RESPONSE OF SOIL

MICROBIAL COMMUNITIES

TO FRACING FLUIDS

By

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Abstract:

Extraction of oil and gas from shale is becoming a significant growing part of domestic energy production. Wastes from drillings include drill cuttings, drilling mud, flow back water during the first 30 days of the well, produced water from 30 days back, and miscellaneous wastes such as spent lubricants. The purpose of this study is to analyze the effect that these fracing fluids pose to soil microbial communities. DNA analysis of soil microorganisms, quantitative real-time PCR was performed to quantify microorganism population, ARISA analysis was done to identify relationships in microorganisms, and a methanogenesis toxicity test conducted in order to determine toxicity of said fracing fluids. Although there was no significant alteration of soil microorganism population, a variation of microbes present at various sampling days, suggests that a unique microorganism exists with the addition of fracing fluids to soil. Future research is essential for the identification of stated microorganism.

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CHAPTER I

INTRODUCTION

Oil and gas from shale has become a significant growing part of domestic energy production. Extraction of oil and natural gas from hard to reach reservoirs is becoming more extensive all over the world, but especially the United States. These methods may pose unique threats to the environment (Entrekin, 2011). Wastes from drillings include drill cuttings, drilling mud, flow back water during the first 30 days of the well, produced water from 30 days back, and miscellaneous wastes such as spent lubricants. Liquid wastes are transported to centralized facilities, landfills, or deep injection disposal wells. There is contamination of surface water and shallow groundwater from spills, leaks, and the disposal of inadequately treated shale gas wastewater. The effects of the chemicals in fracing fluids are known to be toxic, but these chemicals are being used in very dilute quantities and in mixtures, so the effects of these mixtures in unknown.

Much investigation has been done in the possible contamination of groundwater and drinking water sources by various chemical compounds found in fracing fluids. Although, fracing fluids are examined in their original state, it is possible that the chemicals undergo transformations during the fracing operation and under conditions underground (Gordalla, et al., 2013).

The purpose of this study is to investigate the effect of a mixture of common hydraulic fracing fluids on soil and sediment microbial communities. Several experiments will be conducted in order to further understand this effect.

A representation of six fracing fluids were made according to similar compositional types. It is important to mention that the fracing fluids made are only a representation of the actual fluids used for drilling. Microcosms with soil and fracing fluids were set up in order to analyze how the soil microbial communities are affected. A series of experiments were conducted: DNA analysis, chemical oxygen demand to observe if there is any degradation of the organic carbon of the fracing fluids, Automated Ribosomal Intergenic Spacer Analysis (ARISA) to observe if there is a change in structure of the microbial communities in the soil, methanogenesis toxicity to determine if the fracing fluids are toxic, quantitative real-time PCR to see if there is change in population, and the pH and DO were monitored.

CHAPTER II

REVIEW OF LITERATURE

The United States has long sought to decrease its independence on foreign oil by exploring alternative energy sources (Finkel & Law, 2011). Nuclear power and coal have their own sets of problems; therefore, natural gas is increasingly viewed as a viable alternative to meet energy needs (Finkel & Law, 2011). Importantly, relying on natural gas compared to other fossil fuels makes it easier to meet federal air quality standards (Finkel & Law, 2011). Additionally, increasing domestic oil production has also long been a national goal to reduce dependence on oil from unstable regions of the world. Extraction of oil and natural gas from hard to reach reservoirs is becoming more extensive all over the world, but especially the United States, and these new drilling methods may pose unique threats to the environment (Entrekin, 2011). Millions of gallons of water along with a variety of chemicals, which may be toxic, are introduced into the Earth in order to extract the increasingly demanded oil and natural gas in a process called hydraulic fracturing or 'fracing' (Entrekin, 2011). There is a growing concern that rapid and extensive oil and natural gas development could lead to the degradation of natural resources, and often, wells are close to surface waters that could be contaminated by elevated sediment runoff (Entrekin, 2011). The mixtures of fluids used in these processes may also be stored or spilled on the surface with unknown impacts on surface soils and waters.

2.1 Natural Gas Production

Natural gas extraction from unconventional gas reservoirs has significantly expanded through the introduction of horizontal drilling with high volume hydraulic fracturing. These new technological advances have opened vast energy sources, such as low-permeability organic rich shale formations and "tight-sand" reservoirs, altering the domestic energy landscape in the United States (Kargbo et al., 2010). Unconventional hydrocarbon extraction from organic-rich shale formations is now active in more than 15 "plays" in the United States (Vengosh et al., 2014). At the end of 2012, the Marcellus Shale (29% of production), Haynesville Shale (23%), and Barnett Shale (17%) dominated production of natural gas (primarily methane, ethane, and propane) from shales in the U.S., with the remaining 31% of total shale gas production contributed by more than a dozen other basins (Vengosh et al., 2014). The current global estimate of natural gas reserves in unconventional shales is approximately 716 trillion m^3 (2.53×10^{13} MCF) (Boyer et al., 2011) indicating that these new drilling processes will be in use for the foreseeable future.

2.2 Horizontal Drilling and Hydraulic Fracturing ("fracing")

Horizontal drilling and high volume hydraulic fracturing of shale has made oil and gas extraction much more economically feasible (Finkel & Law, 2011). However, the fracing water used, the flowback fluids that come up immediately after fracing, and the produced water then comes up with the hydrocarbons in the long haul potentially pose a threat to the environment and to the public's health (Finkel & Law, 2011).

2.2.1 Process

Hydraulic fracturing involves pumping as much as five million gallons of water mixed with a suite of chemicals and solids, such as sand, under high pressure down and across wells (Finkel & Law, 2011). This pressurized mixture causes the low-permeable rock layer to crack open and create fractures (Granberg, 2013). Sand is usually used as a propellant in order to keep the fissures open and allow a flow path through the tight rock formations to all oil and/or natural gas up the well (Granberg, 2013). Flowback water, which contains a mixture of fracing fluids and the waters native to the formation, is stored often in open pits until disposal and natural gas is piped to market (Granberg, 2013).

2.2.2 Air, Water, and Soil Contamination

It is difficult to assess exactly what goes into fracing fluids because drilling companies are not required by law to list the chemical compounds used in fracing (Finkel & Law, 2011). However, toxic mud and fluid byproducts from the drilling and fracing as well as spills and gas wastes are not uncommon (Finkel & Law, 2011). For example, of the more than 8,600 abandoned wells in Pennsylvania in 2009 alone, taxpayers paid to plug 259 because of leaking natural gas, oil, and acid mine drainage into the groundwater, surface water, and air (Colburn, Kwiatkowski, & Schultz). Post mineral extraction cleanup costs are substantial and include restoration of damaged or contaminated streams and soil, improper handling of wastewater and radioactive materials (Finkel & Law, 2011). Information on the exact ingredients of fracing fluids is essential to better understand the potential health and environmental effects of hydraulic fracturing (Finkel & Law, 2011). In addition, concerns about the contamination of underground water supply have risen (Finkel & Law, 2011). Although regulations are currently being proposed, the disposal of

polluted water used for fracing is sparsely regulated (Finkel & Law, 2011). Soil contamination has not been addressed fully; drilling sludge (a mixture that includes drilling mud and rock cuttings with hydrocarbons, radioactive material, and heavy metals) is spilled on the surface during the drilling phase (Finkel & Law, 2011) and in some states like Oklahoma, they are applied to marginal lands as a method of disposal. Flowback waste fluids and production waters must be disposed of safely because they can potentially contaminate air and soil (Finkel & Law, 2011).

2.2.3 Chemical Residues in Areas of Disposal and Leaks

Rapidly expanding drilling in the Marcellus Shale has significantly increased the volume of produced water that must be managed (Wilson & VanBriesen, 2012). Produced water management may include treatment followed by surface water discharge, such as to a publically owned wastewater treatment plants (POTWs), or centralized brine treatment plants (CWTs) (Wilson & VanBriesen, 2012). Estimates of salt loads associated with produced water and with discharges from water treatment plants in 2008 and 2009, indicate that more than 50% of the total dissolved solids in the produced water generated in those years were released to surface water systems (Wilson & VanBriesen, 2012). Especially during low-flow conditions of 2008 and 2009, these loads would be expected to affect drinking water (Wilson & VanBriesen, 2012). Over time, metals, salts, and organics may build up near wastewater disposal or spill sites. Each respective compound has a fixed reactivity and solubility that varies as a function of pH, temperature, and the occurrence of other components in the soil and water; as a result, the physicochemical conditions of surface waters and the reactivity of each compound will determine how it interacts with particulate matter or river sediments (Vengosh et al., 2014). Ultimately, these properties will

determine the long-term fate of such reactive contaminants; reactive constituents would be absorbed into soil, stream or pond sediments and potentially pose long- term environmental and health risks.

For example, Marcellus wastewaters contain elevated levels of naturally occurring radionuclides (NORM) in the form of radium isotopes (Warner et al., 2013). The elevated radium levels in Marcellus brines is due to the mobilization of radium from uranium-rich source rocks into the liquid phase under high salinity and reducing conditions (Rowan et al., 2011). Disposal of the NORM-rich Marcellus waste fluids to freshwater streams could cause radium absorption onto the stream sediments in disposal or spill sites because radium absorption is inversely correlated with salinity (Krishnaswami et al., 1991).

Disposal of treated wastewater originating from both conventional and unconventional oil and gas production in western Pennsylvania has caused radium accumulation on stream sediments downstream of a disposal site from a brine treatment facility (Warner et al., 2013). The radium accumulated in the stream sediments has $^{228}Ra/^{226}Ra$ ratios identical to those of the Marcellus brines, thus linking this accumulation directly to the disposal of unconventional shale wastewater (Vengosh et al., 2014). The level of radioactivity found in sediments at one brine treatment discharge site exceeded the management regulations in the U.S. for a licensed radioactive waste disposal facility (Warner et al., 2013).

Elevated NORM levels were also found in soils near roads associated with road spreading of conventional oil and gas brines for deicing (Skalak, et al. 2014) and on pond bottom sediments associated with a spill from hydraulic fracturing activities (Warner et al., 2013). High NORM levels were also recorded in soil and sludge from reserve pits used in unconventional

natural gas mining (Rich & Crosby, 2013). Reactive residuals in brines, such as metals and radioactive elements, can accumulate in river and lake sediments and could pose long-term environmental and health effects by slowly releasing toxic elements and radiation in the affected areas (Vengosh et al., 2014).

2.2.4 Public Health Concerns

Little research has been done on the potential adverse health effects of fracing (Finkel & Law, 2011). One study, based on Pennsylvania Department of Environmental Protection and the Susquehanna River Basin Commission Material Safety Data Sheets for 41 products used in fracturing operations, assessed the chemicals used in fracing and found that 73% of the ingredients had between 6 and 14 different adverse health effects including skin, eye, and sensory organ damage; respiratory distress including asthma, gastrointestinal and liver disease; brain and nervous system harms; cancers and negative reproductive effects (Diamanti-Kandarakis et al., 2009). Fracing fluid and flow back fluids contain candidate endocrine disruptors, but because of the lack of disclosure information by the drilling companies of the exact chemical compounds used in fracing fluids, it is difficult to truly assess their potential adverse effects (Finkel & Law, 2011). With ill characterized toxicities and little certainty regarding exposure pathways the long-term health risk from fracing is not known (Finkel & Law, 2011). The information that does exist on hydraulic fracing fluid compositions is compiled by FracFocus, the national hydraulic fracturing chemical registry. It is managed by the Ground Water Protection Council and Interstate Oil and Gas Compact Commision whose missions both revolve around conservation and environmental protection. The webpage was created to provide the public access to reported

chemicals used for hydraulic fracturing. It lists chemical compositions and volumes of water used in fracing in selected states, including Oklahoma, though many chemicals are still listed as 'proprietary' (GWPC & IOGCC, 2015)

2.2.5 The Economic Aspect

The economic benefit for drilling natural gas is potentially huge for landowners and industry (Finkel & Law, 2011). There are estimates of more than $500 billion in recoverable natural gas in Pennsylvania alone (Jacquet., 2009). For example, the Marcellus Shale, a black shale formation that lies up to 10,000 feet below ground surface extending over 54,000 square miles primarily in New York and Pennsylvania, contains between 168 trillion to 516 trillion cubic feet of natural gas (Anon, 2011).

