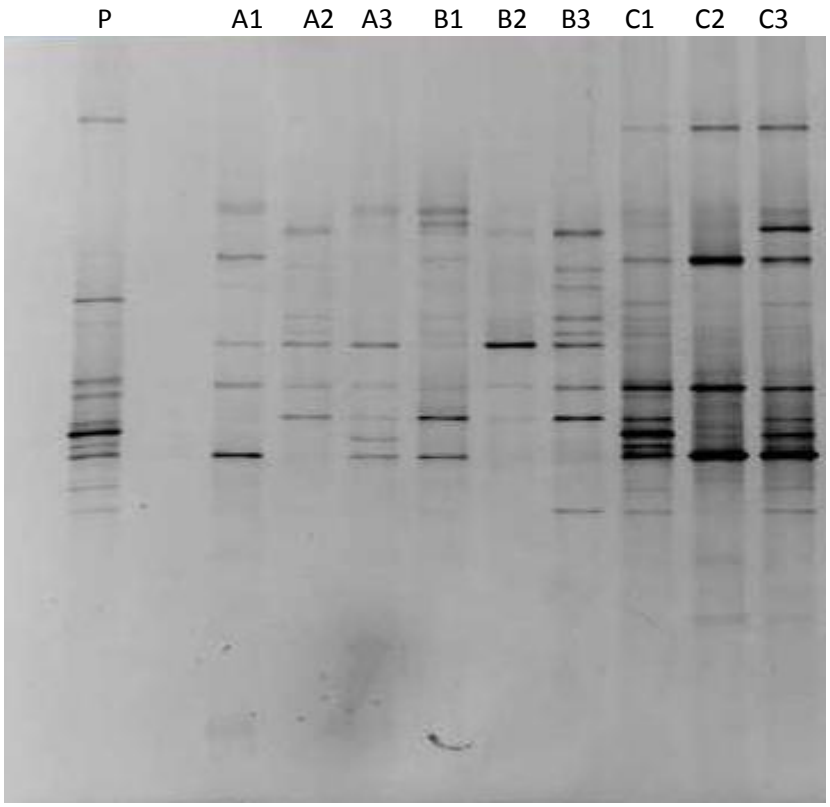


Figure 4.2.4 DGGE band patterns of methanogenic archaeal PCR products from TEST 2, Paper Degradation with Presence of Dissolved Inhibitors. A represents Aqueous Coating with BioLithe™(B); B, Aqueous Coating with BioLithe™(C); C, Untreated Control (in triplicates); L, ladder; P, digester sludge as positive control.

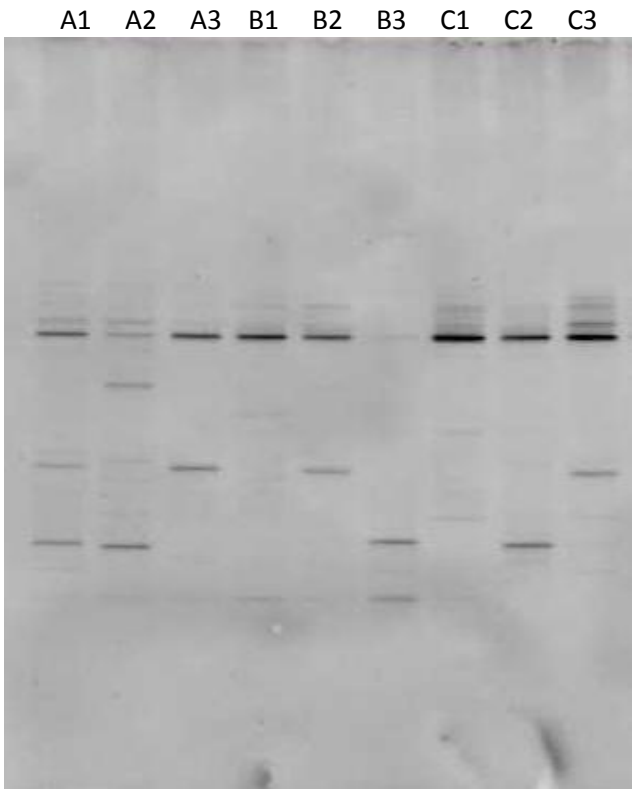


Figure 4.2.5 DGGE band patterns of bacterial PCR products from TEST 2, Paper Degradation with Presence of Dissolved Inhibitors. A represents Aqueous Coating with BioLithe™(B); B, Aqueous Coating with BioLithe™(C); C, Untreated Control (in triplicates).

4.3 TEST 3, Degradation of Paper Saturated with Inhibitors II

4.3.1 Biogas and methane measurements

The cumulative volume of methane produced during Test 3 is shown in Figure 4.3.1. During the 98 days of operation, standard deviations in some treatments were quite substantial. High deviations resulted from inconsistent behaviors among triplicates, making data more difficult to interpret. The gas generation curves showing substantial standard deviations included BioLithe™(B) in Low Concentration, BioLithe™(C) in High Concentration, and BioLithe™(C) in Low Concentration. The high variability among triplicates could be suggestive of inconsistent inoculation, poor bottle set up, or perhaps, inconsistently occurring toxicity caused by uneven distribution of the solutions on the paper. To investigate this phenomenon, genomic DNA of methanogenic archaea in bioreactors was analyzed to determine whether bottles exhibiting outlier behavior with respect to methane production also exhibited a different microbial community than other bottles.

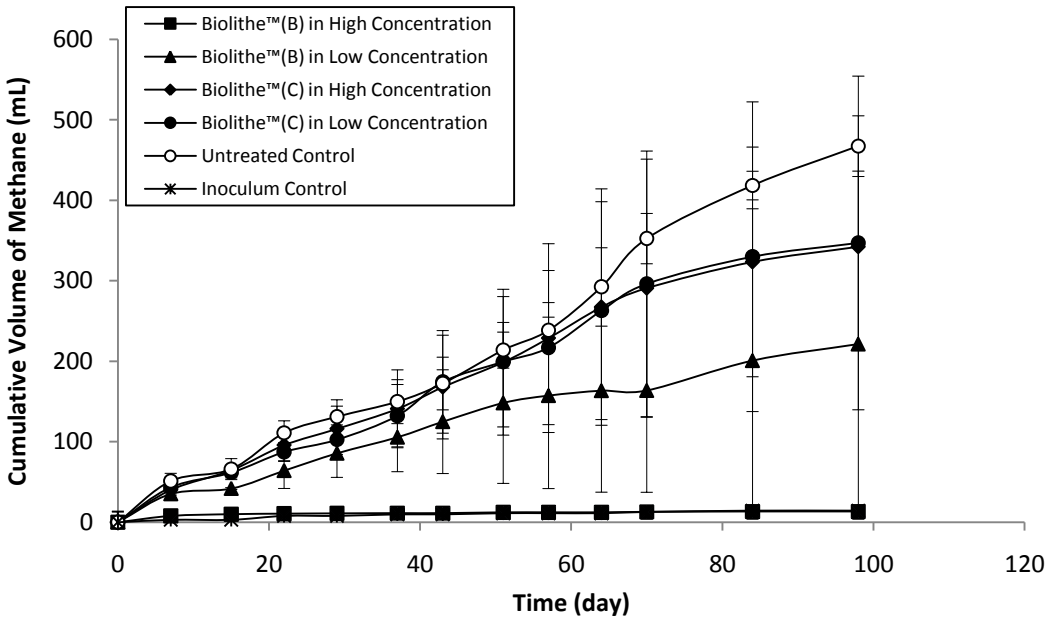
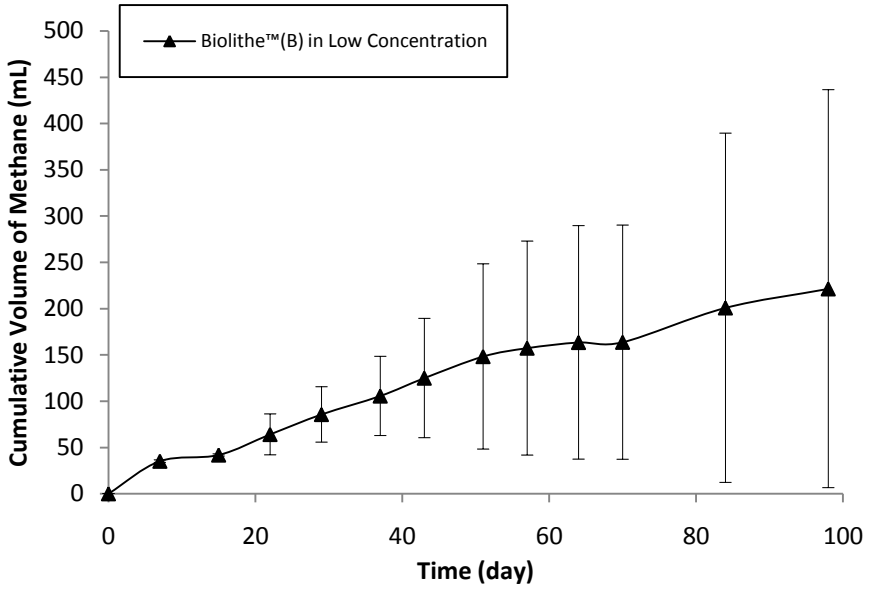


Figure 4.3.1 Cumulative volume of Methane produced by different treatments during TEST 3, Degradation of Paper Saturated with Inhibitors II. Symbols are averages of triplicate bottles and error bars are one standard deviation.

Figure 4.3.2, Figure 4.3.3 and Figure 4.3.4 show cumulative methane production and corresponding DNA band patterns on DGGE gels from Bottle Set B (BioLithe™(B) in low concentration), C (BioLithe™(C) in high concentration) and D (BioLithe™(B) in low concentration), respectively, with primers 0348 aF-GC and 0619 R (Table 3.4.1). The methanogenic archaeal community in each bioreactor was expected to be complex and abundant, because inocula utilized for this experiment was anaerobic digester sludge. However, among banding patterns in Bottle Set B, only B1 displayed a clear resolution and good separation of DNA bands, while B2 had only one distinct band and B3 had bands that were rather light and thin and difficult to distinguish. C1 and C3 had similar band patterns while C2 had only a single distinct band. Distinctive and clear patterns were observed for D1 and D2, but no bands could be seen in D3. The patterns shown in

B2, B3, C2 and D3 indicated quite different microbial community conditions from their corresponding replicates. Also, these four bioreactors had rather low cumulative biogas and methane production, which were more than 50% reduced compared to their triplicates and thus resulted in high standard deviation.



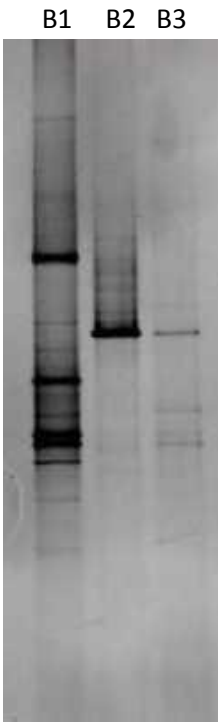
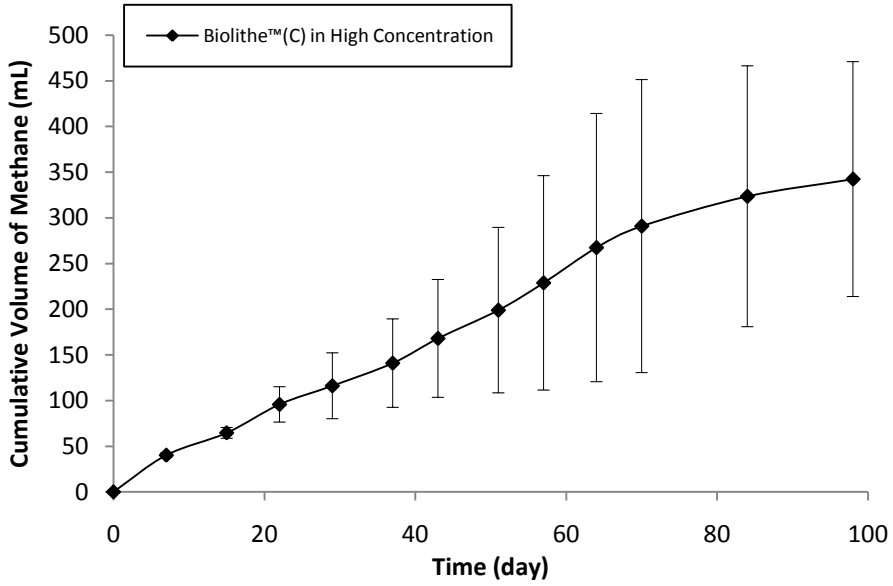


Figure 4.3.2 Cumulative Methane (top) and DGGE band patterns (top right) on day 98 of Bottle Set B (BioLithe™(B) in low concentration)



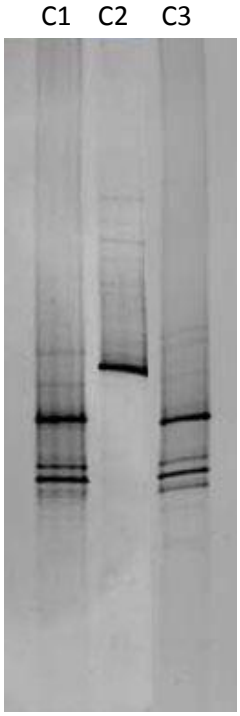
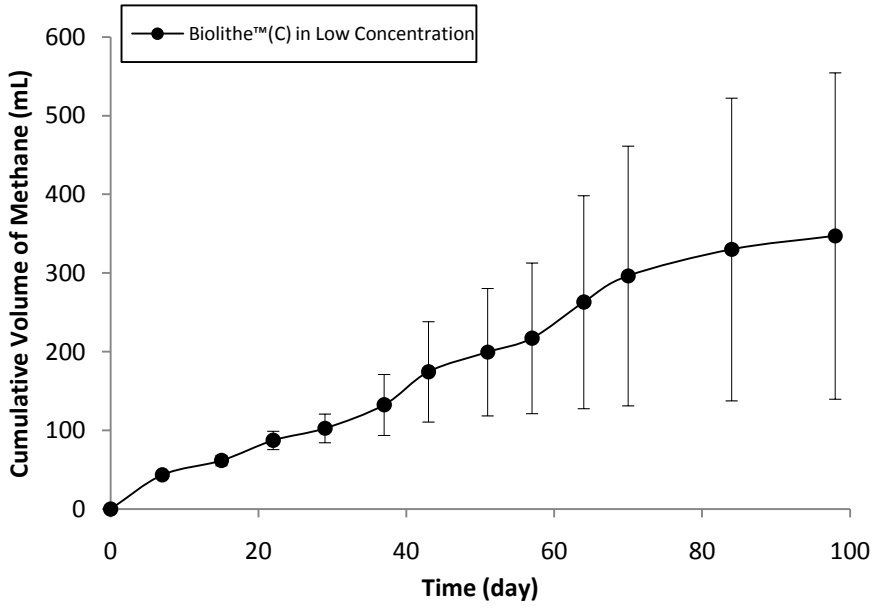