2.2.6 Future Drilling

From 2000 to 2008, the number of active wells drilled in New York State nearly doubled from 6845 to 13,687 and over the next decade, another 80,000 wells could be drilled (Sickle., 2009). Industry estimates indicate that over the next 20 to 30 years an additional 300,000 new wells could be drilled using fracing technology (Pennsylvania Department of Environmental Protection, 2015).

2.3 Research

Much investigation has been done in the possible contamination of groundwater and drinking water sources by the various chemical compounds found in fracing fluids. Although, fracing fluids are examined in their original state, it is likely that the chemicals undergo transformations during the fracing operation and under conditions underground (Gordalla et al., 2013). Furthermore, microbial transformations might occur, when the efficiency of the biocide has decayed (Gordalla et al., 2013). A detailed analysis of possible degradation or transformation products would be necessary (Gordalla et al., 2013). More research is being done in the potential fate and toxicity of biocides used in hydraulic fracturing operations. A study was performed to identify the following physicochemical and toxicological aspects, as well as knowledge gaps that should be considered when selecting biocides: (1) uncharged species will dominate in the aqueous phase and be subject to degradation and transport whereas charged species will sorb to soils and be less bioavailable; (2) many biocides are short-lived or degradable through abiotic and biotic processes, but some may transform into more toxic or persistent compounds; (3) understanding of biocides' fate under downhole conditions (high pressure, temperature, and salt and organic matter concentrations) is limited; (4) several biocidal alternatives exist, but high cost, energy demands, and/or formation of disinfection byproducts limits their use (Kahrilas, 2015).

2.4 Conclusion

Further investigation is essential in order to fully understand the impacts of the suite of chemical compounds used for fracing on the microbial life on soils due to spills (unintentional or intentional) and in earthen fracing fluid holding facilities. The purpose of this experiment is to further understand the environmental effects of six unique representative fracing fluid mixtures

on surface soil microorganism communities as a step towards assessing the toxicity and/or biodegradability of these fluid mixtures on soil microbial communities.

CHAPTER III

METHODOLOGY

3.1 Fracing Fluid Preparation

The list of all constituents of different fracing fluids that were used to drill 76 wells in Payne County, OK from fall 2012 to May 2014 were obtained from public records (FracFocus, 2015). The constituents of each fracing fluid and their concentration were transferred into Excel and though a wide variation existed well to well, six major compositional types were found to recur. From this data, six 'representative' fracing fluids, labeled A – F, were calculate from the average concentration of the constituents from within each group. It is important to mention that the 'representative' fracing fluids made do not contain exactly the ingredients found in the actual fracing fluid compositions available to the public in the form of concentration and volume employed. The ingredients that were used to make the experimental fracing fluids were meant to be representative of the actual fracing fluids and were chosen due to their similarity with the actual constituents. Importantly, sand was not included (sand is inorganic and not likely to impact the analysis), and motor oil was used as a substitute for components labeled as 'petroleum distillates' and similar crude petroleum-like constituents. Additionally, 'trade secret' ingredients could not be included; however, many of these "trade secrets" are ingredients such as "alcohols" not likely to be major contributions to microbial toxicity due to the very low concentrations used. Regardless, these compositions represent a range of mixtures of biocides, surfactants, and other

toxic components used in the fracing process. The fracing fluids were produced from original chemicals at a stock concentration of 10 times (10X) the average concentration found to be used in fracing water (referred herein as 1 × concentration). The following ingredients were added to a liter of water to make each 10X fracing fluid.

Table 3.1 Fracing Fluid A (10X)

Fracking Fluid A	
0.0269g	1-(Benzyl) quinolium Chloride
0.0456mL	1,2,4-trimethylbenzene
0.0887mL	acetic acid
104mL	acetic anhydride
0.674mL	tersitol
7.35mL	Clidox
1.068g	citric acid
3.05mL	ethanol
2.4mL	motor oil
221mL	isopropanol
0.123mL	methanol
0.2124g	naphthalene
0.3351g	sodium pentetrahyde

Table 3.2 Fracing Fluid B (10 ×)

Fracking Fluid B	
0.008mL	1-decanol
0.00864mL	1-octanol
0.0192mL	2-butyethanol
0.401mL	2-ethylexanol
0.003124mL	acetaldehyde
0.52475mL	acetic acid
0.00735mL	Clidox
0.21895mL	citric acid
0.00368mL	dioxane
0.715662mL	dodecylbenzenesulfuric acid
0.008159mL	tergitol
0.13548mL	ethylene glycol
0.22057mL	ethylene oxide
0.03049mL	isopropanol
0.0285159mL	N-N, dimethylformaldehyde
0.238739mL	gencol
0.01561mL	motor oil
0.006543mL	triethylphosphide

Table 3.3 Fracing Fluid C (10 ×)

Fracking Fluid C	
0.2113g	2-2 bromo-3 nitrilopropane
0.378mL	2-ethyl-exanol
0.6192mL	sodium dodecylbenzenesulfoneate
0.0745mL	ethylene glycol
0.022 mL	genpol X080
1.275mL	poloxamer
1.343mL	polyethyleneglycol

Table 3.4 Fracing Fluid D (10 ×)

Fracking Fluid D	
0.2613g	2-2-bromo-3-3-nitrilopropiate
0.006mL	2-mercaptoethanol
0.094mL	adipic acid
0.019mL	citric acid
0.0286mL	tergitol
0.064mL	formic acid
0.046mL	glycolic acid
2.231mL	motor oil
0.163mL	isopropanol
0.139mL	methanol
0.826667g	polyethylene glycol

Table 3.5 Fracing Fluid E (10 ×)

Fracking Fluid E	
0.073mL	2-butyl ethanol
0.0243mL	ethylene glycol
1.83mL	motor oil
0.088mL	isopropanol
0.485mL	methanol
0.0317mL	N-N-dimethyl formate

Table 3.6 Fracing Fluid F (10 ×)

Fracking Fluid F	
7.35mL	clidox
0.1479g	citric acid
0.3767mL	tergitol
0.05563mL	gelatin
0.9675mL	motor oil
0.006629mL	glutaraldehyde
0.9877mL	methanol
0.00368mL	propargyl alcohol
0.142626mL	SPAN 80

A minimal mineral media was also made for dilution purposes of fracing fluids consisting of (per L) 1000 mg NaCl, 500 mg $MgCl_2$*$6H_2O$, 200 mg KH_2PO_4, 300 mg NH_4Cl, 300 mg KCl, 15 mg $CaCl_2$*$2H_2O$, 1 mL of trace element A and 1 mL of trace element B (Shelton and Tiedje, 1984).

3.2 Experiment set up

Approximately two kilograms of soil at a depth of 0-2 inches were collected from Stillwater, OK on June 17, 2014. Soil collected was from an undeveloped oak-pecan forest soil. A total of 21 bench top microcosms were set up for triplicates of each fracing fluid mixture and a control consisting of mineral media in lieu of fracing fluid. Each microcosm was a 250 mL Erlenmeyer flask containing 100 cm^3 of loose soil sample and 1X concentration of fracing fluid to a total volume of mixed fluid/soil to 150 mL. Each reactor was open to atmospheric conditions, covered with glass wool and was left to stand statically for the duration of the experiment.

3.3 Sample collection

Samples were collected at Days 0, 6, 13, 20, 30, 41, 52, 64, and 78. For COD analysis,2 mL of the liquid supernatant were transferred using glass Pasteur pipettes to a clean 2 mL plastic tube without disturbing microcosm contents (for a liquid only sample). A total of 168 samples for COD analysis were collected and samples were stored at -20°C until analysis. For DNA analysis, each microcosm was vigorously stirred to homogenize the slurry. Approximately 1.5 mL of slurry was transferred with cut-off glass Pasteur pipettes to micro centrifuge tubes. A total of 168 samples for DNA analysis were taken and samples were stored at -20°C until DNA extraction.

3.4 Sample Analysis

3.4.1 Dissolved Oxygen (DO) and pH

Before taking samples, DO and pH measurements were taken by using YSI 5100 dissolved oxygen meter and Mettler Toledo Seven Compact pH/Ion probe according to manufacturer's instructions.

3.4.2 Chemical Oxygen Demand (COD) Analysis

For COD analysis, 1 mL of sample collected was transferred to HACH COD vials containing digestion solution and 1mL of tap water was added. The tube was vigorously shaken and placed in a HACH COD reactor at 150°F for 2 hours. Samples were cooled down to ambient

temperature and placed in HACH DR 5000 spectrophotometer to measure absorbance at λ=620nm. External standards using potassium hydrogen phthalate between the concentrations of 20 mg/Land 1000 mg/L were used to quantify the COD (APHA et al., 2012).

3.4.3 DNA analysis

Microcentrifuge tubes containing sample were brought to ambient temperature and centrifuged at 10,000 rcf for 1 min. The supernatant was discarded and the pellet was transferred to bead beating tubes for DNA extraction using PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, California). DNA was extracted by following the manufacturer's instructions. A total of 168 DNA extractions were done. DNA extracts were frozen at -20 °C until further analysis.

3.4.4 Automated Ribosomal Intergenic Spacer Analysis (ARISA)

ARISA was performed for community analysis similarly as described by McNamara and Krzmarzick (2013). Briefly, the interspacer region between the 16S and 23S sections of the rRNA gene were amplified with polymerase chain reaction. Each PCR reactor (25 µL) contained 1 µL of DNA extract, 1.0 mM of magnesium chloride, 1X DNA GoTaq buffer (Promega), 1 µg of bovine serum albumin (BSA), 1.6 mM of dNTPs, 0.25 µM of 6FAM-ITSF primer and 0.25 µM ITS-ReubR primer (Cardinale et al., 2004), and 0.625 U of GoTaq DNA polymerase (Promega). PCR was performed in a T100 Thermal Cycler (BioRad) with thermocycling conditions described previously (McNamara & Krzmarzick, 2013).

Each DNA extract was amplified in triplicate, and triplicate amplifications were combined in equal volumes. Combined amplifications were mixed with MapMarker1000 (with ROX) fragment size standard (BioVentures, Boston, Massachusetts), denatured with HiDi Formamide (Life Technologies, Grand Island, New York) and sent to the DNA/Protein Core Facility at Oklahoma State University for fragment size analysis on an ABI Model 3730 DNA Analyzer.

3.4.5 Microorganism Community Analysis

ARISA fragment data was analyzed with Peak Scanner v1.0 software (Life Technologies, Grand Island, New York). The base pair sizes and the peak area for the 168 samples was transferred to an Excel worksheet. Fragment sizes that were smaller than 50 bp and larger than 1000 bp were discarded because they were assumed to be outliers, as the expected range is 250-800. Peaks with areas less than 0.5% of the remaining total peak area of the sample were discarded as well because they were not significant contributors.

3.4.6 Quantitative real time PCR (qPCR)

For bacteria 16S rRNA, primers 341F and 534R (Muyzer et al., 1993) were used as previously described (Krzmarzick et al., 2012). Each qPCR mixture totaled of 10 µl using 5 µl of 2 × iTaq SyberGreen Supermix with Rox master mix (BioRad), 300 nM of each primer, and 1.0 µl of undiluted DNA extract or standard. Analysis was on a CFX Connect Real Time System (Bio-Rad Laboratories) with Bio-Rad CFX Manager software. Thermocycling protocol for each analysis was 95 ^{o}C for 3 min followed by 40 cycles of 95 ^{o}C for 15 s and 60 ^{o}C for 30 s. A

melting curve analysis was performed after each complete run to ensure that primer-dimers were not amplified and that the amplification was specific. Standards for each qPCR were prepared from known concentrations of plasmid extracts containing the 16S rRNA gene of interest. Each sample was analyzed with qPCR in duplicate, the duplicates were $\log_{10}$ transformed and averaged. Triplicate microcosms were then averaged and these means and standard deviations.

3.4.7 Methanogenesis Toxicity Test

This test was performed to determine microbial toxicity of fracing fluids for methanogens using acetate as substrate and electron donor. The methanogenic activity is determined by the chemical oxygen demand of methane produced per day (mg CH_4-COD/day) per gram of volatile suspended sludge (g VSS). This reaction is carried out under anaerobic conditions for chemoorganotrophic (acetate as substrate, acetoclastic) methanogens.

A liter of mineral media was composed of: 3,843.84 mg of CH_3CO_2K, 312.6 mg of K_2HPO_4, 12.6 mg of $CaCl_2.2H_2O$, 125 mg of $MgSO_4.7H_2O$, 125 mg of $MgCl_2.6H_2O$, 350 mg of NH_4Cl, 125 mg of yeast extract, 1.2 mL of trace element solution and right before using mineral media, 5000 mg of $NaHCO_3$ is also added.

On Day 0, in an anaerobic chamber, 500 mg of anaerobic digester sludge were added to each serum bottle plus 20 mL of anaerobic medium. The bottles were then crimp capped and left to sit overnight to acclimate to the warmth. On Day 1, 25 mL of media and fracing fluid was added by using volumetric pipettes. Dilutions of 0.05X, 0.1X, 0.25X, 0.5X, 0.75X, and 1X of the fracing fluids were made. Also, control vials were also made containing only the anaerobic media and the anaerobic digester sludge. Each dilution for each fracing fluid and the control

were done in duplicate for a total of 74 flasks. This set up was repeated for the B, C, D, E, and F fracing fluids.

Table 3.7 Methanogenesis Toxicity Experiment Set Up

Serum Flask	Final concentration	Media vol. (mL)	Fracing fluid vol (mL)	Total Vol (mL)
A1	0.05X	0.125	4.875	25
A2	0.05X	0.125	4.875	25
A3	0.1X	0.25	4.75	25
A4	0.1X	0.25	4.75	25
A5	0.25X	0.625	4.375	25
A6	0.25X	0.625	4.375	25
A7	0.5X	1.25	3.75	25
A8	0.5X	1.25	3.75	25
A9	0.7X	1.875	3.125	25
A10	0.75X	1.875	3.125	25
A11	1X	2.5	2.5	25
A12	1X	2.5	2.5	25
1-control	0	0	5	25
2-control	0	0	5	25

Bottles were crimp capped, taken out of the anaerobic chamber, and placed in a 30° C room. The methane content in the serum flask was measured at 1 hour and every 3 hours after that using the gas chromatograph by injecting 100 µL of head space. Duplicate GC measurements were taken for each sample. Bottles must be stored at 30 °C. Methane measurements were taken for 4 days.

CHAPTER IV

FINDINGS

4.1 Dissolved Oxygen (DO), pH, and Chemical Oxygen Demand (COD) of microcosms

The dissolved oxygen was measured throughout the duration of the experiment. Over time, it can be observed in Figure 4-1, that the measured dissolved oxygen levels increased. This can be accounted to the fact that the bottles were open to the atmosphere and oxygen consumption due to degradation did not exceed reaeration, with the exception of the first 20 days in the control, and Fluids E, F and D amended microcosms when oxygen was decreased and between Days 20 and 30 in the Fluid A microcosm.

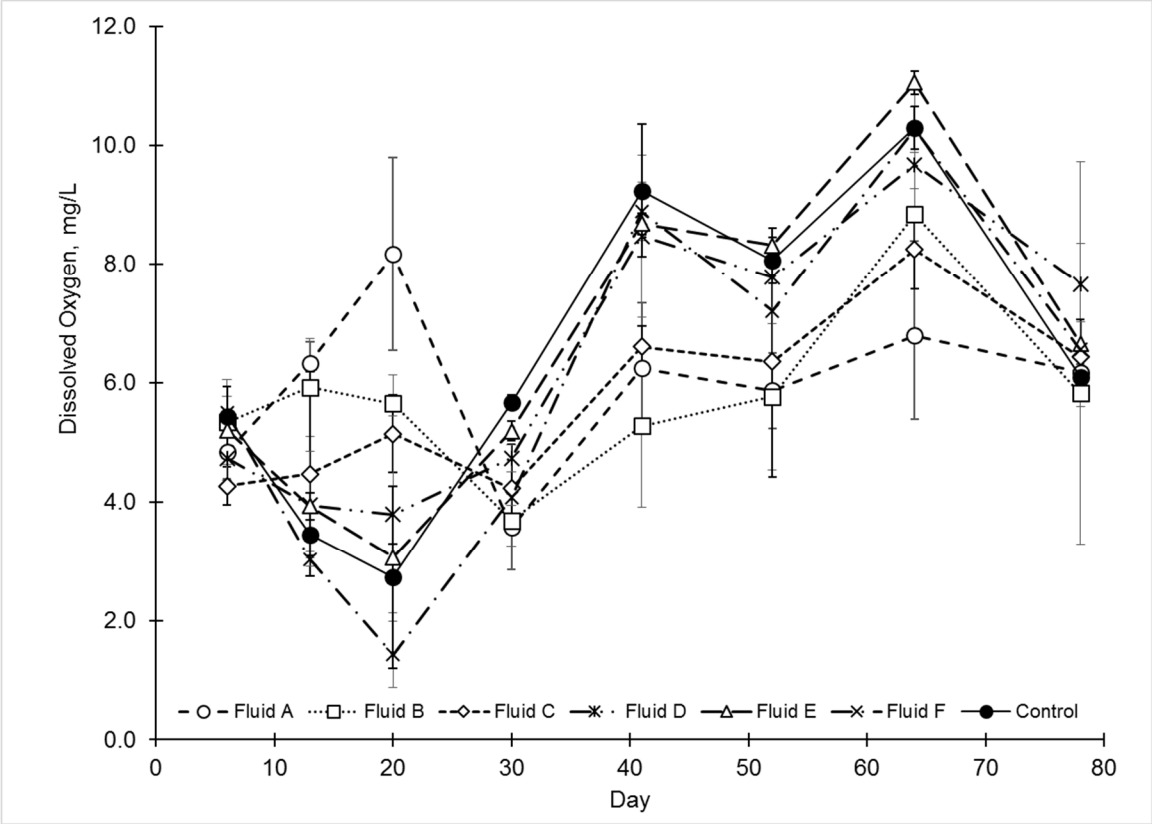

Figure 4.1 Dissolved Oxygen Measurements

On Day 0 of the experiment, the pH was adjusted to a neutral 7.2. For the duration of the experiment, the pH ranges from 5.73-8.45, which is considerably neutral. As illustrated in Table 4.1, on Days 41 and 52 the pH significantly increased in all reactors to a range of 6 -10 mg/L, but on Day 64 and 78, it starts stabilizing close to a neutral pH.

Table 4.1 Measured pH of microcosms containing fracing fluids and control

	pH								
Reactor	Day 0	Day 6	Day 13	Day 20	Day 30	Day 41	Day 52	Day 64	Day 78
A1	7.2	7.32	7.73	8.09	8.02	8.15	8.18	7.7	7.67
A2	7.2	7.41	7.87	8.14	8.08	8.15	8.18	7.69	7.67
A3	7.2	7.28	7.7	8.04	8.1	8.24	8.28	7.69	7.74
B1	7.2	7.28	7.83	7.92	8.03	8.28	8.45	7.99	7.97
B2	7.2	5.73	6.14	7.38	7.88	8.18	8.44	8.13	8.24
B3	7.2	7.18	6.18	7.3	7.81	8.19	8.44	8.07	8.19
C1	7.2	7.3	7.74	7.92	7.96	7.91	7.73	7.19	7.35
C2	7.2	7.32	7.74	7.88	7.95	7.99	7.9	7.3	7.46
C3	7.2	7.26	7.77	7.84	7.96	8.04	8	7.38	7.39
D1	7.2	7.41	7.74	7.88	7.56	7.82	7.96	7.49	7.48
D2	7.2	7.35	7.78	7.85	7.91	7.93	7.97	7.54	7.55
D3	7.2	7.55	7.95	7.92	7.91	8.02	8.08	7.48	7.56
E1	7.2	7.47	7.85	7.82	7.86	7.88	7.94	7.43	7.5
E2	7.2	7.51	7.9	7.84	7.9	8.02	8.04	7.5	7.78
E3	7.2	7.48	7.89	7.77	8.05	7.97	7.99	7.51	7.54
F1	7.2	7.62	7.98	7.97	7.98	8.07	8.01	7.37	7.6
F2	7.2	7.61	7.96	7.9	8.03	8.04	7.98	7.5	7.67
F3	7.2	7.6	7.92	7.88	7.9	7.93	7.93	7.51	7.64
G1	7.2	7.55	7.94	7.83	7.87	8.04	8.03	7.52	7.69
G2	7.2	7.51	7.89	7.72	7.82	7.91	7.99	7.51	7.49
G3	7.2	7.55	7.89	7.7	7.93	8.14	8.06	7.56	7.61

By conducting COD analysis, the concentration of chemicals in the fracing fluids over time was calculated. This determination was made by relating the concentration of COD to absorbance read on the spectrophotometer. All fracing fluid concentrations are shown in Figure 4.2. It is evident that fracing fluid B has a significantly higher concentration of COD, with concentration ranging from 4,500 to 20,100 mg/L. This range of COD is an abnormality and cannot be accounted to the fact that fracing fluid B has relatively more ingredients than the rest of the fracing fluids. In order to better differentiate the concentration of fracing fluid A, C, D, E, F,

and the control, fracing fluid B was removed from the plot in Figure 4.3. It can be seen that the concentration of COD in these fluids ranges from 700 to 4000 mg/L.