Figure 4.3.3 Cumulative Methane (top) and DGGE band patterns (top right) on day 98 of Bottle Set C (BioLithe™(C) in high concentration)



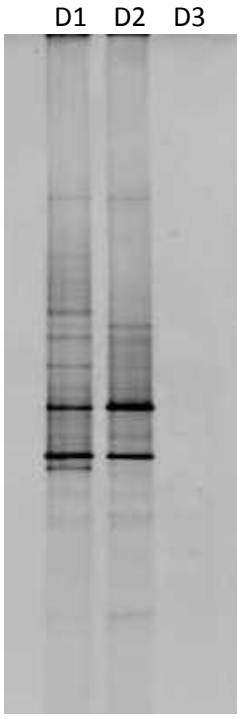


Figure 4.3.4 Cumulative Methane (top) and DGGE band patterns (top right) on day 98 of Bottle Set D (BioLithe™(C) in low concentration)

To determine if differences in methane production in each bottle set corresponded to (or were caused by) deviations in pH, bioreactor bulk liquid was also analyzed for pH. Many studies point out that growth of methanogens is closely associated with the pH in their bulk environment. Usually the pH of digester in a sewage treatment plant is 7 to 7.5, and these values are generally thought to be the best pH range for digesters in general (Fry, 1973).

Experiments conducted by Fry indicated a series of pH changes through the methane digestion process (Fry, 1973). During the initial acid phase of digestion, which may last about two weeks, the pH may drop to 6 or lower, while a great deal of CO_2 is given off. This is followed by about three months of a slow decrease in acidity during which volatile fatty acids and nitrogen-containing organic compounds are digested, and ammonia compounds are formed. As digestion proceeds, less CO_2 and more methane is produced and the pH rises slowly to about 7. As the mixture becomes less acidic, methane fermentation takes over. The pH then rises above the neutral point (pH = 7), to between pH 7.5 and 8.5. After digestion has stabilized, the pH is expected to remain around 8.0 to 8.5 (Fry, 1973). Some research also pointed out that a small decrease in pH resulting from the introduction of acidic materials significantly decreased CH_4 production. A slight increase in soil pH, however, resulted in an enhancement of CH_4 production (Wang et al., 1993).

The pH of each bottle set was thus measured and the results are shown in Table 4.3.1. Bottle Set A (BioLithe™(B) in high concentration) had a pH near neutrality (7.3) and other groups had a pH of near 8, except for replicates B2, B3, C2, and D3, whose pHs were approximately 4, much lower than expected. Bioreactors with a pH around 8

suggest that organic matter degradation was close to the latter phase and that digestion had been stable, while those with lower pH (pH about 4) had accumulated acids, a negative environment for digestion and methanogens were inactive.

Table 4.3.1 pH of each bottle set in the test of "TEST 3, Degradation of Paper Saturated with Inhibitors II" on day 98

BioLithe™(B) in high concentration		**BioLithe™(B) in low concentration**		**BioLithe™(C) in high concentration**		**BioLithe™(C) in low concentration**		**Untreated Control**		**Inoculum**	
A1	7.2	B1	8	C1	8	D1	8.2	E1	8.4	F1	8.4
A2	7.3	B2	4	C2	4.5	D2	8.2	E2	8.4	F2	8.4
A3	7.3	B3	4	C3	8	D3	4	E3	8.4	F3	8.4

It is difficult to determine whether B2, B3, C2 and D3 were functioned by BioLithe formulae so that the system remained in the initial acidic phase and methanogens were inhibited; or whether they simply were not properly functioning bioreactors from the beginning because of some other unfavorable digestion condition. To evaluate the possibility of inhibition by the BioLithe formulae in Test 3, the amount of BioLithe chemicals used in Test 2 (Table 3.1.5) was compared to that used in Test 3. The applied mass of BioLithe™(B) and BioLithe™(C) (0.1 g/100 mL) during Test 2 was similar to that used in groups BioLithe™(B) in Low Concentration (0.13 g/100 mL) and BioLithe™(C) in Low Concentration (0.12 g/100 mL), as indicated as Table 3.1.6. It then could be deduced that BioLithe™(B) and BioLithe™(C) at a concentration or approximately 0.1 g/100 mL is incapable of reducing biogas and methane production by a substantial portion, 50 %, take the case of B2, B3, C2 and D3 as example. Moreover, for the purpose of keeping the coherence of triplicate bioreactors, B2, B3, C2 and D3 were removed from figures and the following statistical analysis. The figures entitled

"Cumulative Volumes of Biogas" and "Cumulative Volumes of Methane" were thus replotted with these outliers removed as shown in Figure 4.3.5 and Figure 4.3.6.

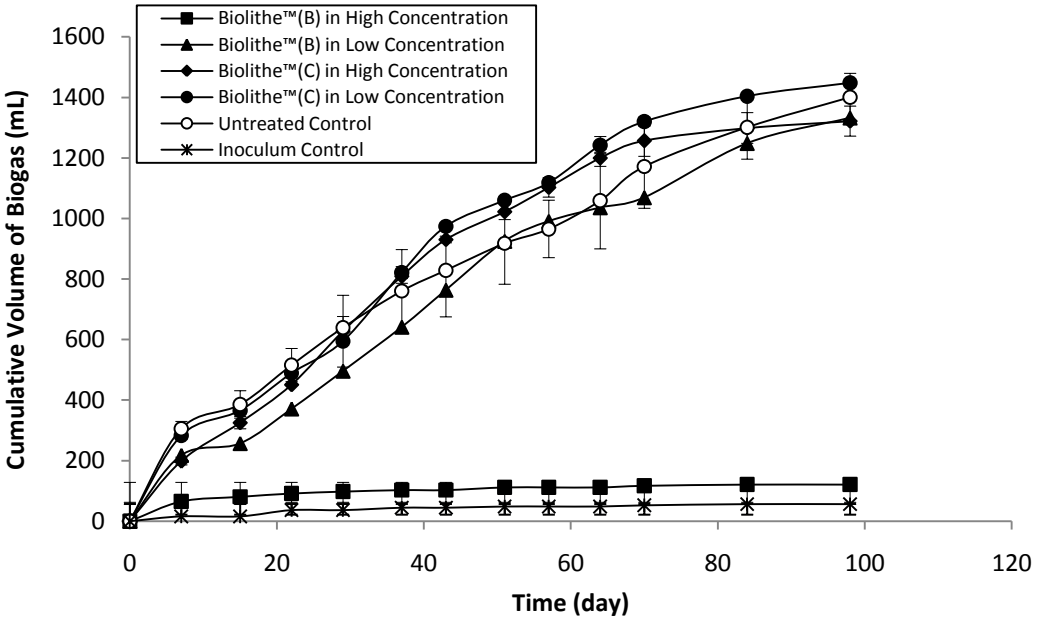


Figure 4.3.5 Cumulative volume of Biogas produced by different treatments during TEST 3, Degradation of Paper Saturated with Inhibitors II. Symbols are averages of triplicate bottles and error bars are one standard deviation.

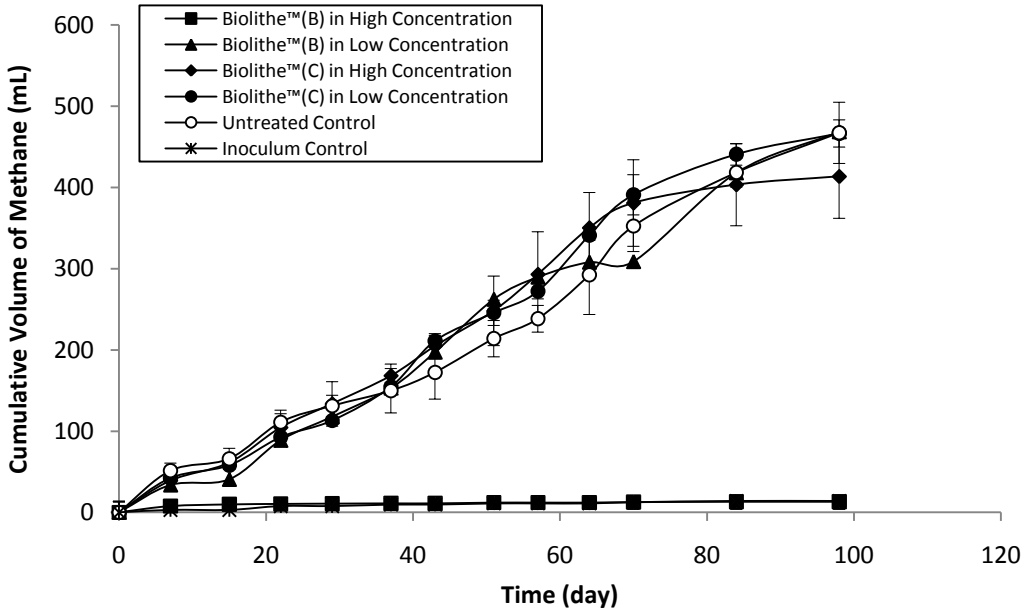


Figure 4.3.6 Cumulative volume of methane produced by different treatments during TEST 3, Degradation of Paper Saturated with Inhibitors II. Symbols are averages of triplicate bottles and error bars are one standard deviation.

Until day 98, bioreactors with BioLithe™(C) in Low Concentration had produced the most biogas, followed by the Untreated Control, then followed by BioLithe™(B) in Low Concentration and BioLithe™(C) in High Concentration (Figure 4.3.5). BioLithe™(B) in High Concentration, which produced only slightly more biogas than Inoculum Control, had the lowest cumulative volume of biogas among all treatments.

In methane production, BioLithe™(C) in High Concentration, BioLithe™(C) in Low Concentration and Untreated Control showed quite similar results and had higher methane production than BioLithe™(B) in Low Concentration, as shown in Figure 4.3.6. BioLithe™(B) in Low Concentration, BioLithe™(C) in High Concentration, BioLithe™(C) in Low Concentration and Untreated Control, all exhibited steep

cumulative gas production curves over the entire 98 days of operation. BioLithe™(B) in High Concentration, however, exhibited a rather different tend in methane generation which leveled off with little additional gas production beyond Day 7. Indeed, BioLithe™(B) in High Concentration produced the lowest volume of methane which was even slightly lower than the Inoculum Control. Paper degradation in the other four treatments were in progress while in BioLithe™(B) in High Concentration paper degradation appeared to be entirely impeded. Visual observation of changes in the bioreactors confirmed these results since the paper samples in BioLithe™(B) in High Concentration were observed to be intact while in other bottles the paper pieces appeared disintegrated or even completely dissolved.

4.3.2 Statistical analyses

TEST 3, Degradation of Paper Saturated with Inhibitors II: Four bioreactors, including B2, B3, C2 and D3, which all had unexpected DGGE patterns and low pH, were excluded from the statistical analysis. BioLithe™(B) and BioLithe™(C) were tested in different concentrations for the purpose of determining the relationship between methane reduction and BioLithe concentration. Both cumulative biogas and methane production in the test showed statistically significant differences by means of ANOVA, indicated by a P-value < 0.0001 highlighted in Table 4.3.2. It was also concluded that BioLithe™(B) in High Concentration reached about 91%, and 97% reduction in biogas and methane production, respectively, compared to the Untreated Control. Tukey's Studentized Range (HSD) Test further suggested that BioLithe™(B) in High Concentration was significantly different from Untreated Control both in biogas production and in methane production. BioLithe™(C) in High Concentration also

decreased methane by 11.5%; however this difference was not statistically significant by Tukey's Studentized Range (HSD) Test. BioLithe™(B) in Low Concentration, BioLithe™(C) in High Concentration and BioLithe™(C) in Low Concentration had no significant differences with Untreated Control as shown in Table 4.3.

It was indicated by statistical data that BioLithe™(B) in High Concentration was incapable of slowing paper degradation, and accordingly of substantially decreasing methane emissions. Neither BioLithe™(C) in High Concentration nor BioLithe™(C) in Low Concentration could significantly effect a reduction in methane production. There was no statistical differences between BioLithe™(B) in Low Concentration and Untreated Control in either biogas or methane production. One reason could be that two bioreactors were removed from the former group so that no triplicate values were available to provide enough confidence for statistical comparison.

Table 4.3.2 Results of TEST 3, Degradation of Paper Saturated with Inhibitors II

Comparison Group	Biogas			Methane		
	ANOVA	HSD[b]	Biogas Reduction[c] (%)	ANOVA	HSD[b]	Methane Reduction[c] (%)
Treated groups[a] and Untreated Control	**P-value < 0.0001**			**P-value < 0.0001**		
BioLithe™(B) in High Concentration and Untreated Control		**SD**	91.35		**SD**	97.17
BioLithe™(B) in Low Concentration and Untreated Control		NSD	4.88		NSD	-
BioLithe™(C) in High Concentration and Untreated Control		NSD	5.65		NSD	11.51
BioLithe™(C) in Low Concentration and Untreated Control		NSD	-		NSD	0.16

[a]BioLithe™(B) in High Concentration, BioLithe™(B) in Low Concentration, BioLithe™(C) in High Concentration, BioLithe™(C) in Low Concentration and Untreated Control

[b]SD means significantly different; NSD means not significantly different

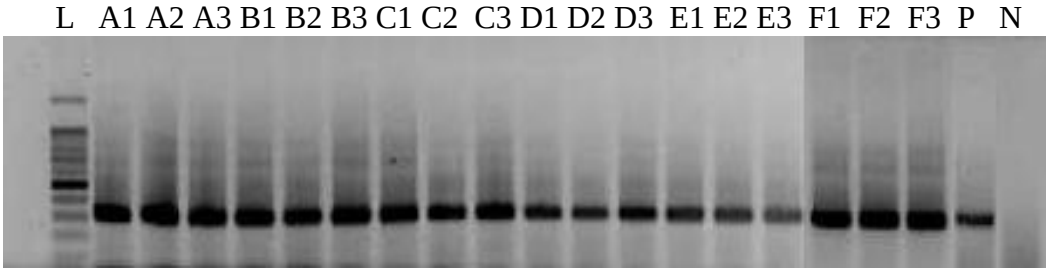
[c]Based on average gas production of each group

4.3.3 Analysis of genomic DNA of methanogenic archaea and bacteria by DGGE

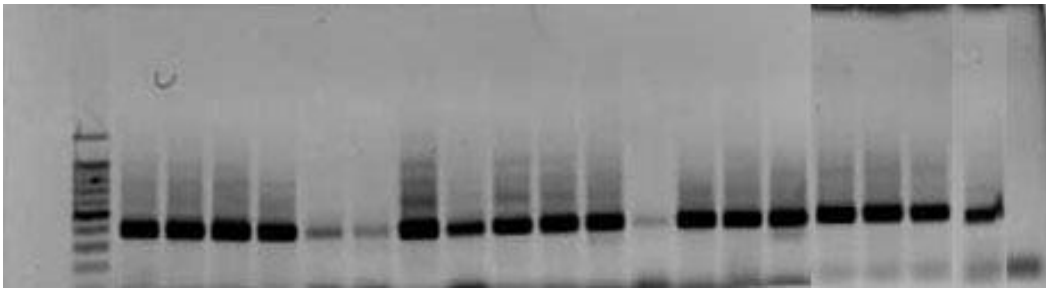
Bioreactors were sampled on day 7, day 51 and day 98 to track structural changes in the microbial community under the effect of the BioLithe formulae. Archaeal methanogenic communities and bacterial communities were analyzed by using primer pair 0348 aF-GC and 0619 R, and primer pair 27F and 519 F respectively (Table 3.4.1).

Over the course of the experiment, DGGE analysis of the amplified 16S rRNA genes showed differences in the methanogenic community (Figure 4.3.7). All samples exhibited similar band patterns on day 7, while B2, B3, C2, and D3 had bands that were either faded out or absent by day 51. Indeed, previously observed bands in B3 and D3 disappeared on day 98, indicating an unfavorable microbial condition in these bioreactors. This finding confirmed the results of a similar prediction according to an acidic environment (pH about 4, Table 4.3.1). In contrast to the community analysis performed for the other two BioLithe Tests (Test 2 and Test 4), the forward primer, 27F, was used instead of 338 F (see Table 3.4.1), for amplification of bacterial 16S rRNA genes. This resulted in amplified fragments approximately 500 bp length as shown in Figure 4.3.8.

(a)



(b)



(c)

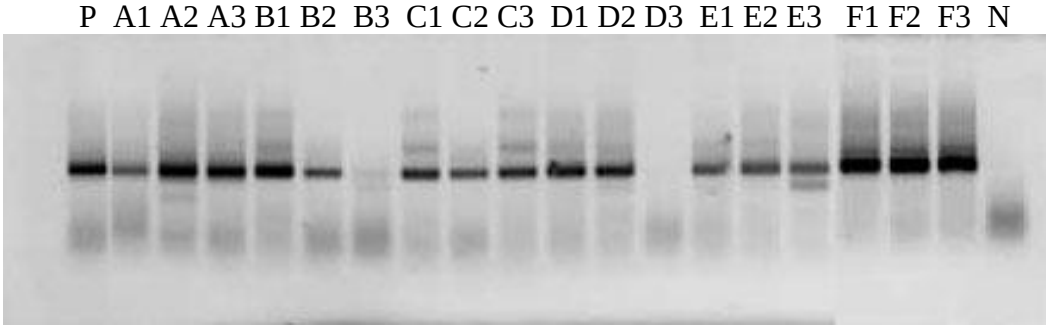
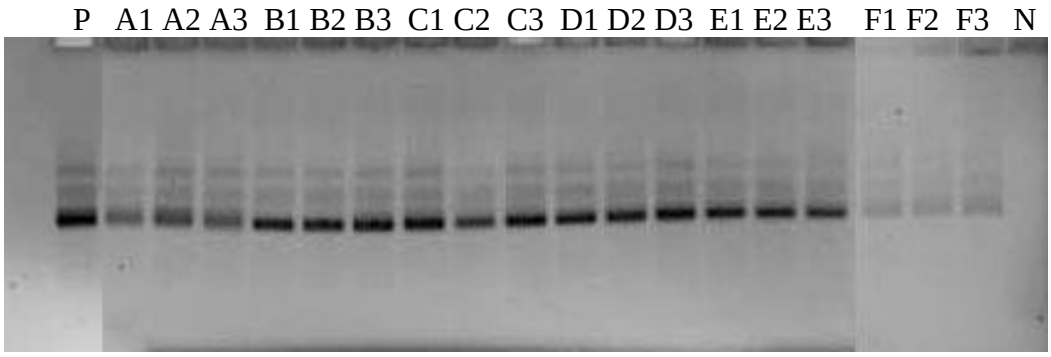
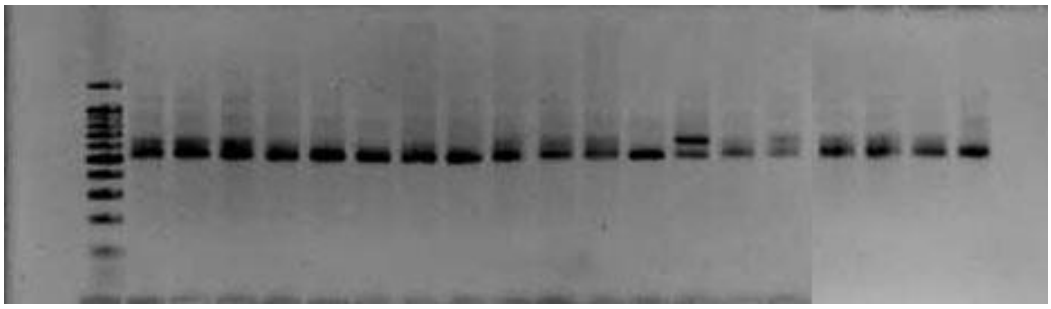


Figure 4.3.7 PCR products of methanogenic archaea in TEST 3, Degradation of Paper Saturated with Inhibitors II. (a) sampling on day 7; (b) sampling on day 51; (c) sampling on day 98. A represents BioLithe™(B) in high concentration; B, BioLithe™(B) in low concentration; C, BioLithe™(C) in high concentration; D, BioLithe™(C) in low concentration; E, Untreated Control, F, Inoculum Control (in triplicates); L, ladder; P, digester sludge as positive control; N, negative control (PCR products without DNA template).

(a)



(b)



(c)

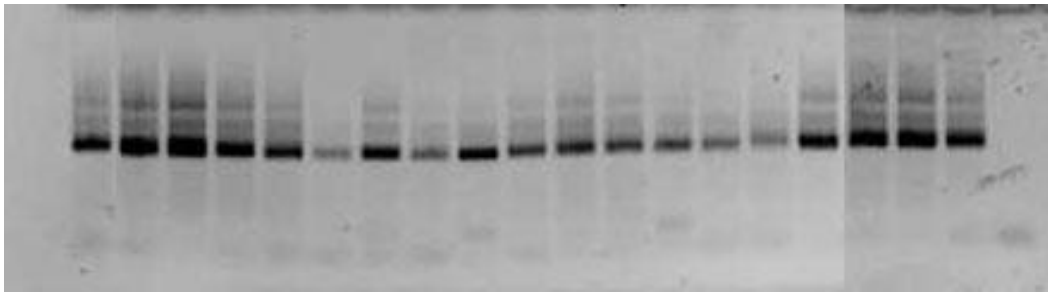


Figure 4.3.8 PCR products of bacteria in TEST 3, Degradation of Paper Saturated with Inhibitors II. (a) sampling on day 7; (b) sampling on day 51; (c) sampling on day 98. A represents BioLithe™(B) in high concentration; B, BioLithe™(B) in low concentration; C, BioLithe™(C) in high concentration; D, BioLithe™(C) in low concentration; E, Untreated Control, F, Inoculum Control (in triplicates); L, ladder; P, digester sludge as positive control; N, negative control (PCR products without DNA template).

Band patterns of amplified 16S rRNA fragments from archaea showed good resolution and separation on DGGE gels, as seen as Figure 4.3.9, Figure 4.3.10 and Figure 4.3.11. Figure 4.3.9 indicates the archaeal methanogenic community in the early phase, showing that band patterns of every column match with each other, including column P (the inoculum source from digester sludge) as a positive control. It is apparent that the dominant methanogens were quite similar not only in triplicates but also among groups. Thereafter, the microbial communities began to shift and develop into different patterns which could be found on the DGGE gel figure of the second sampling (day 51). For example, band 1 (as shown on Figure 4.3.10) was clear and dark in columns A (BioLithe™(B) in High Concentration) and F (Inoculum Control), but relatively thinner in columns B (BioLithe™(B) in Low Concentration), C (BioLithe™(C) in High Concentration), and D (BioLithe™(C) in Low Concentration). Further, band 2 was thick and dark in columns B1, C1, C3, D1 D3 and columns E (Untreated Control), but lighter in A and F. After progression into the later phase of the experiment (day 98), fragments from methanogenic archaea displayed a more complex diversification on the DGGE gel (Figure 4.3.11). Columns A (BioLithe™(B) in High Concentration) developed a more complex banding pattern; B (BioLithe™(B) in Low Concentration), C (BioLithe™(C) in High Concentration), and D (BioLithe™(C) in Low Concentration) had dominant bands shifted; F, Inoculum Control, had a stable microbial community which maintained the same major bands at the start of the test; and other column sets had bands diminished but distinct. The structural change of methanogenic communities with time scale provides a good observation that columns B2, B3, C2, and D3 had band patterns decreasing in intensity and/or gradually disappearing altogether.

With a substituted primer pair producing 500 bp fragments from bacteria, DGGE figures exhibited a completely different result. At the first phase of DGGE analysis, bands in columns B, C, D, E and P (positive control), compared to A and F which had very light intensity bands (Figure 4.3.12). As the test progressed the bacterial community patterns of all columns became quite disparate while communities in F had almost entirely disappeared, as shown in Figures 4.3.13 and 4.3.14. Indeed, clear bands were not obtained from bacterial PCR products despite attempts using a wider range of PCR primers. The images were blurry and undistinguishable, probably as a result of the amplified fragments being too long for good separation.

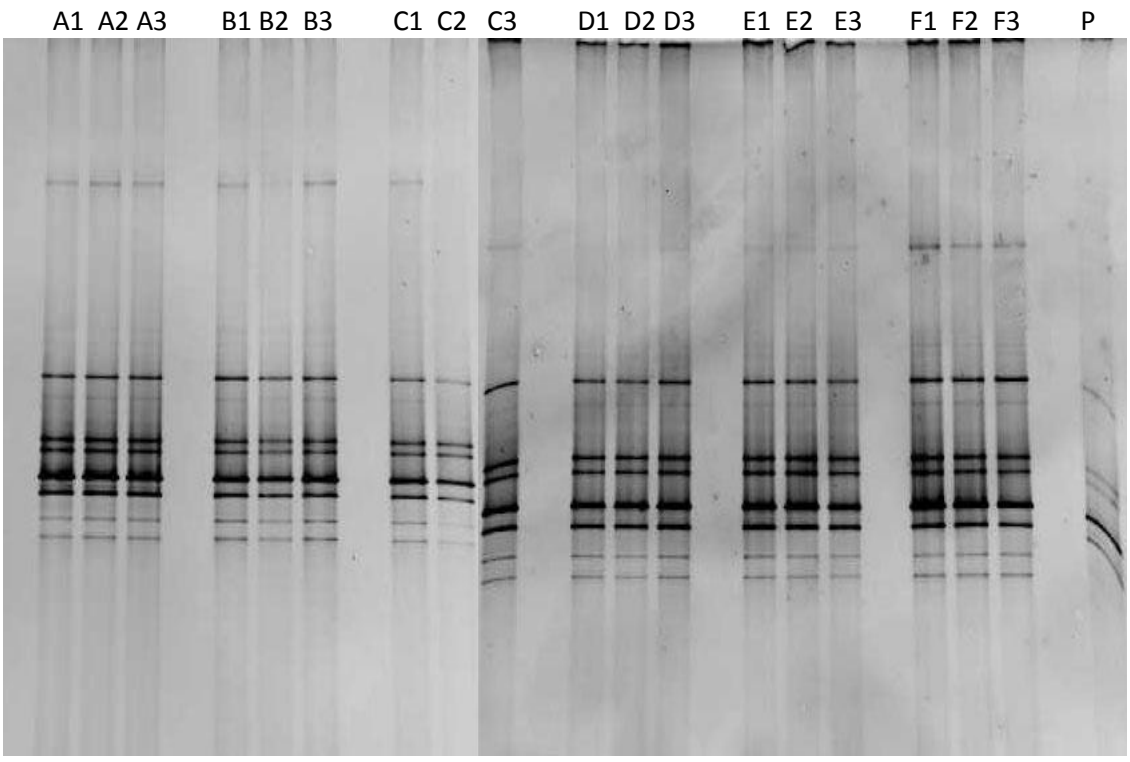


Figure 4.3.9 DGGE band patterns of methanogenic archaeal PCR products from TEST 3, Degradation of Paper Saturated with Inhibitors II- sampling on day 7. A represents BioLithe™(B) in high concentration; B, BioLithe™(B) in low concentration; C, BioLithe™(C) in high concentration; D, BioLithe™(C) in low concentration; E, Untreated Control, F, Inoculum Control (in triplicates); P, digester sludge as positive control.