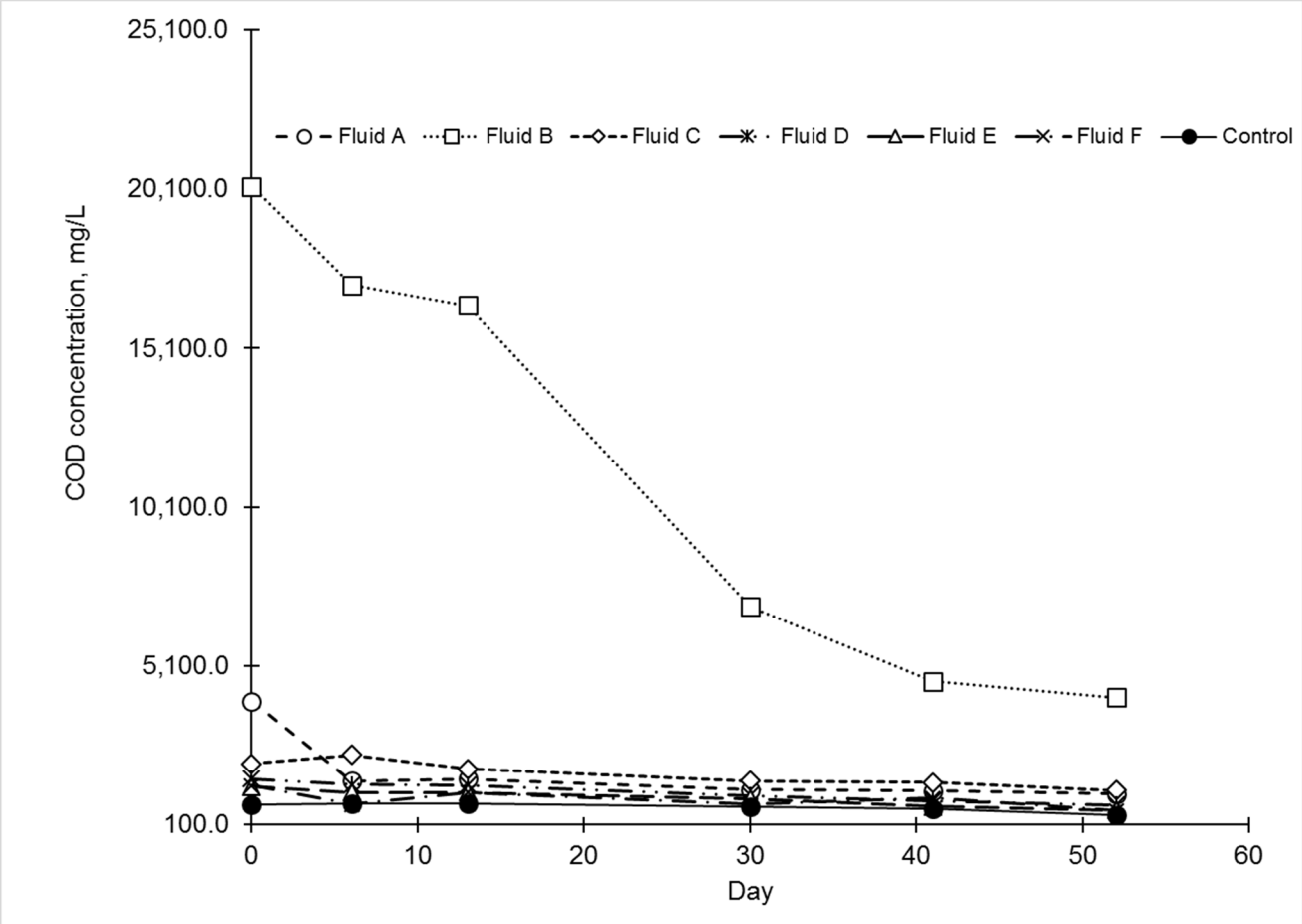

Figure 4.2 Chemical oxygen demand versus time, all microcosms containing all fracing fluids and control

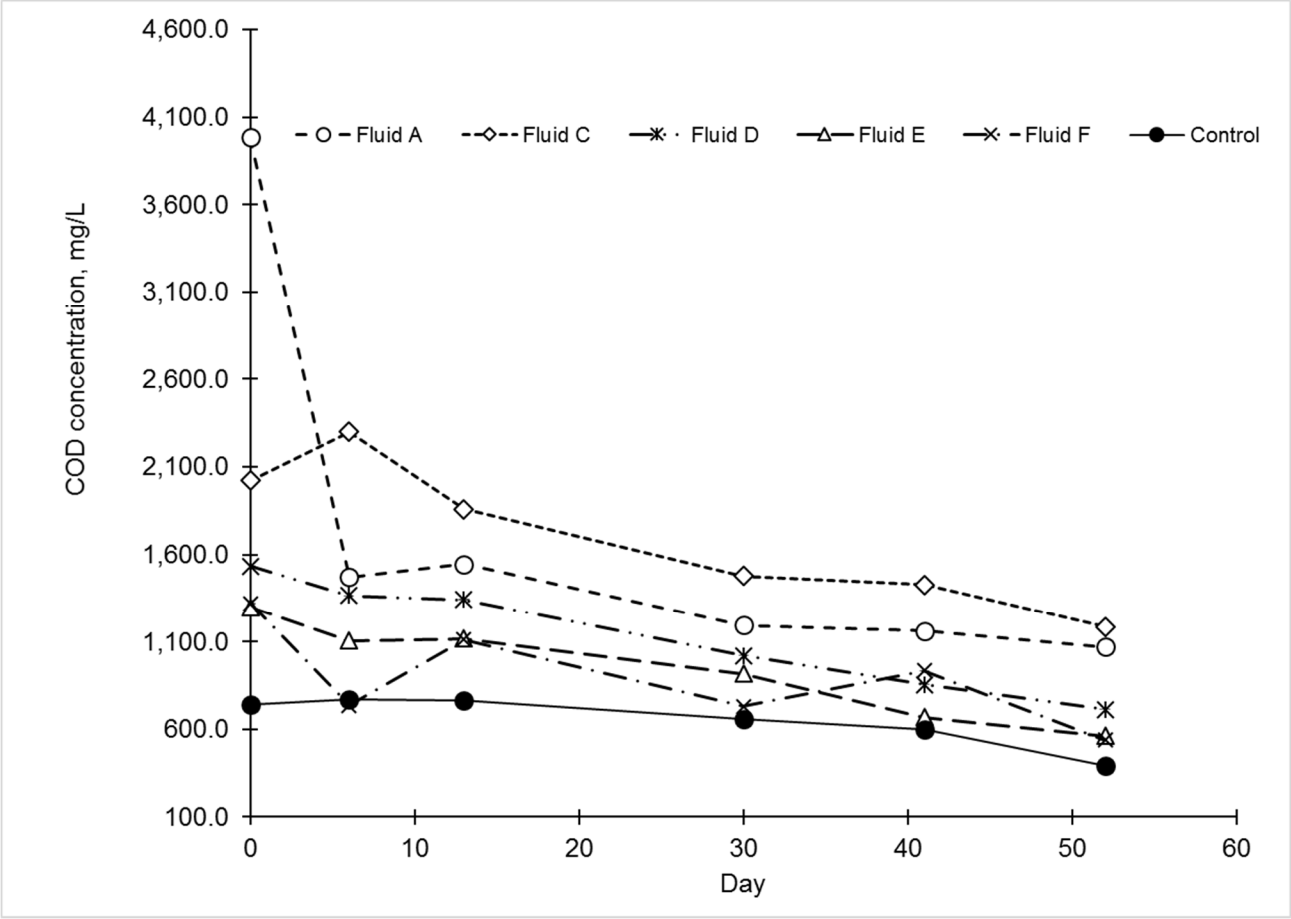

Figure 4.3 Chemical oxygen demand versus time, microcosms containing fracing fluids A,C,D,E,F, and control. Microcosm with fracing fluid B was omitted in order to illustrate lower COD concentrations

Overall, from Day 0 to Day 52, it was observed that the concentrations of all fracing fluids decreased. This was expected due to the fracing fluids decreasing in organic matter over time. The control does not contain organic carbon from the fracing fluid, its only source of organic matter comes from the soil. The control illustrates what is expected, which is a lower COD concentration since Day 0.

4.2 qPCR of microcosms

Quantitative real time PCR was conducted to observe changes in soil microbial communities. Generally the number of communities compared to the control was less and/or decreasing over time, except for microcosms containing fracing fluid A. No logical reason was found to account this result. It is expected that the number of microorganisms in each microcosm will decrease immediately or over time due to the addition of the suite of chemicals of fracing fluids. Although there is a decrease in microbial communities compared to the microbial quantity in the control, for most of the microcosms, there is no radical change in population quantity. It can be said that the soil microbial population remained somewhat constant for the duration of the experiment.

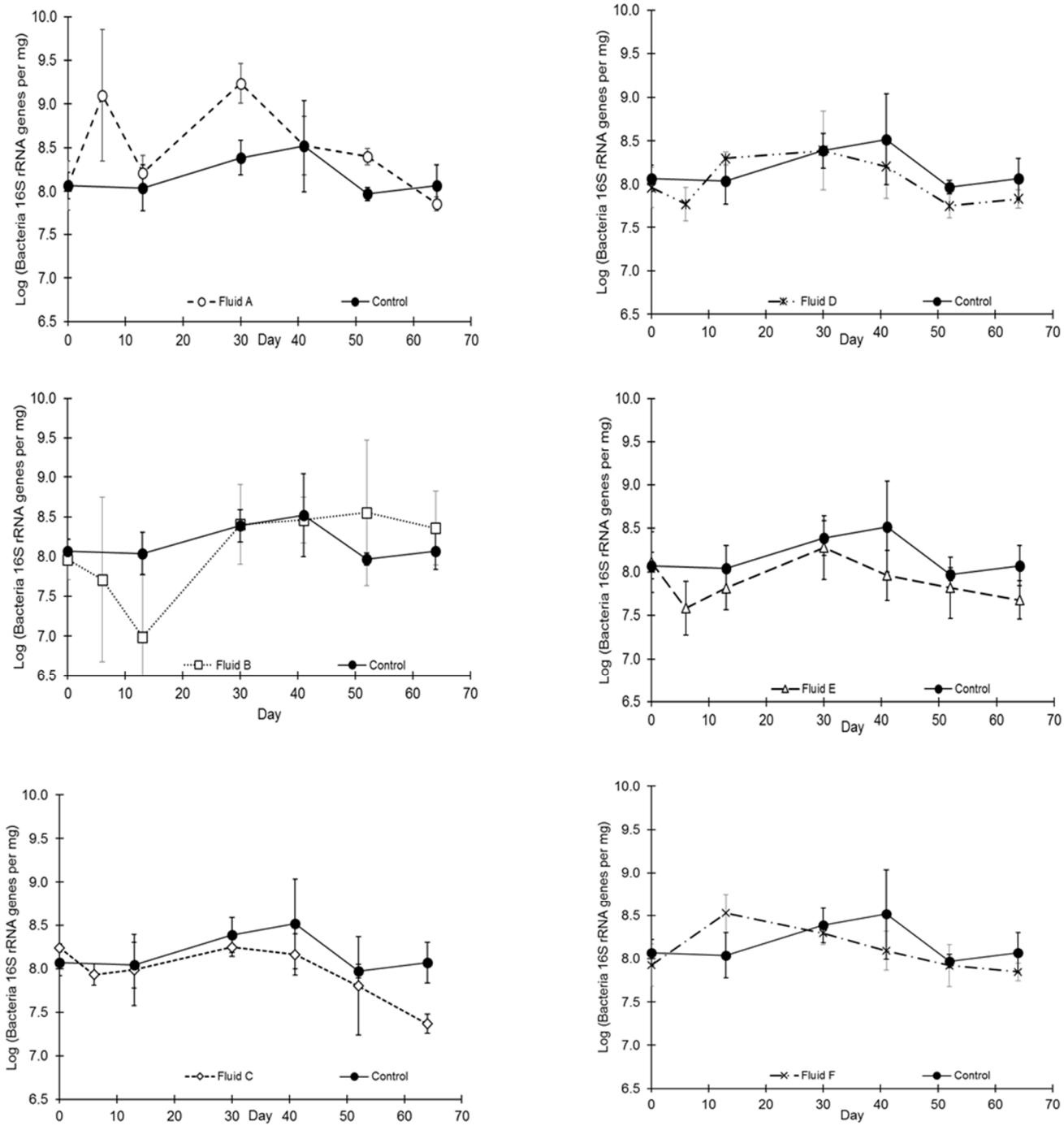

Figure 4.4 qPCR of microcosms. Log of bacteria 16s rRNA genes per mg versus time compared to the bacteria 16s rRNA genes per mg versus time of the control microcosms

4.3 ARISA

Although the microbial community quantity did not show significant or radical changes in quantity, with the ARISA analysis conducted, it was possible to detect a set of unique microorganisms present in various microcosms.

On Day 13 and Day 30, there is a significant difference between the bacteria that is present in microcosms with fracing fluid A (Figure 4.5).

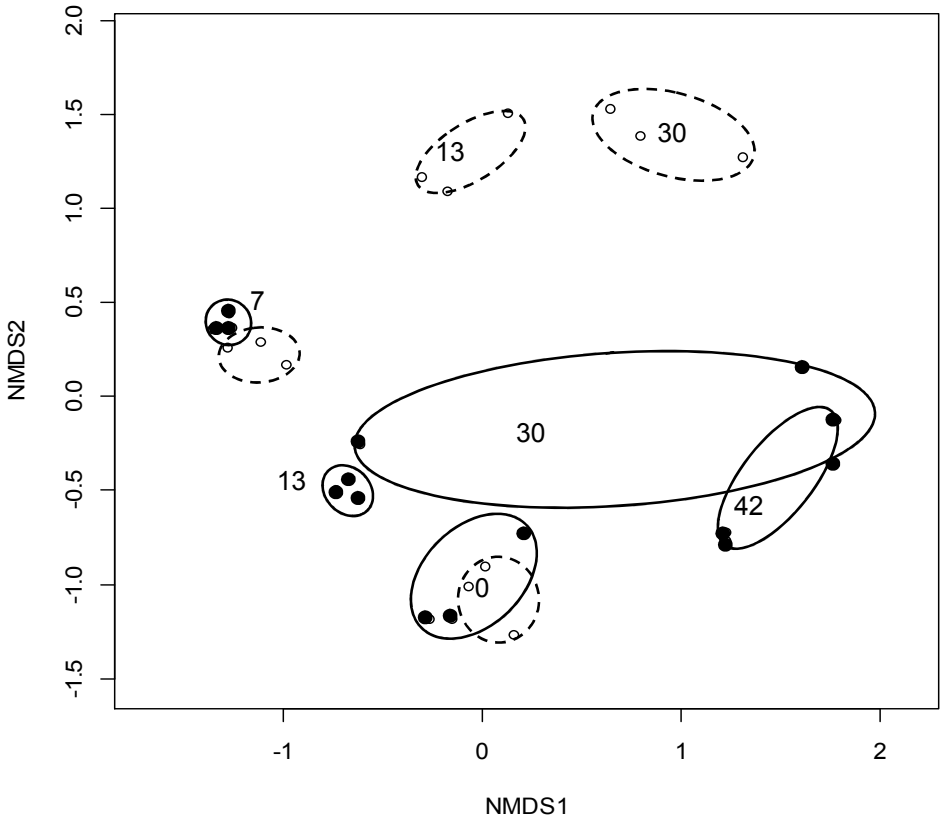

Figure 4.5 The NMDS of the community is shown between fracing fluid A microcosms (solid lines, solid circles) and the control microcosms (dotted lines, open symbols). The triplicate microcosms were grouped by an ellipse for clarity and the sampling day is shown in the numbers.

Again, for Days 13 and 30, there seems to be a change in microorganisms present in microcosms with fracing fluid B. The rest of the sampling days, the microorganisms present are closely related to those of the control. See Figure 4.6.

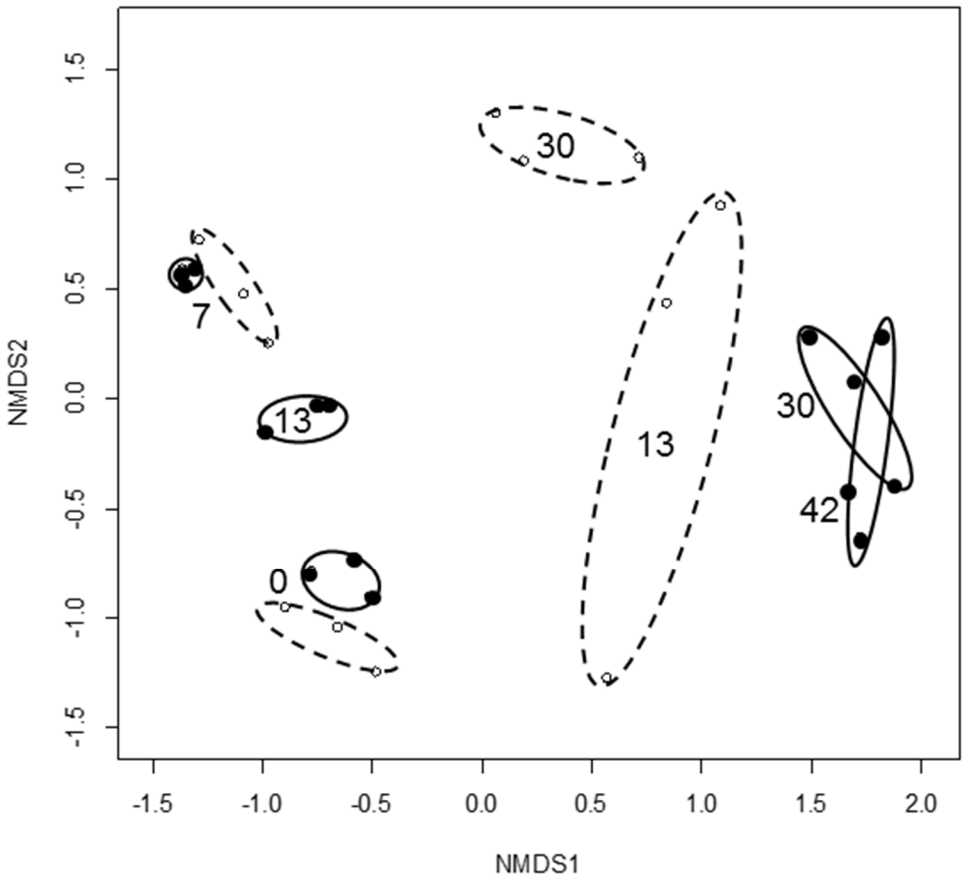

Figure 4.6 The NMDS of the community is shown between fracing fluid B microcosms (solid lines, solid circles) and the control microcosms (dotted lines, open symbols). The triplicate microcosms were grouped by an ellipse for clarity and the sampling day is shown in the numbers.

Microcosms with fracing fluid C is illustrating the same trend as with the microcosms with fracing fluids A and B. On Days 13 and 30, there is a significant difference in relationship between microbes in microcosms with fracing fluid C and control microcosms. The remaining sample days show a close relationship between microorganisms present in the two microcosms (fracing fluid C and control). Refer to Figure 4.7.

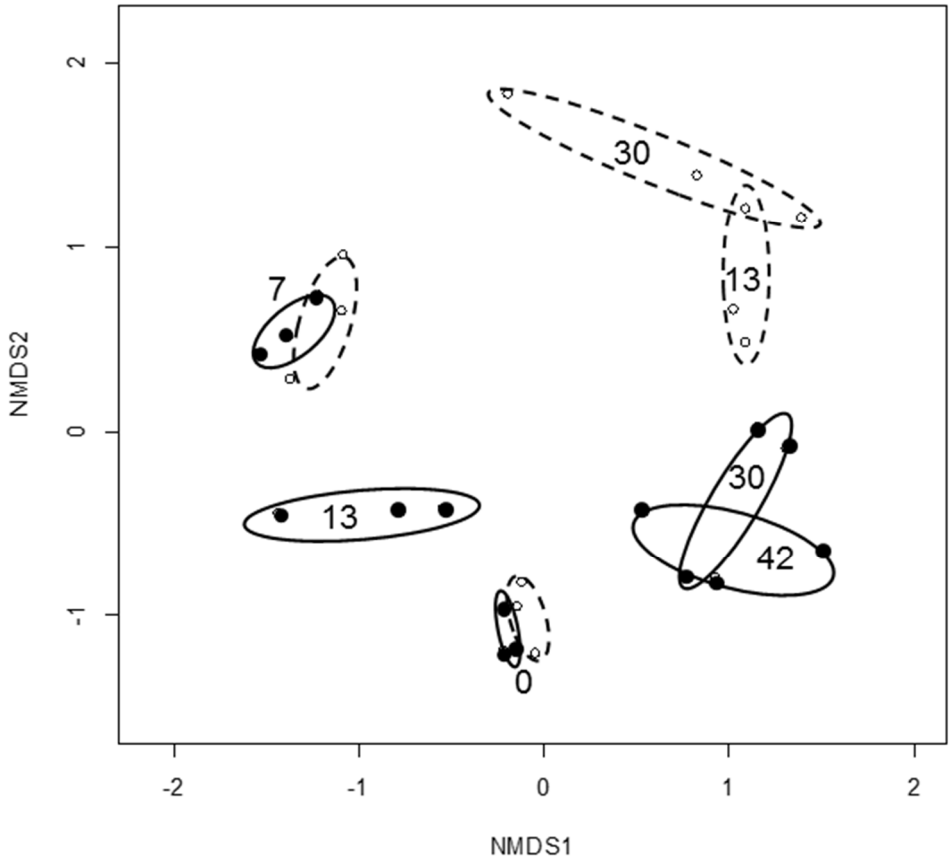

Figure 4.7 The NMDS of the community is shown between fracing fluid C microcosms (solid lines, solid circles) and the control microcosms (dotted lines, open symbols). The triplicate microcosms were grouped by an ellipse for clarity and the sampling day is shown in the numbers.

The comparison of microorganisms in the microcosm containing fracing fluid D and the control is shown in Figure 4.8. There is a significant difference on Day 30. Day 13 also displays a difference but it is not as significant as Day 30. Overall, the microorganisms present remain somewhat constant.