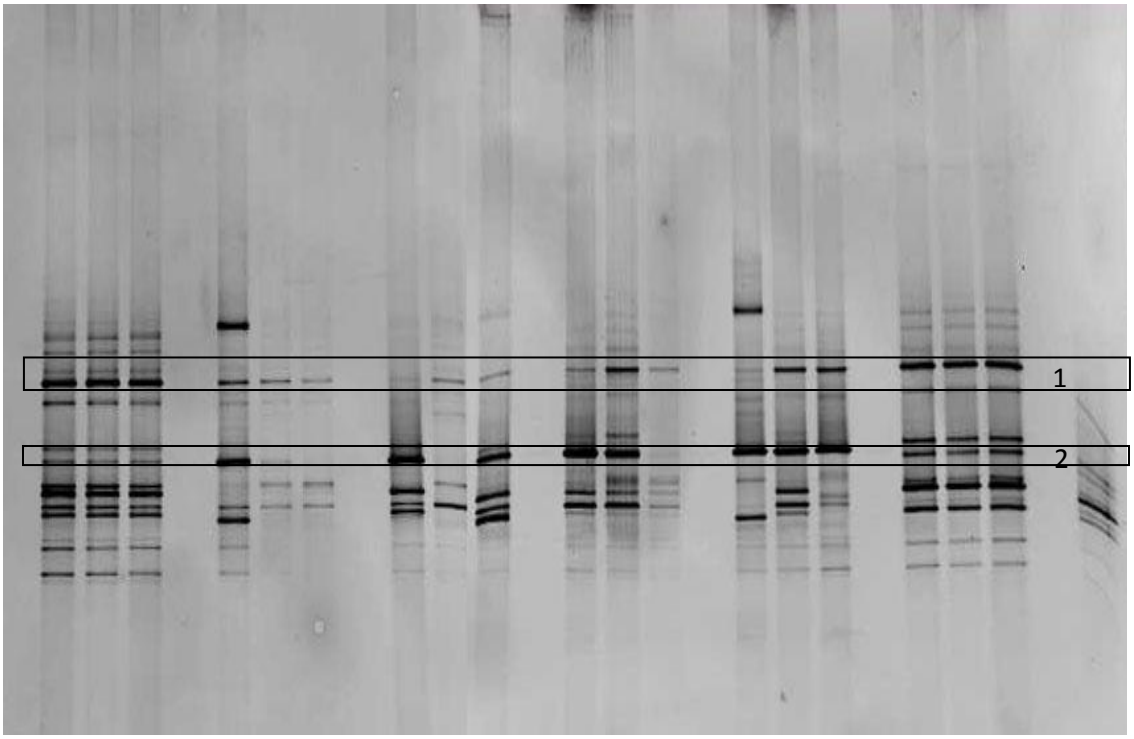


Figure 4.3.10 DGGE band patterns of methanogenic archaeal PCR products from TEST 3, Degradation of Paper Saturated with Inhibitors II- sampling on day 51. A represents BioLithe™(B) in high concentration; B, BioLithe™(B) in low concentration; C, BioLithe™(C) in high concentration; D, BioLithe™(C) in low concentration; E, Untreated Control, F, Inoculum Control (in triplicates); P, digester sludge as positive control.

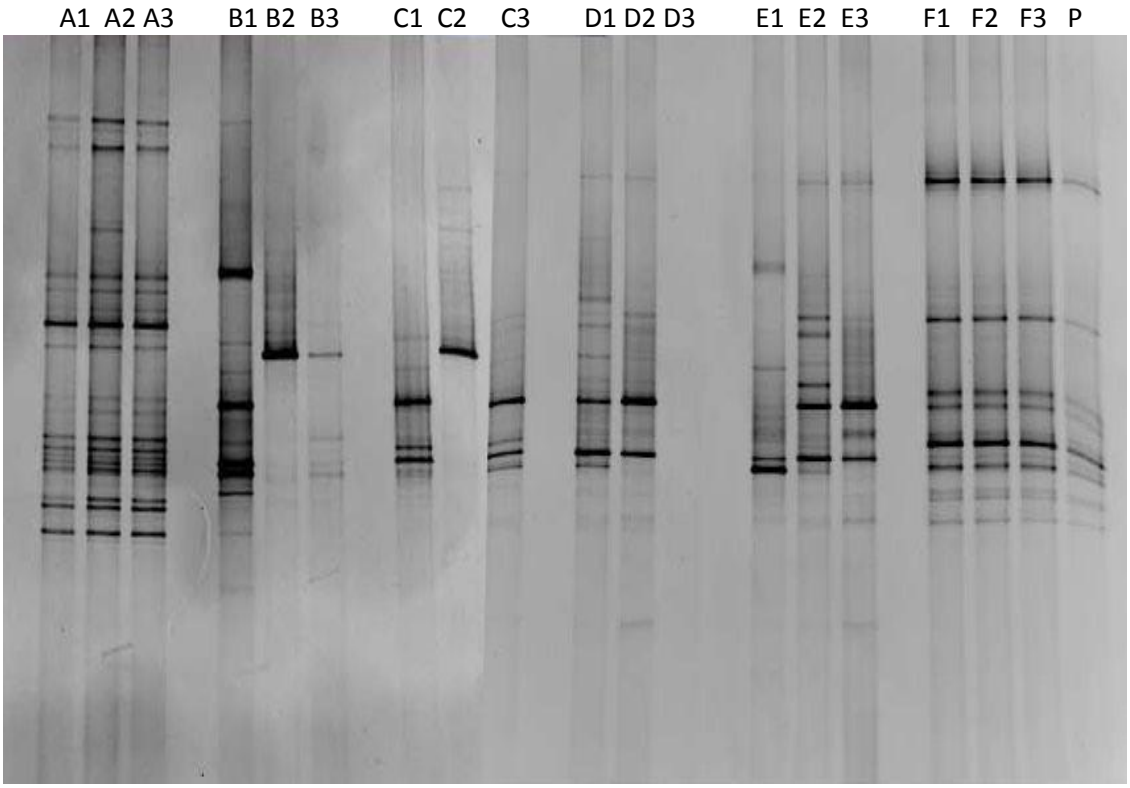


Figure 4.3.11 DGGE band patterns of methanogenic archaeal PCR products from TEST 3, Degradation of Paper Saturated with Inhibitors II- sampling on day 98. A represents BioLithe™(B) in high concentration; B, BioLithe™(B) in low concentration; C, BioLithe™(C) in high concentration; D, BioLithe™(C) in low concentration; E, Untreated Control, F, Inoculum Control (in triplicates); P, digester sludge as positive control.

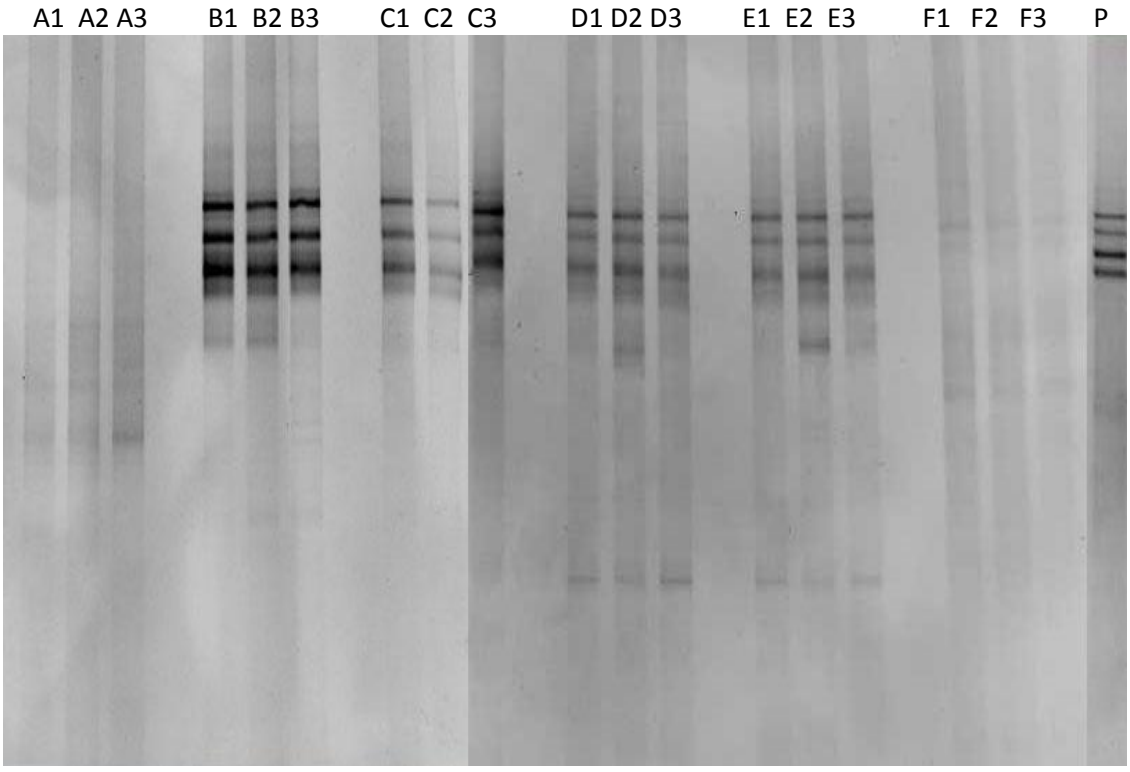


Figure 4.3.12 DGGE band patterns of bacterial PCR products from TEST 3, Degradation of Paper Saturated with Inhibitors II- sampling on day 7. A represents BioLithe™(B) in high concentration; B, BioLithe™(B) in low concentration; C, BioLithe™(C) in high concentration; D, BioLithe™(C) in low concentration; E, Untreated Control, F, Inoculum Control (in triplicates); P, digester sludge as positive control.

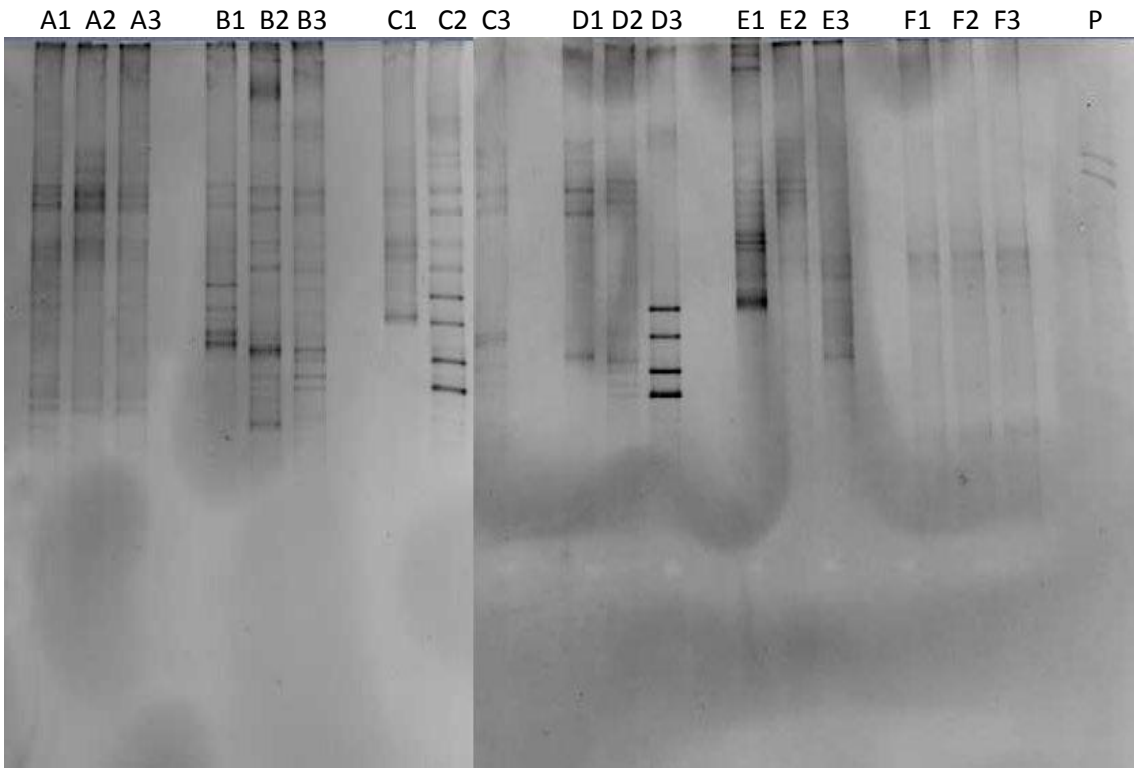


Figure 4.3.13 DGGE band patterns of bacterial PCR products from TEST 3, Degradation of Paper Saturated with Inhibitors II- sampling on day 51. A represents BioLithe™(B) in high concentration; B, BioLithe™(B) in low concentration; C, BioLithe™(C) in high concentration; D, BioLithe™(C) in low concentration; E, Untreated Control, F, Inoculum Control (in triplicates); P, digester sludge as positive control.

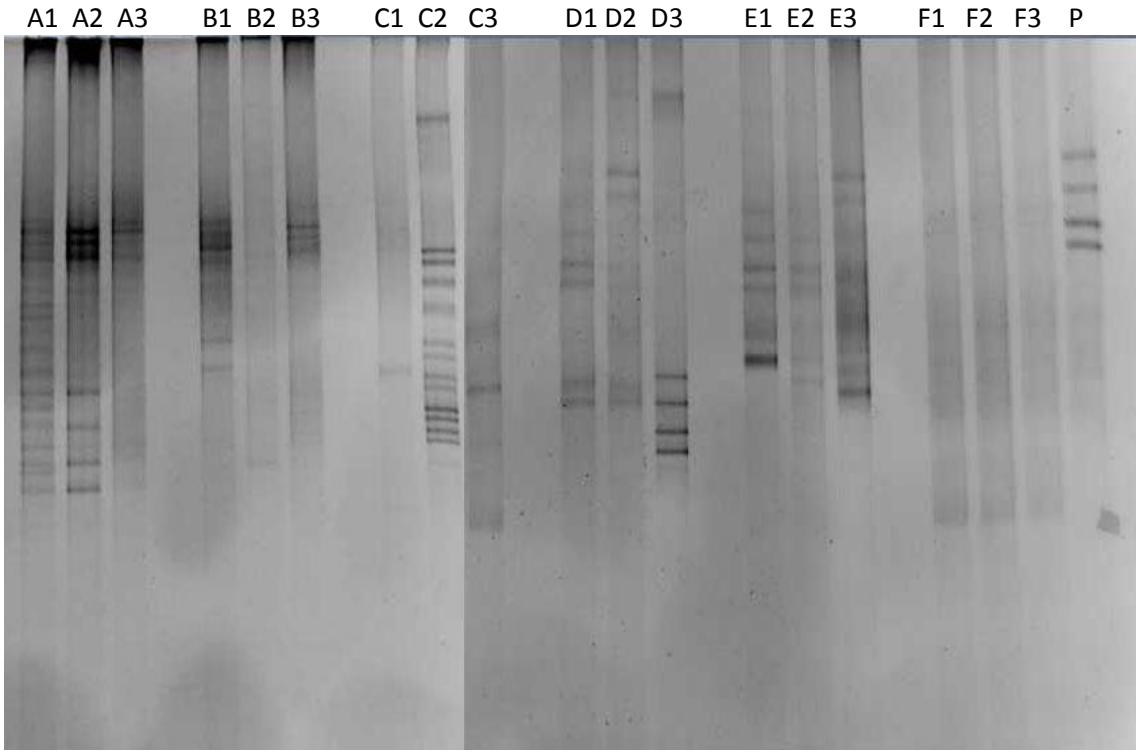


Figure 4.3.14 DGGE band patterns of bacterial PCR products from TEST 3, Degradation of Paper Saturated with Inhibitors II- sampling on day 98. A represents BioLithe™(B) in high concentration; B, BioLithe™(B) in low concentration; C, BioLithe™(C) in high concentration; D, BioLithe™(C) in low concentration; E, Untreated Control, F, Inoculum Control (in triplicates); P, digester sludge as positive control.

4.4 TEST 4, Degradation of Paper Coated with Inhibitors in an Industrial Process

4.4.1 Biogas and methane measurements

In the coated paper experiment, paper samples were coated with two BioLithe solutions (BioLithe™(B) and BioLithe™(C)) mixed with aqueous coating to simulate the industrial paper processing technique (Experimental protocol see Table 3.1.7). By day 117 of the test, Bottle Set Untreated Control had generated the largest volumes of biogas as shown in Figure 4.4.1. Aqueous Coating with BioLithe™(C) and Virgin Aqueous Coating had similar biogas production, followed by Aqueous Coating with

BioLithe™(B), which was the lowest biogas producer among all treatments. Untreated Control had substantially increased biogas production after day 60, after exhibiting a relatively low yield prior to Day 60. This phenomenon was a result of disparate performances among triplicates which can be seen as the substantial variability on the progression curve of the Untreated Control. In the middle of the experiment, however, bioreactors which had exhibited a lag in methane production began to catch up and produce gas intensely. As a result the methane production curve of Untreated Control subsequently exhibited less variability among the triplicates as indicated by the smaller error bars towards the end of the test, and exceeded the gas production exhibited by other groups. Aqueous Coating with BioLithe™(B) maintained the lowest biogas yield of all the treatment groups, except the Inoculum Control, until day 117.

Results similar to those observed for cumulative biogas production were observed for cumulative methane as shown in Figure 4.4.2. Aqueous Coating and Aqueous Coating with BioLithe™(C) demonstrated similar methane production trends, and were approaching the total produced by the Untreated Control, which had the highest cumulative methane production. The Untreated Control began to catch up when the variability between triplicates had decreased after day 60, which indicated that methane generation was tightly associated with the biogas producing process. Aqueous Coating with BioLithe™(B) had demonstrated a slightly lower methane potential around day 20 and kept the tendency to the end, thus cumulatively producing the least methane (Inoculum Control excluded).

Unlike the previous tests where differences were observed between some treated groups and the control group in gas production, Test 4 seemed to have relatively smaller

differences among groups in both biogas and methane production. It is necessary to point out that the amount of BioLithe chemicals attached on paper was substantially lower for Test 4 than other tests (indicated in Table 3.1.7), resulting from the industrial coating technique. Statistical analysis was used to investigate the significance of the observed differences between treated and untreated groups.

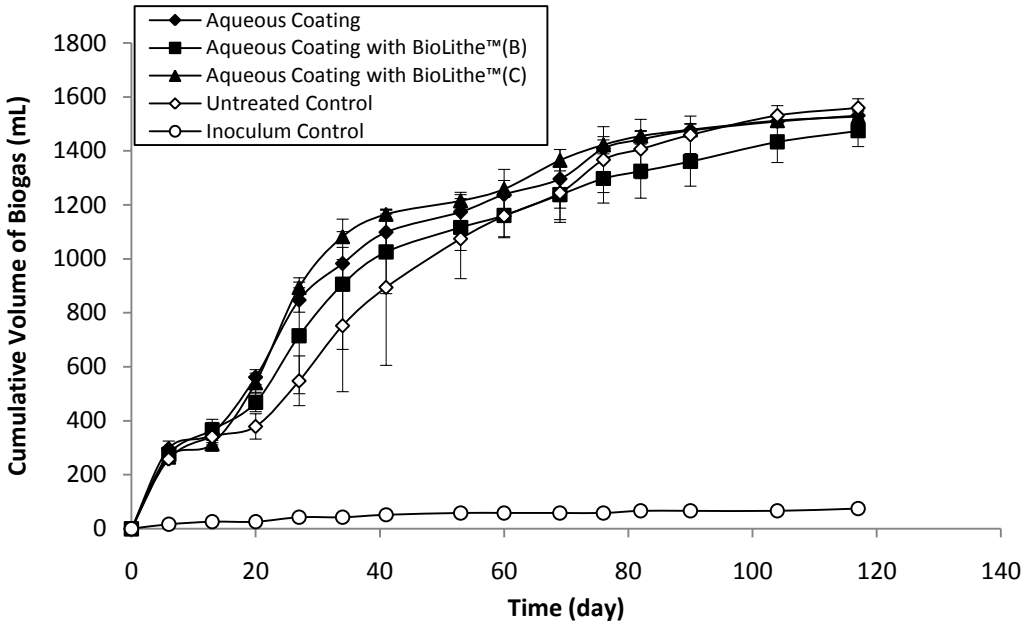


Figure 4.4.1 Cumulative volumes of biogas produced by different treatments during TEST 4, Degradation of Paper Coated with Inhibitors in an Industry Process. Symbols are averages of triplicate bottles and error bars are one standard deviation.

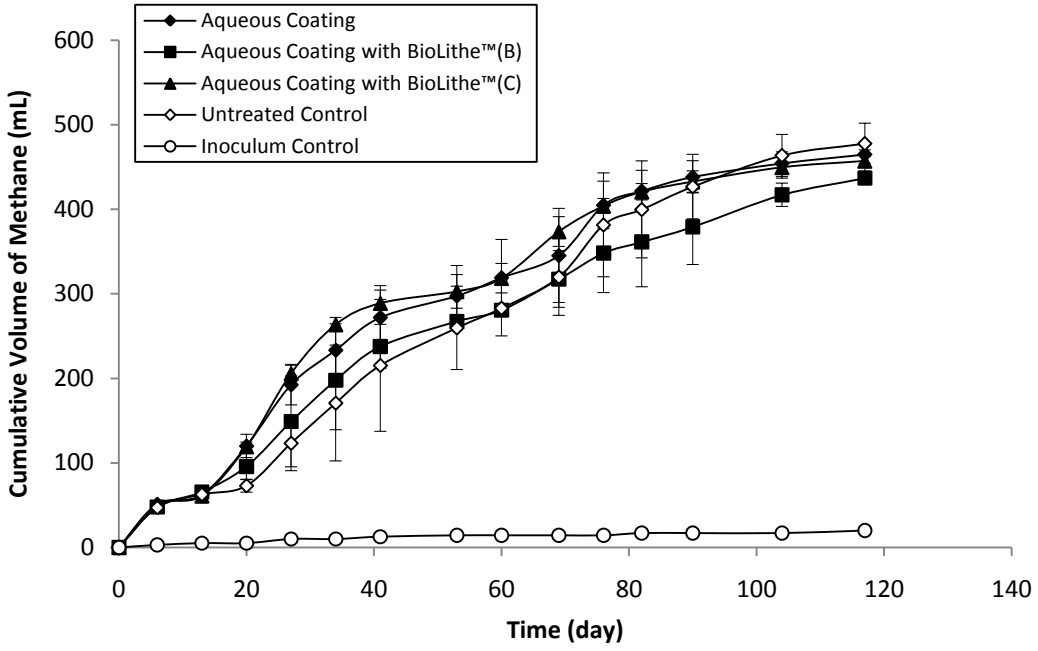


Figure 4.4.2 Cumulative volumes of methane produced by different treatments during TEST 4, Degradation of Paper Coated with Inhibitors in an Industry Process. Symbols are averages of triplicate bottles and error bars are one standard deviation.

4.4.2 Statistical Analyses

TEST 4, Degradation of Paper Coated with Inhibitors in an Industrial Process: The test was carried out with BioLithe solutions coated on paper via an industrial technique and was quite close to a practical industrial process. ANOVA indicated that there were no significant differences in cumulative volumes of biogas or methane between treatments, with P-values all greater than 0.05 as shown in Table 4.4.1. Aqueous Coating, Aqueous Coating with BioLithe™(B) and Aqueous Coating with BioLithe™(C) were then individually compared with the Untreated Control using Tukey's Studentized Range (HSD) Test, again indicating that Aqueous Coating and Aqueous Coating with BioLithe™(C) were not significantly different from the Untreated Control in either biogas or methane production. Also, there was no statistical significance in cumulative

biogas production between Aqueous Coating with BioLithe™(B) and the Untreated Control. However, there was a significant different in cumulative methane production between Aqueous Coating with BioLithe™(B) and the Untreated Control as highlighted in Table 4.4.1.

Aqueous Coating with BioLithe™(C) decreased methane production by 4.32% but failed to be shown statistically significant (investigated by HSD). Aqueous Coating with BioLithe™(B) was shown to result in statistically lower methane generation than the Untreated Control with an 8.53% reduction. The test also validated that Aqueous coating, the usual coating material applied on paper did not influence biogas or methane production in the normal coating amounts utilized.

Table 4.4.1 Results of TEST 4, Degradation of Paper Coated with Inhibitors in an Industrial Process

Comparison Group	Biogas			Methane		
	ANOVA	HSD[b]	Biogas Reduction[c] (%)	P-value	HSD[b]	Methane Reduction[c] (%)
Treated groups[a] and Untreated Control	P-value > 0.05			P-value > 0.05		
Aqueous Coating and Untreated Control		NSD	1.83		NSD	2.66
Aqueous Coating with BioLithe™(B) and Untreated Control		NSD	5.47		**SD**	8.53
Aqueous Coating with BioLithe™(C) and Untreated Control		NSD	2.06		NSD	4.32

[a] Aqueous Coating, Aqueous Coating with BioLithe™(B) and Aqueous Coating with BioLithe™(C)

[b]SD means significantly different; NSD means not significantly different

[c]Based on average gas production of each group.

4.4.3 Analysis of genomic DNA of methanogenic archaea and bacteria by DGGE

Total DNA was sampled from each bioreactor on day 61, and then primer pair 0348 aF-GC and 0619 R was applied for archaea and primer pair 338 F-GC and 519 R was used for bacteria, since the bacteria primer pair 27 F and 519 R produced a fragment that failed to provide good separation on DGGE gel (see PCR primers in Table 3.4.1). The amplified fragments having a 271 bp length for archaea and 181 bp length for bacteria are shown on Figures 4.4.3 and Figure 4.4.4.

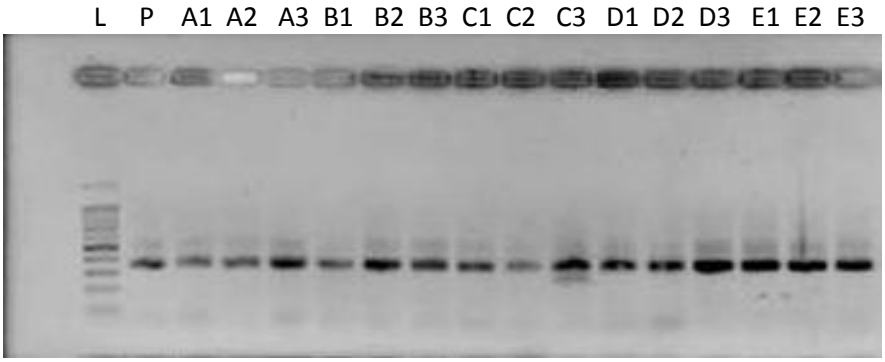


Figure 4.4.3 PCR products of methanogenic archaea in TEST 4, Degradation of Paper Coated with Inhibitors in an Industry Process. A represents Aqueous Coating; B, Aqueous Coating with BioLithe™(B); C, Aqueous Coating with BioLithe™(C); D, Untreated Control; E, Inoculum Control (in triplicates); L, ladder; P, digester sludge as positive control.