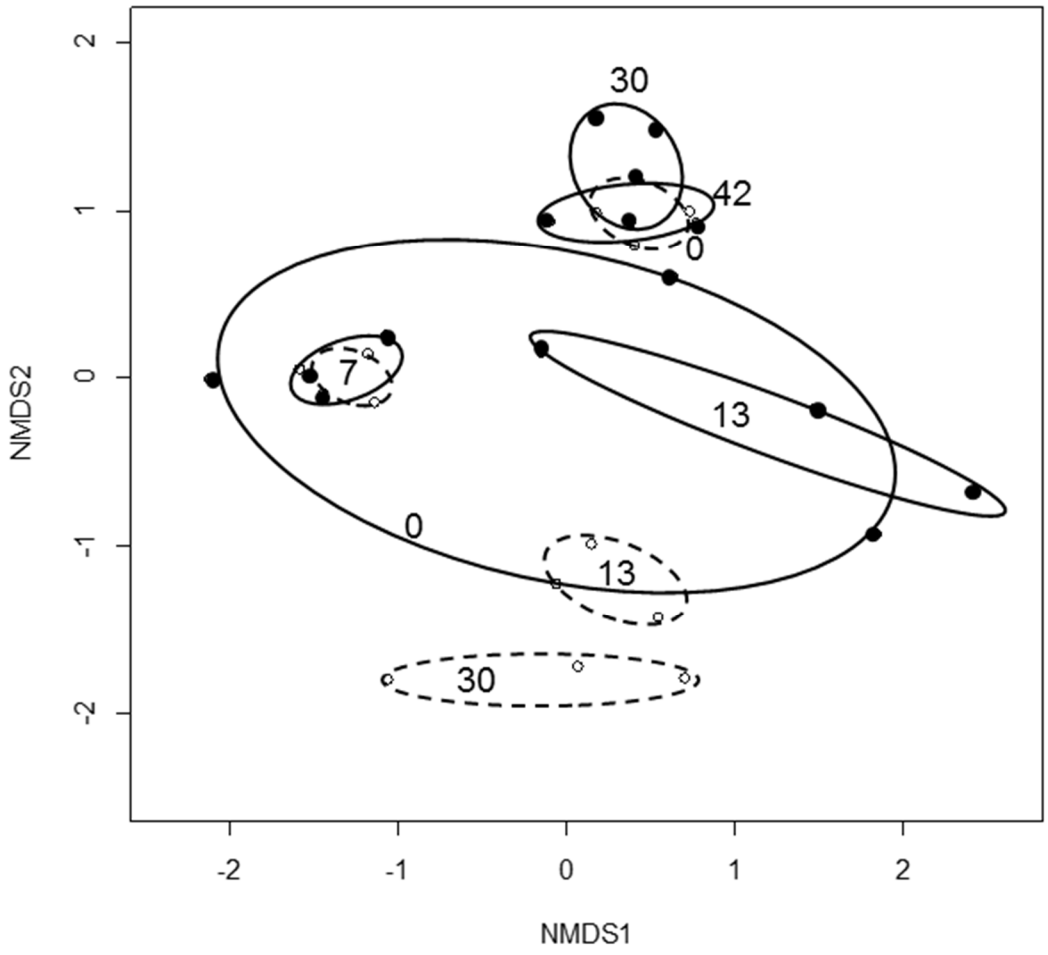

Figure 4.8 The NMDS of the community is shown between fracing fluid D microcosms (solid lines, solid circles) and the control microcosms (dotted lines, open symbols). The triplicate microcosms were grouped by an ellipse for clarity and the sampling day is shown in the numbers.

Figure 4.9 illustrates the same trend that has been portrayed for fracing fluids A,B,C, and D. Day 13 and Day 30 appear to have a difference, not as significant as the previous fracing fluids, in the microorganisms present. The rest of the sampling days, show a close relationship between fracing fluid E and control microcosm microorganisms present.

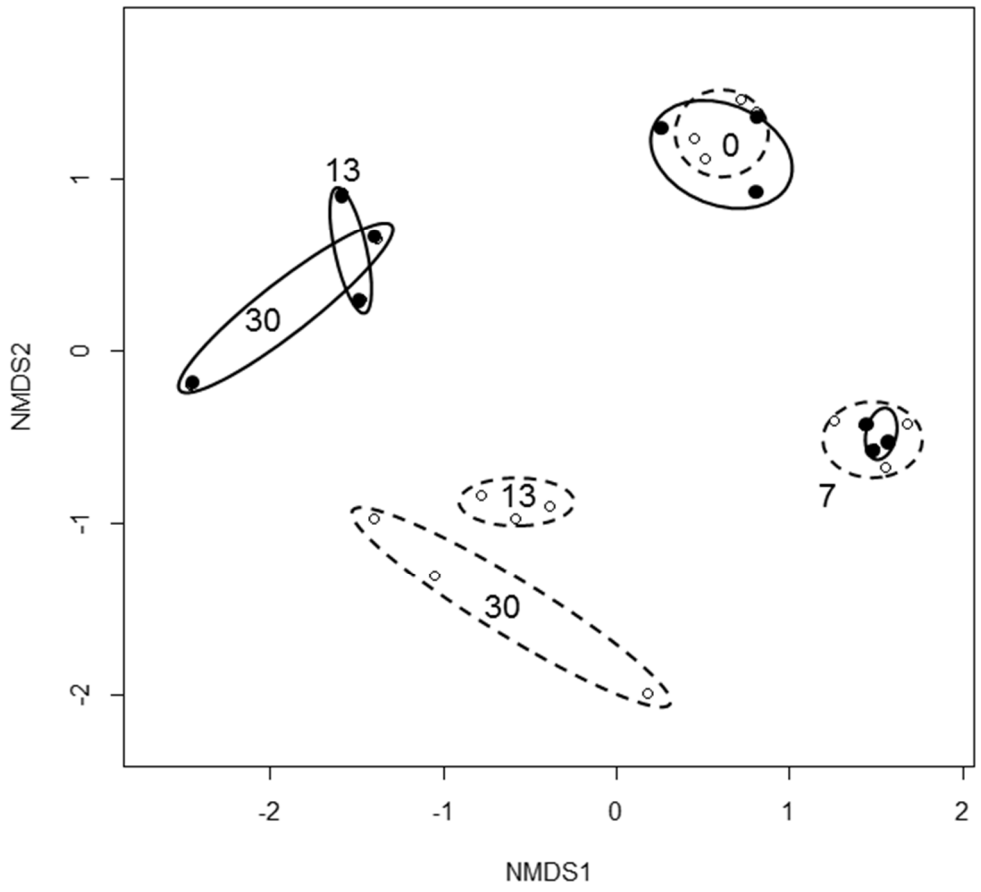

Figure 4.9 The NMDS of the community is shown between fracing fluid E microcosms (solid lines, solid circles) and the control microcosms (dotted lines, open symbols). The triplicate microcosms were grouped by an ellipse for clarity and the sampling day is shown in the numbers.

Although, there are two sampling dates with a significant difference in microbial relationship, sampling Day 13 of microcosm with fracing fluid F displays a larger difference in soil microbial communities than Day 30, as shown in Figure 4.10. The rest of the sampling days show a close relationship between soil microorganism in microcosms containing fracing fluid F and control microcosm

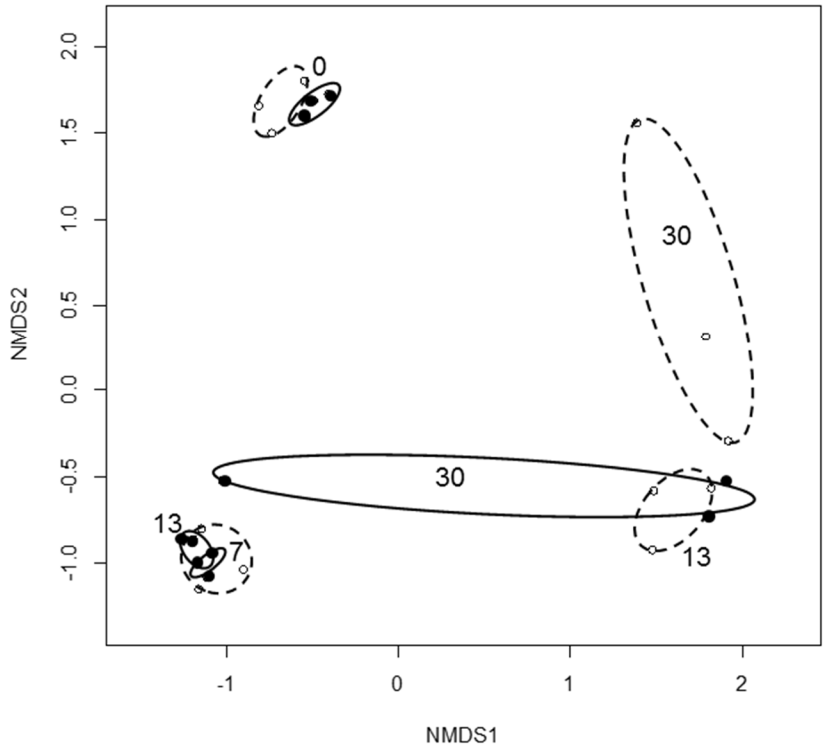

Figure 4.10 The NMDS of the community is shown between fracing fluid F microcosms (solid lines, solid circles) and the control microcosms (dotted lines, open symbols). The triplicate microcosms were grouped by an ellipse for clarity and the sampling day is shown in the numbers.

4.4 Methanogenesis Toxicity Test

By conducting the methanogenesis test, it was possible to observe if there was inhibition of the methane production by the fracing fluids. It was probable that due to the quality of the anaerobic sludge, production of methane was not very high when conducting the methanogenesis toxicity test. However, it was still possible to illustrate that the suite of chemicals used in fracing fluids was toxic to soil microbes. Production of methane by methanogenic bacteria in a microcosm containing a fracing fluid was compared to a microcosm without the fracing fluid. Essentially, it can be observed that fracing fluid E is nontoxic, thus the high production of methane, which is close to the level of production to the control. On the other hand, fracing fluid C was toxic at all levels, there was no methane production. The remaining fracing fluids (A,B,D,F) were more or less similar with approximately 40-70% of methane compared to the control at dilution 0.05X and fully inhibitory of methanogenesis at concentration of 1X.

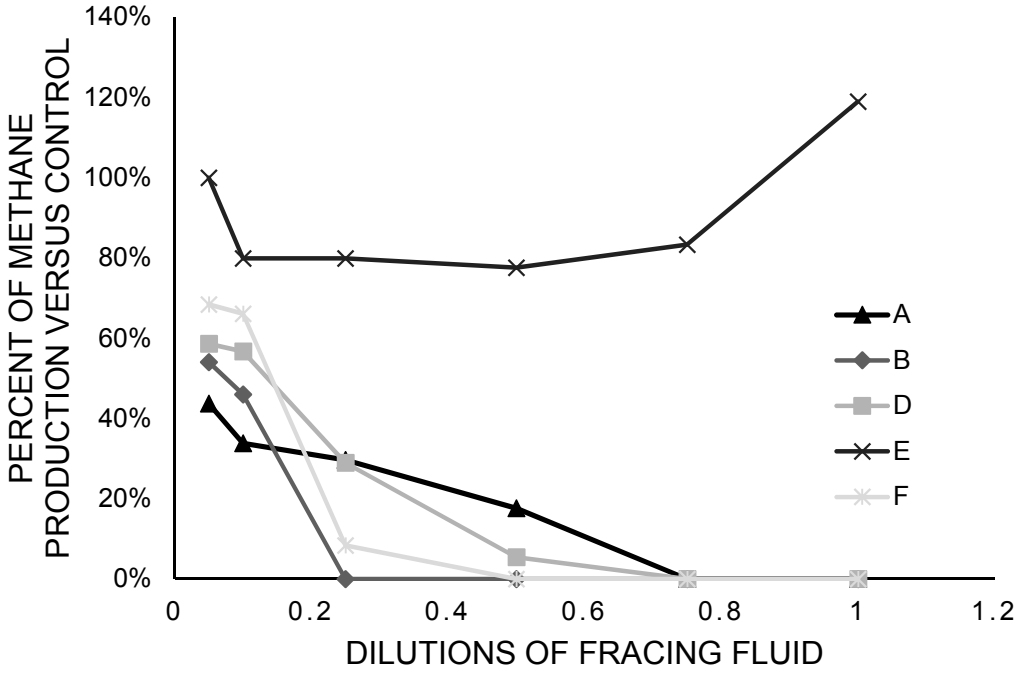

Figure 4.11 Percent of methane production versus control methane production against concentration of fracing fluid. Fracing fluid C was omitted from plot due to 0% methane production.

CHAPTER V

CONCLUSION

The series of experiments conducted is indicative of the potential effects on soil microorganism communities of the fracing fluids used for drilling oil and gas from shale. It is essential to understand the impacts that these fracing fluids have on the environment.

Although fracing fluids are being used at a low concentrations, by conducting the methanogenesis toxicity test, it was determined that there is in fact toxicity involved with these fluids employed. Fracing fluid E was the less toxic of all, on the other hand fluid C was the most toxic, thus inhibiting production of methane. The rest of the fluids (A,B,D, and F) showed toxicity as well, but they are not as toxic as fluid C.

Quantitative real-time analysis showed a small decrease in microbial population when compared to the population of the control microcosms. This is not a very substantial decline in population. This was expected due to the toxicity of the fracing fluids.

Although the quantitative real-time PCR exhibited no change in quantifiable soil microbial population, ARISA results revealed a unique type of soil microorganisms existing at sampling Day 13 and Day 30 for most of the microcosms containing fracing fluids. This can either indicate that a new microorganism is growing or that an existing microorganism that was dormant is showing more activity and is degrading the organic carbon in these fracing fluids. Future studies are essential for the identification of these existent microorganisms and analysis to determine what chemicals are being degraded.

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APPENDICES

Appendix A: pH measurements

Table A1. pH measurements

	pH								
Reactor	Day 0	Day 6	Day 13	Day 20	Day 30	Day 41	Day 52	Day 64	Day 78
A1	7.2	7.32	7.73	8.09	8.02	8.15	8.18	7.7	7.67
A2	7.2	7.41	7.87	8.14	8.08	8.15	8.18	7.69	7.67
A3	7.2	7.28	7.7	8.04	8.1	8.24	8.28	7.69	7.74
B1	7.2	7.28	7.83	7.92	8.03	8.28	8.45	7.99	7.97
B2	7.2	5.73	6.14	7.38	7.88	8.18	8.44	8.13	8.24
B3	7.2	7.18	6.18	7.3	7.81	8.19	8.44	8.07	8.19
C1	7.2	7.3	7.74	7.92	7.96	7.91	7.73	7.19	7.35
C2	7.2	7.32	7.74	7.88	7.95	7.99	7.9	7.3	7.46
C3	7.2	7.26	7.77	7.84	7.96	8.04	8	7.38	7.39
D1	7.2	7.41	7.74	7.88	7.56	7.82	7.96	7.49	7.48
D2	7.2	7.35	7.78	7.85	7.91	7.93	7.97	7.54	7.55
D3	7.2	7.55	7.95	7.92	7.91	8.02	8.08	7.48	7.56
E1	7.2	7.47	7.85	7.82	7.86	7.88	7.94	7.43	7.5
E2	7.2	7.51	7.9	7.84	7.9	8.02	8.04	7.5	7.78
E3	7.2	7.48	7.89	7.77	8.05	7.97	7.99	7.51	7.54
F1	7.2	7.62	7.98	7.97	7.98	8.07	8.01	7.37	7.6
F2	7.2	7.61	7.96	7.9	8.03	8.04	7.98	7.5	7.67
F3	7.2	7.6	7.92	7.88	7.9	7.93	7.93	7.51	7.64
G1	7.2	7.55	7.94	7.83	7.87	8.04	8.03	7.52	7.69
G2	7.2	7.51	7.89	7.72	7.82	7.91	7.99	7.51	7.49
G3	7.2	7.55	7.89	7.7	7.93	8.14	8.06	7.56	7.61

Appendix B: Dissolved Oxygen

Table B1. DO measurements

	DO							
Reactor	Day 6	Day 13	Day 20	Day 30	Day 41	Day 52	Day 64	Day 78
A1	4.8	6.66	10.04	2.8	5.38	5.18	6.34	6.03
A2	5.3	5.94	7.28	4.21	7.47	6.43	8.36	6.28
A3	4.4	6.38	7.17	3.7	5.88	5.98	5.67	6.21
B1	4.7	5.15	5.95	4.03	6.76	6.78	9.2	8.7
B2	5.2	5.83	5.89	3.83	4.06	4.4	8.96	3.97
B3	6.1	6.78	5.1	3.2	5	6.11	8.35	4.78
C1	4.4	4.98	5.85	4.66	6.4	4.26	7.5	6.41
C2	4.5	5.87	5	3.37	7.01	8.07	8.81	6.47
C3	3.9	2.54	4.57	4.65	6.4	6.72	8.41	6.41
D1	5	3.06	1.87	4.99	8.8	8.28	9.74	6.71
D2	4.3	3.92	4.78	4.59	6.96	7.23	8.34	6.23
D3	4.9	4.85	4.7	4.61	9.64	7.81	10.93	10.02
E1	4.9	3.68	2.8	5.21	8.74	8.42	11.25	6.21
E2	5.4	4.12	3.13	5.01	8.48	8.54	11.06	6.78
E3	5.3	3.98	3.25	5.34	8.82	7.97	10.86	6.98
F1	5.4	3.04	1.17	4.1	8.32	8.07	10.08	6.37
F2	5.3	3.15	2.06	3.92	9.18	6.36	10.01	6.21
F3	5.8	2.9	1.04	4.18	9.15	7.18	10.71	7.08
G1	5.9	3.87	2.37	5.69	10.01	8.42	10.43	5.65
G2	5.5	3.21	1.4	5.54	9.74	8.11	10.57	6.16
G3	4.9	3.28	4.4	5.78	7.96	7.65	9.89	6.44

Appendix C: COD analysis

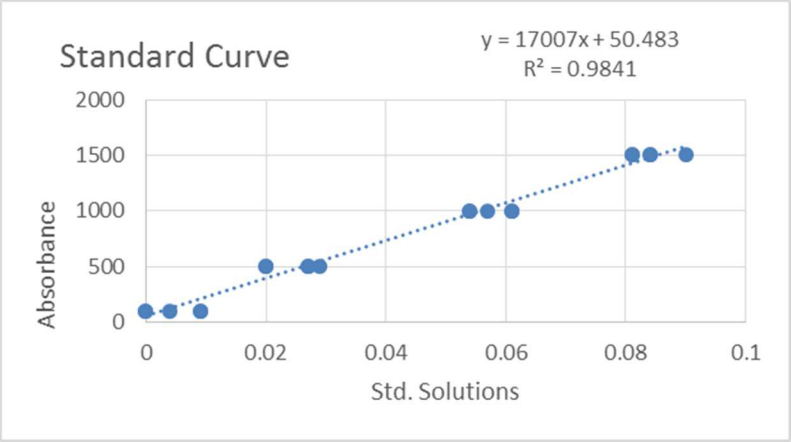

Figure C1. Absorbance Standard Curve for COD

Table C2. Absorbance measurements for COD

	Absorbence							
Reactor	Day 0	Day 6	Day 13	Day 30	Day 41	Day 52	Day 64	Day 78
A1	0.175	0.073	0.075	0.057	0.048	0.05	0.026	0.059
A2	0.205	0.053	0.074	0.06	0.063	0.046	0.063	0.056
A3	0.314	0.125	0.115	0.085	0.085	0.084	0.027	0.076
B1	1.13	1.034	0.97	0.335	0.253	0.239	0.253	0.207
B2	1.252	1.001	0.956	0.514	0.299	0.262	0.337	0.256
B3	1.169	0.971	0.967	0.368	0.251	0.213	0.301	0.22
C1	0.12	0.112	0.089	0.09	0.09	0.066	0.075	0.07
C2	0.118	0.126	0.094	0.078	0.071	0.068	0.118	0.072
C3	0.11	0.159	0.136	0.084	0.082	0.066	0.099	0.076
D1	0.077	0.068	0.065	0.055	0.045	0.036	0.108	0.048
D2	0.093	0.078	0.086	0.058	0.052	0.042	0.113	0.044
D3	0.092	0.086	0.077	0.058	0.045	0.039	0.226	0.059
E1	0.062	0.054	0.061	0.061	0.047	0.037	0.109	0.038
E2	0.074	0.048	0.073	0.054	0.036	0.03	0.18	0.047
E3	0.084	0.084	0.054	0.038	0.026	0.023	0.136	0.035
F1	0.078	0.043	0.087	0.04	0.044	0.031	0.088	0.043
F2	0.073	0.042	0.056	0.037	0.038	0.028	0.103	0.192
F3	0.073	0.036	0.044	0.043	0.074	0.027	0.086	0.038
G1	0.037	0.055	0.043	0.035	0.032	0.02	0.096	0.068
G2	0.036	0.035	0.037	0.037	0.038	0.017	0.107	0.027
G3	0.049	0.037	0.046	0.035	0.027	0.023	0.107	0.029

Appendix D: qPCR analysis

Table D1. Raw qPCR data

Day 0			per mg
7.444297516	0.248686	0.465	7.776845
7.609232724	0.272494	0.186	8.33972
7.690659387	0.083653	0.41	8.077876
7.761200443	0.065371	0.411	8.147359
7.604048273	0.347096	0.348	8.062469
7.147071418	0.106241	0.295	7.677249
7.809343438	0.043158	0.418	8.188167
7.704684038	0.270733	0.274	8.266933
7.511145174	0.100113	0.179	8.258292
7.432840087	0.296219	0.162	8.223325
7.448263404	0.202009	0.462	7.783621
7.565289802	0.061741	0.481	7.883145
7.902063636	0.066332	0.251	8.50239
7.553715531	0.338738	0.375	7.979684
7.631045719	0.361961	0.602	7.851449
7.714326869	0.21252	0.836	7.792121
7.473154687	0.309739	0.494	7.779428
7.764659739	0.140047	0.363	8.204753
7.549971067	0.102873	0.424	7.922605
7.841879289	0.148638	0.6	8.063728
7.7853215	0.109943	0.364	8.22422

Day 6			per mg
8.36537	0.017996	0.072	9.508038
8.027285	0.116512	0.626	8.230711
8.435941	0.085309	0.076	9.555128
8.46325	0.18018	0.378	8.885758
6.54721	0.119741	0.179	7.294357
6.147671	0.188125	0.157	6.951771
7.798238	0.122854	0.867	7.860219
7.511641	0.232718	0.274	8.07389
7.684757	0.080702	0.669	7.859331
7.724394	0.006717	0.753	7.847599
7.365677	0.038261	0.647	7.554773
7.951826	0.18687	1.067	7.923661
7.274203	0.193597	0.425	7.645814
7.871752	0.011094	1.036	7.856392
7.30409	0.825738	1.143	7.246044
6.057887	#DIV/0!	0.791	6.159711
#DIV/0!	#DIV/0!	0.962	#DIV/0!
#DIV/0!	#DIV/0!	1.151	#DIV/0!
#DIV/0!	#DIV/0!	0.691	#DIV/0!
#DIV/0!	#DIV/0!	0.879	#DIV/0!
7.577425	0.516341	1.192	7.501149

Day 13			per mg
7.348914	0.210413	0.189	8.073453
7.933543	0.3481	0.309	8.443584
7.542891	0.057957	0.263	8.122936
5.799554	0.198388	0.158	6.600897
5.870505	0.145886	0.244	6.483115
7.382406	0.041412	0.316	7.882719
7.161391	0.262486	0.121	8.078606
7.229186	0.088268	0.078	8.337092
7.148764	0.080755	0.408	7.538104
4.28443	0.134852	0.116	
7.511484	0.106191	0.144	8.353122
7.231173	0.164349	0.096	8.248902
6.850436	0.145132	0.104	7.833403
7.268671	0.241453	0.165	8.051187
7.050748	0.17022	0.312	7.556593
7.41356	0.236382	0.11	8.372167
4.074146	0.045868	0.217	
7.730122	0.073934	0.111	8.684799
7.178515	0.204487	0.176	7.933002
7.373974	0.047441	0.108	8.34055
7.154295	0.132199	0.208	7.846799