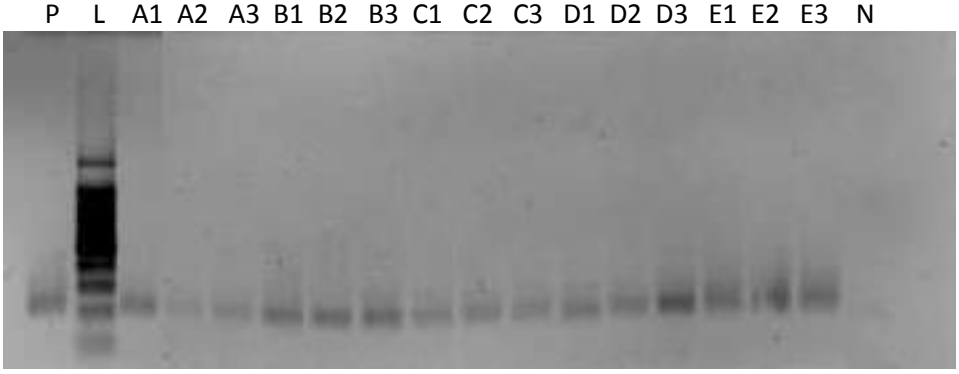


Figure 4.4.4 PCR products of bacteria in TEST 4, Degradation of Paper Coated with Inhibitors an Industry Process. A represents Aqueous Coating; B, Aqueous Coating with BioLithe™(B); C, Aqueous Coating with BioLithe™(C); D, Untreated Control; E, Inoculum Control (in triplicates); L, ladder; P, digester sludge as positive control; N, negative control (PCR products without DNA template).

The 16S rRNA PCR products were all then analyzed by DGGE. Figure 4.4.5 shows the structural community of methanogenic archaea of each treatment bioreactor. Note that the columns of E (Inoculum Control) had a banding pattern most similar to

those in column P (digester sludge as positive control) which was representative of the original inoculum, while the microbial community became more diverse in other treatments. Triplicates in columns A (Aqueous Coating), C (Aqueous Coating with BioLithe™(C)), and E were similar in each group, while B2 (Aqueous Coating with BioLithe™(B)) and D1 (Untreated Control) behaved differently from their triplicates.

Bacterial DGGE analysis is shown on Figure 4.4.6. Band pattern 1 dominated columns A, B and C, but hardly could be seen in columns D.

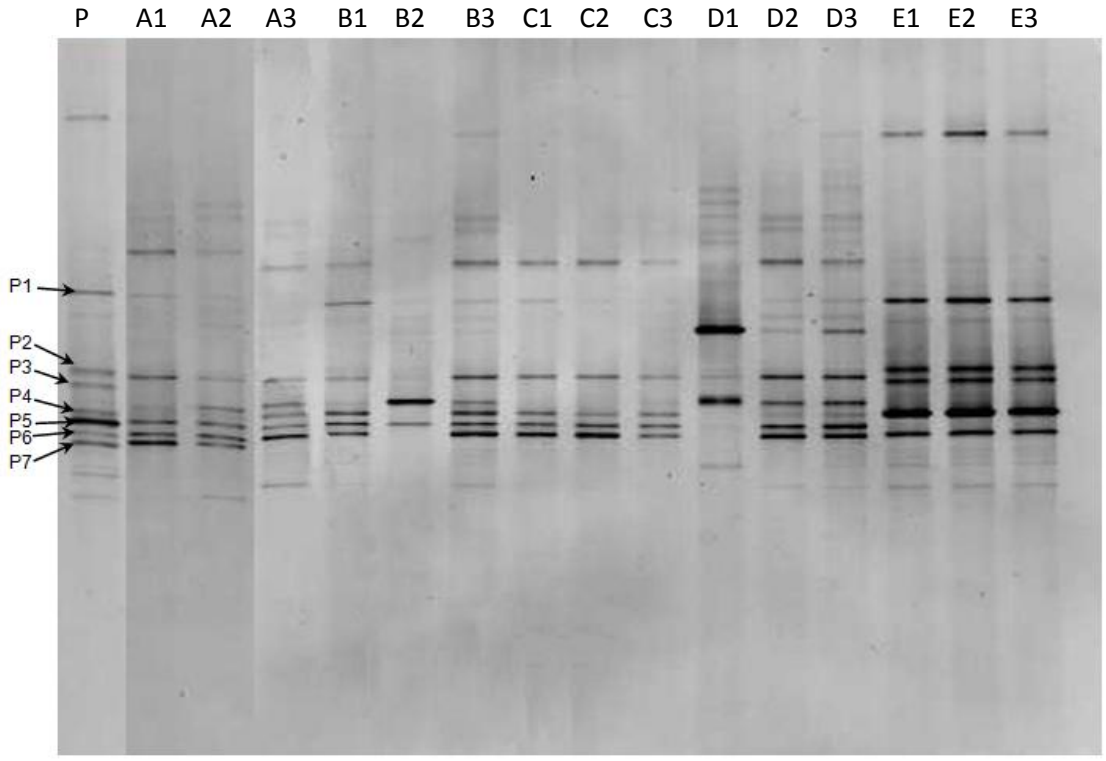


Figure 4.4.5 DGGE band patterns of methanogenic archaeal PCR products from TEST 4, Degradation of Paper Coated with Inhibitors. A represents Aqueous Coating; B, Aqueous Coating with BioLithe™(B); C, Aqueous Coating with BioLithe™(C); D, Untreated Control; E, Inoculum Control (in triplicates); P, digester sludge as positive control. Bands collected from different columns were unified and displayed in column P.

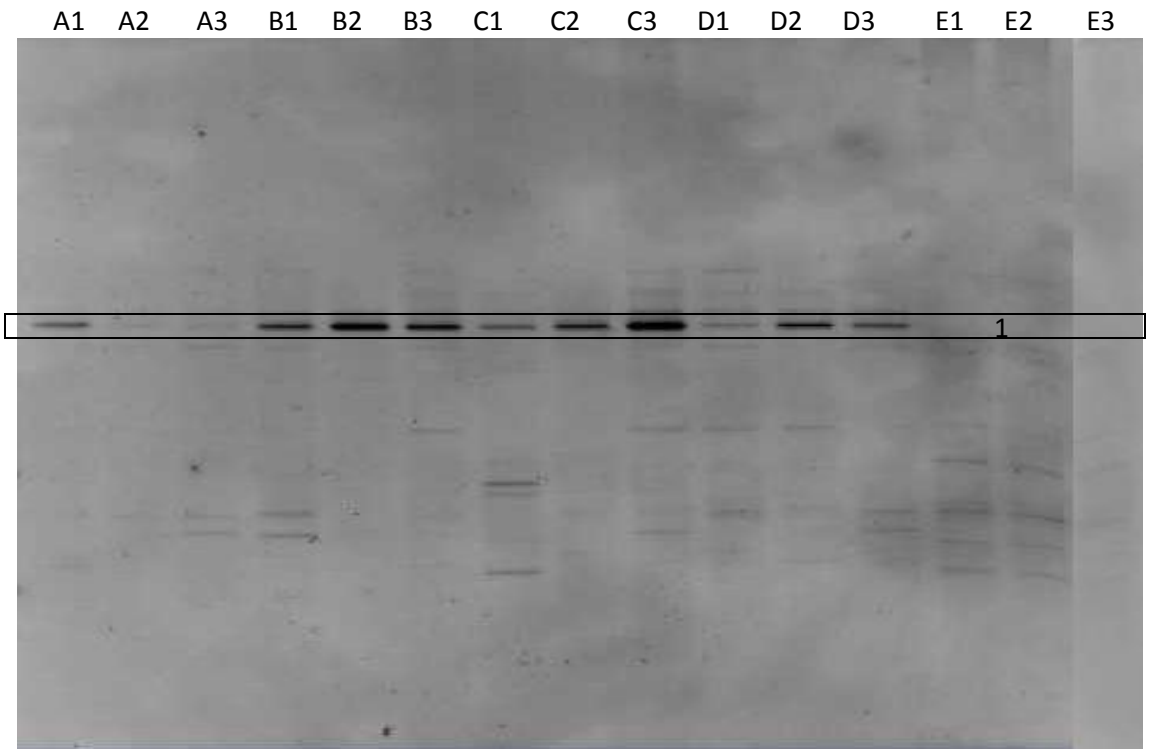


Figure 4.4.6 DGGE band patterns of bacterial PCR products from TEST 4, Degradation of Paper Coated with Inhibitors. A represents Aqueous Coaitng; B, Aqueous Coating with BioLithe™(B); C, Aqueous Coating with BioLithe™(C); D, Untreated Control; E, Inoculum Control (in triplicates).

4.4.4 Sequence results of fragments of methanogenic archaea

Bands selected from each DGGE experiment were excised from both archaeal and bacterial DGGE gels, of which the first batch from the methanogenic archaea of TEST 4 were sent for sequencing. Returned results were unified as marked bands shown in Figure 4.4.5, and described in Table 4.4.2. Base length for sequences was between 100 and 200 bp. Gene segments of bands labeled P1-P7 (Figure 4.4.5) were retrieved and compared to sequences deposited in the GenBank database, and selective closest relatives are displayed in Table 4.4.2. Except P1 whose fragment had a relatively low similarity (80%)

with Uncultured ArcI archaeon, sequence fragments from bands (P2-P7) were closely related to methanogens with a similarity from 97% to 100%. The gene fragment for P7 has a 100% similarity to Uncultured *Methanosarcina* sp., isolated from waste sludge, which is similar to the inoculum applied in these BioLithe Tests.

Table 4.4.2[a] Sequence results from Test 4-Methanogenic archaeal products

DGGE band name	**Closest relative**	**Sequence length (bases)**	**Similarity%**	**Accession**	**Isolated from (Sources)**	**Reference**
P1	Uncultured ArcI archaeon	150-200	88	CU917096.1	Microorganisms involved in anaerobic digestion of sludge	Riviere et al., 2009
P2	Uncultured *Methanosarcina* sp.	100-150	98	CU466563.1	Wastewater treatment plant anoxic basin	Genoscope, 2008
P3	Uncultured *Methanosarcina* sp.	150-200	97	CU466559.1	Wastewater treatment plant anoxic basin	Genoscope, 2008
P4	Uncultured *Methanosaetaceae* archaeon clone OTRo_O1_56	150-200	100	GU257215.1	Archaea in sediments of a drinking water reservoir	Scheer et al., 2010
P5	Uncultured *Methanosarcinales* archaeon clone OP1_7_7_F02	150-200	99	GU120501.1	Microbial community within a natural asphalt lake	Schulze-Makuch., 2009
P6	*Methanosarcina* sp. 48	150-200	99	EF112192.1	Archaeal community of rumen	Evans and Joblin, 2008
P7	Uncultured *Methanosarcina* sp.	150-200	100	AB489230.1	Anaerobic self-degradation of waste-activated sludge	Kobayashi et al., 2009

a Based on BlastN analyzed using GenBank database. Available from: http://blast.ncbi.nlm.nih.gov/Blast.cg

CHAPTER 5

CONCLUSION

5.1 Conclusions of Test Results

The BioLithe project was developed and analyzed through different approaches, including gas measurements and molecular biology analysis, and the obtained numeral data was examined by one-way ANOVA plus Tukey's Studentized Range (HSD) Test statistics. Three types of BioLithe formulae (BioLithe™(A), BioLithe™(B), and BioLithe™(C)) provided by BioLithe LLC underwent a series of tests from initial paper saturated tests to a practical paper coating test. BioLithe™(A) was first eliminated for its unpromising performance on methane reduction in TEST 1. BioLithe™(C) was substituted for BioLithe™(A) for the remainder of the tests and it was found that it was also unable to significantly act on methane inhibition or delay even in high concentration.

TEST 2 later confirmed by statistical analysis that Aqueous Coating with BioLithe™(B) and Aqueous Coating with BioLithe™(C) could significantly reduce biogas production, while the methane reduction was not proved by HSD. However, the two treated groups did impact the microbial community as shown by examination of the DGGE products (Figure 4.2.5 and Figure 4.2.6), and it was apparent that methanogenic archaea were influenced by the presence of BioLithe chemicals plus Aqueous Coating, because the communities were different from the untreated group. Aqueous Coating, the universally applied coating material, was later tested for its potential for abating biogas/methane production and its effect on microbial structure.

TEST 3 showed that BioLithe™(B) in high concentration could highly suppress methane generation and it strongly inhibited paper decomposition. Interestedly, DGGE on archaeal methanogen in BioLithe™(B) in high concentration had developed into most complex patterns while other treated groups had less band diversity. A possible explanation for this finding is that BioLithe™(B) in high concentration inhibited or suppressed bacteria which would function via hydrolysis and fermentation processes, so that it essentially stopped the breakdown of paper at the initial step for anaerobic degradation (Figure 1.1).

TEST 4 was conducted last and the major result was that only BioLithe™(B) could statistically reduce methane production via the industrial coating technique. Aqueous Coating did not impact methane emissions with the usual (light) coating amount on paper.

Taken together, the results of all tests indicate that BioLithe™(B) could effectively control paper degradation in relatively high amounts. Moreover, even with a relatively small amount of coating on paper, BioLithe™(B) was able to decrease methane generation. Microbial communities in anaerobic paper degradation were also influenced by the presence of BioLithe™(B) which might be associated with methane performance. The potential of BioLithe™(B) in methane reduction implies a possibility of putting this formula into industrial process and production.

5.2 Analysis of BioLithe formulae components on methane reduction

BioLithe™(B) was proved for its methane reduction potential and its composition was then analyzed and compared with other BioLithe formulae. The formula of BioLithe™(B) consisted of various compounds, containing metal elements such as iron (Fe^{3+}), copper (Cu^{2+}), zinc (Zn^{2+}), magnesium (Mg^{2+}) and manganese (Mn^{2+}). Effects of metals on inhibition of methanogenesis have been broadly studied (Lorowitz et al., 1992; Sanchez et al., 1996; Zhang et al., 2009), and some recent articles were summarized in Table 1.2. Some metallic compounds in Table 1.2, such as magnesium, ferric iron, copper and zinc are found in the formula of BioLithe™(B). The concentration of metal elements in BioLithe™(B) applied in each test were calculated and are shown in Table 5.1. Although existing differences between the anaerobic inocula used in the various studies (such as methanogen concentration, species, nutrients, etc.) are unknown, it indeed seems evident from examination of the literature that Mg^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} are likely the major inhibitors of methane generation. Note that the BioLithe Tests used paper as the sole major carbon source, while other studies referred to in Table 1.2 did not.

Table 5.1 Concentrations of metal elements in BioLithe™(B) in each test

Metal element in composition of BioLithe™(B)	**Element /Formula (%)**	**Metal Concentration[b,c] (mg/L)**				
		Test 1	**Test 2**	**Test 3**		**Test 4**
				High Conc.	**Low Conc.**	
Iron (Fe^{3+})	7.45	551.30	74.49	655.49	96.83	0.69
Magnesium (Mg^{2+})	3.15	233.10	31.45	276.76	40.89	0.29
Manganese (Mn^{2+})	0.86	63.64	8.62	75.86	11.21	0.08
Copper (Cu^{2+})	1.46	108.04	14.60	128.45	18.98	0.13
Zinc (Zn^{2+})	3.17	234.58	31.73	279.21	41.25	0.29

a. (Element/Compound) *(Compound/Formula)%
b. Assume BioLithe chemicals completely dissolved in medium.
c. Based on mass of BioLithe™(B) in each test- Test 1 (0.74 g/100 mL), Test 2 (0.1 g/100 mL), Test 3 (0.88 and 0.13 g/100 mL), and Test 4 (0.92 mg/100 mL). Sources from Table 3.1.4 to Table 3.1.7.

Interestedly enough, BioLithe™(B) and BioLithe™(A) have a similar chemical recipe (Table 3.1.1 and Table 3.1.2), with the major difference that ferrous sulfate heptahydrate in BioLithe™(A) was substituted for magnesium sulfate heptahydrate in BioLithe™(B). Some study showed that degradation of cellulose and production of methane was stimulated in an environment containing ferrous iron (Fe^{2+}) (Khan et al., 1979). On the other hand, it was reported that the presence of manganese (Mn^{2+}) was able to stimulate activity of methanogenic communities (Lorowitz et al., 1992). A).

Sulfate is another important composition both in BioLithe™ (A) and BioLithe™ (B). Sulfate was added into BioLithe™ (A) as $FeSO_4 \cdot 7H_2O$ and into BioLithe™ (B) as $MgSO_4 \cdot 7H_2O$, in order to encourage the growth of sulfate-reducing bacteria, with the purpose that they would out-compete the methanogens for electron donor available from the breakdown of the paper (e.g., Lovley and Klug, 1983). This "competitive exclusion" should result in less formation of methane, thus channeling the degradation process towards carbon dioxide, a less potent greenhouse gas than methane. However, although present in BioLithe™ (A), sulfate did not apparently exert any inhibitory function on methane generation. It is possible that the presence of Fe^{2+} in the BioLithe™ (A) formula precipitated any sulfide formed during the reduction of sulfate a an Fe-S solid. Because BioLithe™ (B) contained no Fe^{2+}, any sulfide produced would exist in an aqueous or gaseous form that could have an inhibitory effect on methanogenesis.

CHAPTER 6

PROJECT IMPLICATIONS AND FUTURE WORK

6.1 Project Implications

An innovative idea for formulating paper products with incorporated inhibitors or competitors of methanogenesis to reduce or postpone the methane potential of paper during degradation in landfills—termed BioLithe—was examined during this proof of concept study. The potential of one of the tested formulae, BioLithe™(B) (Table 3.1.2) to achieve methane reduction implies a possibility of putting this formula into industrial process and production. In particular, BioLithe™(B) greatly slowed or reduced methane production when compared to untreated controls. The idea of paper with lower greenhouse gas potential is the fundamental starting point for BioLithe technology. Inhibition of methane production through paper treatment could likely be reversed in the landfill, after the paper has been deposited and aged *in situ* and the leachate dissolves and removes the inhibiting compound(s). This would lead to the scenario shown in Figure 2.2 where delayed methanogenesis results in better capture of methane production. If methanogenesis is delayed until the landfill cell is sealed and biogas collection begins, less methane would be emitted to the atmosphere.

New BioLithe formulae should be developed which are cost effective and have better effects. For example, addition of sulfate as part of BioLithe™(A) (Table 3.1.1) and BioLithe™(B) (Table 3.1.2) did not alone appear to effectively shut down methanogenesis through competitive exclusion where sulfate reducers could outcompete methanogens for electron donor (e.g., Lovley and Klug, 1983).

Also, treatment via the industrial coating process may not be the only approach to attach inhibitory chemicals on paper. Inhibitors perhaps could be added during the process of paper manufacturing with the benefit of saving the cost of industrial coating and achieve higher methane reduction by increasing the concentration of inhibitors or competitors. Chipboard and printer paper were the paper samples used in this series of BioLithe experiments. It is also possible that BioLithe formulae could be broadly employed in combination with other types of paper.

The development of BioLithe formulae should take account into two major factors. First, the cost for BioLithe inhibitors should be economic and stable because a high cost for material would not be sustainable economically. For example, gluconate, the composition in BioLithe™(B) with the form combined by heavy metals is more expensive than other commonly used chemical compositions. There is yet no reference to indicate its ability to conclusively achieve methanogen suppression. Second, chemicals selected for BioLithe formulations should not be harmful to humans and should be environmentally friendly. For example toxic volatile organic compounds such as chloroform could not be adopted for testing even though they may have highly inhibitory potential with a relatively small mass. When paper ends up in landfills, BioLithe chemicals would be dissolved and collected in leachate for recycling or treatment, and they should not lead to environmental contamination.

6.2 Future Work

Results obtained from this BioLithe study shows that the experimental design and, experimental and statistical approaches employed were workable and effective in a series of experiments. It could be continually applied for future BioLithe tests. Some following tasks are planned for continuing work and some of them have already been in process.

First, sequence both archaeal and bacterial sequences that eluted as individual bands of DGGE gels. More information about the microbial community could thus be obtained and better analysis of the BioLithe-influenced effect on archaeal methanogens and bacteria could be deduced. For example, it would be interesting to determine if sulfate-reducing bacteria are important community members in the BioLithe™(A) and BioLithe™(B) treated samples.

Second, bioreactors with strong biogas and methane inhibition in previous tests could be continued for degradation experiment, by removing undecomposed paper samples to new bioreactors containing fresh digester sludge. The persistence of BioLithe formula on paper could be determined, to estimate the progression of methane emissions when treated paper ends up in landfill.

Third, the concentration of BioLithe chemicals in dissolved coating material when coated on paper could be changed, and one could observe corresponding changes in methane production to determine the highest BioLithe concentration on coating, which may be associated with the least methane production.

Last, one needed area of work is to develop new BioLithe formulae based on BioLithe™(B), or other chemical formulations, which may have stronger methane

reduction potential or the ability to shift paper transformation to primarily carbon dioxide, rather than a mixture of methane and carbon dioxide. In every case, development of BioLithe formulae should be cost-effective and environmentally friendly.

REFERENCES

Achenbach, L. and Woese, C. (1995) 16S and 23S rRNA-like primers. In: Archaea, A Laboratory Manual, Methanogens (Sowers, K.R. and Schreier, H.J., Eds.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp:521-523.

Amann R. I., Ludwig W., Schleifer K-H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. 59:143–169.

Arsova L., Haaren R.V., Goldstein N., Kaufman S.M., and Themelis N.J. (2008) The State Of Garbage In America. BioCycle 49(12):22. Available from: http://www.jgpress.com/archives/_free/001782.html (Accessed online August 2010)

Asakawa, S., Morii, H., Akagawa-Matsushita, M., Koga, Y. and Hayano, K. (1993) Characterization of Methanobrevibacter arboriphilicus SA isolated from a paddy field soil and DNA-DNA hybridization among M. arboriphilicus strains. Int. J. Syst. Bacteriol. 43: 683- 686.

Barlaz M.A. (1998) Carbon Storage during Biodegradation of Municipal Solid Waste Components in Laboratory-Scale Landfills. Global Biogeochemical Cycles 12:373–380.

Bartacek J., Fermoso F.G., Catena A.B., Lens P.N.L. (2010) Effect of sorption kinetics on nickel toxicity in methanogenic granular sludge. Journal of Hazardous Materials. 180:289-296.

BBC News Website. (2009) Climate Change Glossary. Available from: http://news.bbc.co.uk/2/hi/science/nature/8314501.stm (Accessed online August 2010)

Béguin, P, Lemaire M (1996) The cellulosome: an exocellular, multiprotein complex specialized in cellulose degradation. Crit Rev Biochem Mol Biol 31:201-236.

Bédaich J.P, Tardif C, Bédaich A, Gaudin C (1997) The cellulolytic system of *Clostridium cellulolyticum*. J Biotechnol 57:3-14.

Belevi H. and Baccini P. (1989) Long-term behavior of municipal solid waste landfills. Waste Management and Research. 7(1):43-56.

Bender J, Vatcharapijarn Y, Jeffries TW (1985) Characteristics and adaptability of some new isolates of *Clostridium thermocellum*. Appl Environ Microbiol 49:475-477.

Bernalier A., Fonty G., Bonnemoy F., and Gouet P. (1992) Degradation and fermentation of cellulose by the rumen anaerobic fungi in axenic cultures or in association with cellulolytic bacteria. Current Microbiology. 25: 143-148.

Bogner J., Spokas K., Burton E., Sweeney R. and Corona V. (1995) Landfills as atmospheric methane sources and sinks. Chemosphere. 31(9):4119-4130.

Briones A., Raskin L. (2003) Diversity and dynamics of microbial communities in engineered environments and their implications for process stability, Curr. Opin. Biotechnol. 14:270–276.

Bundt M., Widmer F., Pesaro M., Zeyer J., Blaser P. (2001) Preferential flow paths: biological "hot spots" in soils. Soil Biol. Biochem. 33:729–738.

Casamayor E.O., Massana R., Benlloch S., Ovreas L., Diez B., Goddard V.J., Gasol J.M. (2002) Changes in archaeal, bacterial and eukaryal assemblages along a salinity gradient by comparison of genetic fingerprinting methods in a multipond solar saltern.Environ Microbiol. 4:338–348.

Casserly C., Erijman L. (2003) Molecular monitoring of microbial diversity in an UASB reactor, Int. Biodeter. Biodegr. 52:7–12.

Cahyani V.R., Matsuya K., Asakawa ., Kimura M. (2003) Succession and phylogenetic composition of bacterial communities responsible for the composting process of rice straw estimated by PCR-DGGE analysis. Soil Sci. Plant Nutr. 49: 619-630.

Chae K.J., Choi M.J., Kim K.Y., Ajayi F.F., Park W., Kim C.W., Kim I.S. (2010) Methanogenesis control by employing various environmental stress conditions in two-chambered microbial fuel cells. Bioresource Technology. 101(14):5350-5357.

Chakraborty N, Sarkar G.M., Lahiri S.C. (2002) Biomethanation of a cellulose-based substrate in the presence and absence of cellulolytic bacterium. World Journal of Microbiology and Biotechnology. 18(4):321-324.

Chan A.S.K. and Parkin T.B. (2000) Evaluation of potential inhibitors of methanogenesis and methane oxidation in a landfill cover soil. Soil Biology & Biochemistry 32:1581-1590.

Chen Y., Cheng J.J., Creamer K.S. (2007) Inhibition of anaerobic digestion process: A review. Bioresouce Technology. 99:4044-4064.

Chin, K.-J., Lukow, T., Stubner, S. and Conrad, R. (1999) Structure and function of the methanogenic archaeal community in stable cellulose- degrading enrichment cultures at two different temperatures (15 and 30 ℃). FEMS Microbiol. Ecol. 30:313-326.

Cooper, C.D., Alley, F.C. (2002) Air Pollution Control: A Design Approach, third ed. Waveland Press Inc., Long Grove, IL.

Cummings, S.P., Stewart, C.S. (1994) A study of cellulose degradation in landfills, ESTUB/B2/00198/REP, U. K. ESTU.

Czepiel P., Mosher B., Crill P. and Harriss R. (1996) Quantifying the Effect of Oxidation on Landfill Methane Emissions. Journal of Geophysical Research, 101(D11):16721-16730.

Dumitru R., Palencia H., Schroeder S.D. (2003) Targeting methanopterin biosynthesis to inhibit methanogenesis. Applied and Environmental Microbiology. 69(12):7236-7241.

Eleazer W.E., Odle W.S., Wang Y.S. and Barlaz M.A. (1997) Biodegradability of municipal solid waste components in laboratory-scale landfills. Env. Sci. Tech. 31(3):911-917.

Eleazer, W.E., Odle, W.S., Wang, Y.S., Barlaz, M.A. (1997) Biodegradability of municipal solid waste components in laboratory-scale landfills. Environ. Sci. Technol. 31:911–917.

Evans P.N., Joblin K.N. (2008)Structure of archaeal community of rumen. Applied and Environmental Microbiology. 74(12):3619-3625.

Fey, A., Chin, K.J. and Conrad, R. (2001) Thermophilic methanogens in rice field soil. Environ. Microbiol. 3:295-303.

Fry J.L., Merrill R. (1973) Methane digesters for fuel gas and fertilizer with complete instructions for two working models, pp. Santa Barbara, California.

Forster, P., Ramaswamy, V., Artaxo, P., Berntsen, T., Betts, R., Fahey, D., Haywood, J., Lean, J., Lowe, D., Myhre, G., Nganga, J., Prinn, R., Raga, G., Schultz, M., Dorland, R.V. (2007) Changes in atmospheric constituents and in radiative forcing. Tech. rep., In Climate Change 2007: the Physical Science Basis. 4th Assessment report of the Intergovernmental Panel on Climate Change.

Genoscope.(2008) Environmental 16s rDNA sequence from Evry wastewater treatment plant anoxic basin. Centre National de Sequencage. Available from: http://www.ncbi.nlm.nih.gov/nucleotide/194685773?report=genbank&log$=nucltop&blast_rank=10&RID=8G3VUAJ0013 http://www.ncbi.nlm.nih.gov/nucleotide/194685769?report=genbank&log$=nucltop&blast_rank=11&RID=8G3VUAJ0013 (Access online August 2010)

GeoSyntec Consultants. (2001A) E*valuation of Historical Data at Leachate Recirculating Landfills Report to Waste Management, Inc.* Lombard, Illinois, on

Area A/B Disposal Cell, Central Solid Waste Management Center. Sandtown, Delaware, Project No. ME0184, Columbia MD, USA.

GeoSyntec Consultants. (2001B) Evaluation of Historical Data at DSWA Test Cells. Report to Delaware Solid Waste Authority. Central Solid Waste Management Center, Sandtown, Delaware, Project No. ME0225, Columbia MD, USA.

Godon,J.J., Zumstein,E., Dabert,P., Habouzit,F. and Moletta,R. (1997) Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. Appl. Environ. Microbiol. 63 (7):2802-2813.

Government Engineering. (2006) Landfill design and operation. Available from: http://www.govengr.com/ArticlesMay06/landfilldesign.pdf (Accessed online August 2010)

GroMkopf, R., Janssen, P.H. and Liesack, W. (1998) Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Appl. Environ. Microbiol. 64:960-969.

Gutleben W., Unterholzner V.,Volgger D.,Zemann A. (2004) Characterization of carbohydrates in paper and paper pulps using anion exchange chromatography and principal component analysis. Chemistry and Materials Science. 146(2):111-117.

In S.J., Fukui M., Suwa Y. (1992) Analysis of substrates for methanogenesis in anaerobic sludges using specific inhibitors. Water Science and Technology. 26(3-4):847-856.

IPCC. (2007) Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Available from:
http://www.ipcc.ch/pdf/assessment report/ar4/wg1/ar4-wg1-spm.pdf. (Accessed online August 2010)

IPCC. (2007) Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Available from: http://www.ipcc.ch/pdf/assessment-report/ar4/syr/ar4_syr.pdf (Accessed online August 2010)

ITRC (Interstate Technology & Regulatory Council). (2005) *Characterization, Design, Construction, and Monitoring of Bioreactor Landfills*. ALT-3. Washington, D.C.: Interstate Technology & Regulatory Council, Alternative Landfill Technologies Team.

Janssen P.H., Frenzel P. (1997) Inhibition of methanogenesis by methyl fluoride: Studies of pure and defined mixed cultures of anaerobic bacteria and archaea. Applied and Environmental Microbiology. 63(11):4552-4557.

Kacik F., Kacikova D., Jablonsky M., Katuscak S. (2009) Cellulose degradation in newsprint paper ageing. Polymer Degradation and Stability 94:1505-1514.

Keyser M., Witthuhn R.C., Lamprecht C., Coetzee M.P., Britz T.J. (2006) PCR-based DGGE fingerprinting and identification of methanogens detected in three different types of UASB granules. Syst Appl Microbiol. 29(1):77-84.

Khan A.W. and Trottier T.M. (1978) Effect of sulfur-containing compounds on anaerobic degradation of cellulose to methane by mixed cultures obtained from sewage sludge. Applied and Environmental Microbiology, 35(6):1027-1034.

Khan A.W., Trottier T.M., Patel G.B. and Martin S.M. (1979) Nutrient Requirement for the degradation of cellulose to methane by a mixed population of anaerobes. Journal of General Microbiology, 112:365-372.

Kim S.K., Lee T. (2008) Degradation of lignocellulosic materials under sulfidogenic and methanogenic conditions. Waste Management. 29:224-227.

Kinsella S., Gleason G., Mills V., Rycroft N., Ford J., Sheehan K. and Martin J., (2007) The state of the paper industry monitoring the indicators of environmental performance. Environmental Paper Network. pp:6.

Kleikemper J., Silvina A. Pombo, Martin H. Schroth, William V. Sigler, Manuel Pesaro, and Josef Zeyer. (2005) Activity and diversity of methanogens in a petroleum hydrocarbon-contaminated aquifer. Applied and environmental microbiology. p:149-158.

Kosik M., Surina I., Rucka I., Lapcik L., Resier V. (1983) Thermolytic reaction of cellulose-Dehydration reactions of cellulose. Chem Pap. 37(6):843-850.

Kumar S., Gaikwad. S.A. and Shekdar A.V. (2004) Estimation method for national methane emission from solid waste landfills. Atmospheric Environment. 38(21):3281-3487.

Kugelman., McCarty P.L. (1964) Cation toxicity and stimulation in anaerobic waste treatment. J. Water Pollut. Control. 37:97–116.

L évesque M.J., Guiot S.R. (2004) Positioning of *Methanosaeta* and *Methanosarcina* sp. within the microstructure of carbohydrate-fed anaerobic granules. In: Proceedings of the 10th Anaerobic Digestion Conference. pp:1934–1937.

Lorowitz W.H., Nagle D.P., Tanner R.S. (1992) Anaerobic oxidation of elemental metals coupled to methanogenesis by methanobacterium – thermoautotrophicum. Environmental Science and Techonology. 26(8):1606-1610.

Lovley, D. R.; Klug, M. J., Sulfate reducers can outcompete methanogens at freshwater sulfate concentrations. Applied and Environmental Microbiology 1983, 45, (1), 187-192.

M ackie K.R. and Cooper C.D. (2009) Landfill gas emission prediction using Voronoi diagrams and importance sampling. Environmental Modelling and Software. 24:1223-1232.

Madigan, M.T. and Martinko J.M. (2006) Syntrophy and Methanogenesis, in Brock Biology of Microoranisms, G. Carlson, Editor. Prentice Hall: Upper Saddle River, NJ.

Mancinelli R. and McKay C. (1985) Methane-Oxidizing Bacteria in Sanitary Landfills. Proc. First Symposium on Biotechnical Advances in Processing Municipal Wastes for Fuels and Chemicals, Minneapolis, pp: 437-450.

Mastrandrea M. D. and Schneider S. H. (2005) "Global warming." World Book Online Reference Center. World Book, Inc. Available from: http://www.worldbookonline.com/wb/Article?id=ar226310 (Accessed online August 2010).

Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplifed genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695-700.

Nakatsu, C.H., Torsvik, V. and Ovreas, L. (2000a) Soil community analysis using DGGE of 16S rDNA polymerase chain reaction products. Soil. Sci. Soc. Am. J.64:1382-1388.

NJ Department of Environmental Protection. (2010) New Jersey energy master plan. Available from: http://www.state.nj.us/globalwarming/ (Accessed online August 2010)

Oremland, R.S., Taylor, B.F., 1975. Inhibition of methanogenesis in marine sediments by acetylene and ethylene: validity of the acety- lene reduction assay for anaerobic microcosms. Applied Microbiology. 30: 707-709.

Øvreås L., Forney L., Daae F.L., Torsvik V. (1997) Distribution of bacterioplankton in Meromictic Lake Sælenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA, *Appl. Environ. Microbiol.* 63:3367–3373.

Parameswaran P., Torres C.I., Lee HS., Brown R.K., Rittmann B.E. (2009) Syntrophic interactions among anode respiring bacteria (ARB) and non-ARB in a biofilm anode: Electron Balances. Biotechnology and Bioengineering. 103(3):513-523.

Park S., Brown K. W., Thomas J. C., Lee I.C. and Sung K. (2009) Comparison study of methane emissions from landfills with different landfill covers. Environmental Earth Science. 60(5):933-941.

Pei C.X, Mao S.Y., Cheng Y.F., Zhu W.Y. (2010) Diversity, abundance and novel 16S rRNA gene sequences of methanogens in rumen liquid, solid and epithelium fractions of Jinnan cattle. The Animal Consortium 2009. 4(1): 20–29.

Power Partners[SM] Resources Guide (PPRG). (2009) Landfill methane energy recovery. http://www.uspowerpartners.org/Topics/SECTION6Topic-LandfillMethane.htm (Accessed online August 2010)

RISI (Resource Information Systems Inc). (2004) North American graphic paper forecast, 4(3). Bedford, MA.

Riviere,D., Desvignes,V., Pelletier,E., Chaussonnerie,S.,Guermazi,S., Weissenbach,J., Li,T., Camacho,P. and Sghir,A.(2009) Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. ISME J 3 (6):700-714.

Rozej A., Montusiewicz A., Lebiocka M. (2008) Characteristics of microbial communities in biomethanization processes. Archives of Environmental Protection. 34(3):299-307.

Sanchez J.M., Valle L., Rodriguez F., Morinigo M.A., Borrego J.J. (1996) Inhibition of methanogenesis by several heavy metals using pure cultures. Letters in Applied Microbiology. 23(6):439-444.

Scharff H, Jacobs J. (2006) Applying guidance for methane emission estimation for landfills. Waste Management. 26(4):417-429.

Scheer M., Liebner N., Roeske I., Roeske K. (2010) Particular diverse archaeal communities in sediments of floodplains and a drinking water reservoir.(Unpublished). Available from: http://www.ncbi.nlm.nih.gov/nucleotide/285818735?report=genbank&log$=nucltop&blast_rank=1&RID=8G6AC5HS01P (Access online August 2010)

Schulze-Makuch D., Haque S., Hallam S.J., Antonio M., Ali D., Hossein R., Song Y.C., Yang J., Beckles D., Guinan E., Lehto H.(2010) A Microbial Community within a Natural Asphalt Lake.(Unpublished). Available from: http://www.ncbi.nlm.nih.gov/nucleotide/296881246?report=genbank&log$=nucltop&blast_rank=21&RID=8G72RADG01P (Access online August 2010)

Shelton D.R., Tiedje J.M. (1984) General method for determining anaerobic biodegradation potential. Applied and Environmental Microbiology. 47(4):850-857.

U.S. Department of Energy. (2005) Forest products: Industry of the future. Annual report fiscal year 2004.Industrial Technologies Program. Available from: http://www.eere.energy.gov/industry/about/pdfs/forest_fy2004.pdf / (Accessed online August 2010)

U.S. EPA. (2009) Inventory of U.S. greenhouse gas emissions and sinks: 1990-2007. Washington DC.

U.S. EPA. (2010) Inventory of U.S. Greenhouse Gas Emissions and Sinks:1990-2008. Tech. Rep. EPA 430-R-10-006. U.S. Environmental Protection Agency, Washington, DC.

Varel V.H. and Wells J.E. (2007) Influence of thymol and a urease inhibitor on coliform bacteria, odor. Journal of Environmental Quality. 36(3):773-779.

Weber, S., Lueders, T., Friedrich, M.W. and Conrad, R. (2001) Methanogenic populations involved in the degradation of rice straw in anoxic paddy soil. FEMS Microbiol. Ecol. 38:11-20.

Zhang L., Keller J., Yuan Z. (2009) Inhibition of sulfate-reducing and methanogenic activities of anaerobic sewer biofilms by ferric iron dosing. Water Research. 43(17):4121-4132.

Zhao H.X., Yang D.C., Woese C.R. (1996) Anaerobic bioprocessing of organic waste. World Journal of Microbiology and Biotechnology. 12(3):221-238.

Zinder S.H., Koch M. (1984) Non-acetoclastic methanogenesis from acetate: Acetate oxidation by a thermophilic syntrophic coculture. Arch Microbiol. 138(3):263–272.

Zou X, Gurnagul N, Uesaka T, Bouchard J. (1994) Accelerated aging of papers of pure cellulose: mechanism of cellulose degradation and paper embrittlement. Polym Degrad Stab. 43:393–402.

APPENDIX

A.1 Sequence of methanogenic archaeal products in Test 4, Degradation of Paper Coated with Inhibitors in an Industrial Process

DGGE band name	**Sequence fragment**
P1	CGGGAATCCCGAGTGCTCGTGTATTGTTGGGCTGTTCTTTGGTCTAAAAAACCA TAGGAGTAAGGGTGGGCANACCGGTGNCNCCNCCGCGGNAATACCGGCAGCT CAAGTGGTAACCNCGATTATTGNGCCTAAAACGTTCGTAGCCGGATAAGTAANT CTTTGTTTAAATCCTGCGACTCAACCGGGGGAA
P2	ACAAATTCTGGCTTGTCGTGATGCCTAAAAAGCATTATATAGCAAGGGCCGGGC AAGACCGGTGCCAGCCGCCGCGGTAACACCGGCGGCTCGAGTGGTAACCGTTA TTATTGGGTCTAAAGGGTCTGTA
P3	ACAAATTCTGGCTTGTCGTGATGCCTAAAAAGCATTATATAGCAAGGGCCGGGC AAGACCGGTGCCAGCCGCCGCGGTAACACCGGCGGCTCGAGTGGTAACCGTTA TTATTGGGTCTAAAGGGTCTGTA
P4	TAATCCAGGACCCCCGGGTGCCAGGTTACAAATCAGGCTGTCGTGATGCCTAAA AAGCATTATATAGCAAGGGCCGGGCAAGACCGGTGCCAGCCGCCGCGGTAACA CCGGCGGCTCGAGTGGTAACCGTTATTATTGGGTCTAAAGGGTCTGT
P5	AATCAGGCTGTCGTGATGCCTAAAAAGCATTATATAGCAAGGGCCGGGCAAGA CCGGTGCCAGCCGCCGCGGTAACACCGGCGGCTCGAGTGGTAACCGTTATTATT GGGTCTAAAGGGTCTGTAGCCGGCCGGATAAGTCTCTTGGGAAATCTGGCAGC TTAACTG
P6	GTGATCAAGGAACCTCGAGTGCCAGGTTACAAATCTGGCTGTCGTGATGCCTAA AAAGCATTATATAGCAAGGGCCGGGCAAGACCGGTGCCAGCCGCCGCGGTAAC ACCGGCGGCTCGAGTGGTAACCGTTATTATTGGGTCTAAAGGGTCTGTA
P7	CAGGGACACCGAGTGCTAGCATCATATGCTGGCTGTCCAGGTGTGTAAAATACA CCTGTTAGCAAGGGCCGGGCAAGACCGGTGCCAGCCGCCGCGGTAACACCGGC GGCCCGAGTGGTGATCGTGATTATTGGGTCTAAAGGGTCCGTAGCCGGTTTGG TCAGTCTTCC
P8	CAAGGGCCGGGCAAGACCGGTGCCAGCCGCCGCGGTAACACCGGCGGCCCGA GTGGTGATCGTGATTATTGGGTCTAAAGGGTCCGTAGCCGGTTTGGTCAGTCCT CCGGGAAATCTGACAGCTCAACTG