Day 30			per mg
8.181668	0.124024	0.061	9.396338
8.23417	0.084516	0.182	8.974098
8.442974	0.183989	0.127	9.33917
8.169439	0.248721	0.194	8.881657
8.221656	0.190703	0.594	8.44787
7.375459	0.452793	0.307	7.886321
7.511677	0.234197	0.141	8.362457
7.358078	0.081088	0.141	8.208859
7.366041	0.09575	0.159	8.164644
7.228402	0.287169	0.223	7.880097
7.572725	0.176636	0.067	8.74665
7.712411	0.171148	0.15	8.53632
7.050922	0.509099	0.121	7.968137
7.602792	0.078949	0.083	8.683714
7.381057	0.215515	0.158	8.1824
7.27684	0.049094	0.134	8.149735
7.5222	0.212975	0.135	8.391866
7.577861	#DIV/0!	0.173	8.339814
7.654248	#DIV/0!	0.194	8.366447
7.450467	0.45758	0.179	8.197614
7.822915	0.078142	0.168	8.597606

Day 41			per mg
7.829721	0.223491	0.311	8.336961
7.75342	0.354168	0.269	8.323668
8.415078	0.167054	0.322	8.907222
8.334989	0.171461	0.434	8.697499
7.9858	0.259148	0.275	8.546467
7.32263	0.080961	0.153	8.137938
7.472182	0.094579	0.314	7.975253
7.36667	0.081504	0.193	8.081113
7.729939	0.239143	0.2	8.428909
7.8819	0.342351	0.218	8.543444
7.690466	0.049663	0.271	8.257496
7.349245	0.1241	0.337	7.821615
7.655643	0.171754	0.769	7.769717
7.639654	0.142197	0.664	7.817486
7.828876	0.055529	0.346	8.2898
7.693441	0.003227	0.224	8.343193
7.490928	0.228522	0.383	7.90773
7.47701	0.084496	0.283	8.025224
7.260022	0.060515	0.219	7.919578
7.931883	0.020167	0.115	8.871185
7.829233	0.22409	0.117	8.761047

Day 52			per mg
8.288163	0.077219	0.859	8.35417
7.592512	0.087052	0.18	8.337239
7.732511	0.754048	0.168	8.507202
8.259416	0.057732	0.13	9.145473
8.479935	0.263592	0.288	9.020542
7.108801	0.165197	0.414	7.491801
7.345747	0.230109	0.412	7.73085
7.142921	0.235293	0.735	7.276633
7.750029	0.126605	0.224	8.399781
7.365281	0.135598	0.446	7.715946
7.316833	0.013965	0.257	7.9069
7.308904	0.086418	0.468	7.638658
7.365749	0.228515	0.342	7.831723
7.641052	0.53631	0.302	8.161045
7.029466	0.122854	0.371	7.460092
7.233232	0.337215	0.112	8.184014
7.159579	0.182736	0.286	7.703213
7.029225	0.353317	0.141	7.880006
7.176792	0.138688	0.191	7.895759
7.168795	0.264806	0.132	8.048221
7.366234	0.148488	0.25	7.968294

Day 64			per mg
7.389584	0.43836	0.273	7.953421
7.334086	0.361496	0.327	7.819538
7.333742	0.632176	0.338	7.804826
7.966132	0.233496	0.175	8.723094
7.888681	0.351894	0.238	8.512104
7.197882	0.050077	0.228	7.839947
6.972473	0.152323	0.524	7.253142
7.065196	0.156228	0.394	7.469699
6.94581	0.185164	0.369	7.378783
7.103556	0.131063	0.233	7.7362
7.440095	0.332984	0.31	7.948733
7.335979	0.140939	0.328	7.820105
7.095036	0.198789	0.467	7.425719
7.140469	0.21905	0.212	7.814133
7.157864	0.307507	0.233	7.790508
4.012162	0.037145	0.492	
7.620376	0.328648	0.501	7.920539
7.472079	0.408593	0.498	7.77485
7.587789	0.396771	0.53	7.863513
7.583344	0.090863	0.183	8.320893
7.424143	0.225412	0.251	8.02447

Day 78			per mg
7.589884	0.324477	0.144	8.431522
7.675891	0.346417	0.478	7.996463
7.849337	0.404362	0.378	8.271845
7.863347	0.358124	0.346	8.324271
7.984426	0.199959	0.253	8.581306
7.519227	0.317265	0.441	7.874788
7.25688	0.022285	0.341	7.724126
7.225141	0.133136	0.35	7.681073
7.592027	0.165977	0.417	7.971891
7.443806	0.263087	0.37	7.875604
7.508398	0.15059	0.42	7.885149
7.234959	0.120855	0.338	7.706042

Appendix E: ARISA analysis

Table E1. Fragment sizes and their relative abundance (% of total) for the triplicate microcosms amended with fracing fluid A.

Fragment Size	Day 0			Day 7			Day 13			Day 30			Day 42		
231.4	1.1	1.2	1.4	4.9	1.0	0.7	5.6			0.9					
234.8	11.0	12.4	11.7	4.4	5.2	5.0				8.8		0.8	1.4	1.4	0.6
253.2	4.3	7.2	6.6		0.7					1.3					
284.3				10.0	10.1	12.0	4.8	5.0	6.9	9.4					
285.3				8.7	9.9	9.9	8.7	7.5	10.3	8.6					
288.3				6.2	6.0	6.7	2.4	3.0	3.5	5.2					
289.4		0.8		3.7	2.9	3.9	5.8	5.7	4.6	3.5			1.4	1.3	
291.8				0.9			16.5	17.8	5.5	2.2					
294.0	5.9	4.4	5.0		1.5	0.8				2.3					
296.0							3.1	5.0	2.9						
296.9			1.3		1.1	0.6	4.1	5.0	5.8	1.0	2.0	1.0	1.5	1.5	0.8
309.6				3.7	4.0	4.1	3.7	5.5	3.1	2.4					
326.3	7.0	5.4	3.7	1.2	2.1	1.3				2.2					
335.2	7.1	6.2	6.7	1.4	2.2	1.3				2.6					
578.8											9.4	1.5	2.7	2.9	2.1
579.7											9.2	7.5	5.7	6.2	14.3
593.9													8.2	8.2	
689.8													4.9	5.4	
710.9				1.2								24.5			12.0
781.7	1.1			3.2											33.5
782.6									0.8		38.3	52.9	45.8	45.2	29.0

Table E2. Fragment sizes and their relative abundance (% of total) for the triplicate microcosms amended with fracing fluid B. Shown are fragment sizes which exceed 5% of the total area in at least 1 sample.

Fragment Size	Day 0			Day 7			Day 13			Day 30			Day 42		
223.0		0.8		0.8			8.4								
234.8	13.6	13.1	16.6	9.8	11.6	10.2		0.6							
253.2	7.9	4.7	3.6	0.9	1.7	1.8									
284.3				10.2	8.3	8.4	13.0	6.7	3.6						
285.3				8.8	3.1	2.6	13.1	10.3	2.4						
286.3				2.3	3.1	3.5	1.8	6.1	4.3						
288.3				5.2	3.3	2.9	1.2	2.5	1.2						
289.4				2.8			6.2	4.7							
291.8					4.2	4.2		4.5	21.2						
294.0	4.4	5.4	5.1	2.2	2.2	2.4									
296.0							1.6	2.4	6.9						
296.9			1.0	0.8	1.9	1.8	3.3	10.0	6.5						
309.6		1.2		3.8	5.5	4.2	3.3	6.2	7.8						
326.3	5.6	5.9	6.8	2.7	4.1	3.5			0.6						
335.2	6.2	7.6	7.2	1.5	5.7	4.4									
363.6								0.6		1.2	1.9	8.3	1.1		
408.5							2.3								5.8
425.4										6.0	1.3	1.0	3.9	1.6	6.0
428.5				1.5						11.9	5.6	16.4	7.6	23.0	14.9
464.8										4.1			8.3		0.6
564.0	5.9														
591.0															7.6
601.3				2.3	7.7	7.8		1.2	1.2						
602.7											1.7	30.8		4.7	2.7
629.8							3.6	0.5			18.8	16.2	0.8	5.0	
686.9										8.2			1.3	3.7	0.7
719.3											33.3			2.0	
744.6				0.8					0.7	28.3	0.9	3.5	34.6	16.9	18.9
745.1				0.7						14.8	2.7	1.9	22.1	11.1	12.0
782.6				1.3						15.1	22.6	9.7	8.7	23.9	21.3

Table E3. Fragment sizes and their relative abundance (% of total) for the triplicate microcosms amended with fracing fluid C. Shown are fragment sizes which exceed 5% of the total area in at least 1 sample.

Fragment Size	Day 0			Day 7			Day 13			Day 30			Day 42		
223.0	0.9							5.8							
234.8	15.0	12.5	14.2	10.9	11.5	7.7				0.9	2.2	1.8		1.2	2.8
253.2	4.2	6.0	3.5	1.8						1.4	3.8	1.5	1.0	2.9	2.6
284.3				4.8	11.5	3.9	4.4	8.9	12.1						
285.3				2.9	7.5	1.4	2.9	5.9	12.2						
291.8				4.7		2.2	20.8	7.7	1.4			0.8			
296.0							7.6	4.5	1.4						
296.9	1.1	1.1	1.2	1.9		0.9	6.9	5.6	2.9			0.7			
307.0	2.1	1.4	2.7			1.8			0.8		3.4	6.6		2.9	10.8
309.6			1.4	4.9	4.3	3.9	7.6	4.7	3.6						
326.3	5.7	6.6	5.6	5.3	3.6	3.7				1.3	2.9	1.6	1.0	1.8	3.0
335.2	7.9	6.8	5.5	5.9	4.5	2.3				0.9	2.6	1.1	0.6	1.6	3.0
363.6								0.9	1.1	5.1			0.9	0.7	
368.8								0.7	1.1					1.1	9.6
421.0										1.3	6.4		0.9	2.7	
428.5										7.0		10.1	1.1		9.0
429.6										5.9	1.3	4.3	3.9	1.1	
432.1										38.4	3.0	5.2	30.2	1.2	5.2
465.8											1.1	6.6		1.0	4.3
529.3								6.9	7.4						
594.9				3.4	17.0	13.6	1.0	3.2	8.6						
601.3				6.2	3.7	2.7	1.5	1.3	1.8				0.8	28.5	
607.4				1.8									11.4		
610.6				1.4	12.7			2.9		2.1			0.9		
618.5										13.9	22.1	10.9	37.8	26.9	24.5
744.6												12.8			3.6
745.1							0.7					9.0			

Table E4. Fragment sizes and their relative abundance (% of total) for the triplicate microcosms amended with fracing fluid D. Shown are fragment sizes which exceed 5% of the total area in at least 1 sample.

Fragment Size	Day 0			Day 7			Day 13			Day 30			Day 42		
91.0		1.1							7.3						
223.0	7.0	0.7					1.4								
230.1	2.4	2.2	1.9						5.2	1.4					0.6
234.8	16.4	13.6	8.6	3.7				1.3	10.9	2.3	1.4	0.7	0.9	2.9	0.9
253.2	4.8	6.3	1.9						4.0	0.8	0.8				
270.8										3.7		1.2	11.3		3.6
282.1				1.1						7.8	2.2	2.9	22.0	3.7	4.6
284.3				9.4		1.3	9.8			1.0			0.7		
285.3				6.1		1.6	8.6			1.7	1.1	1.7			0.8
288.3	0.9			6.2	1.4	5.5	5.2								
289.4			2.7	2.9		0.8	5.0			6.9	4.3	3.7	17.3	21.1	7.7
291.8						1.2	4.0			5.2	22.6	29.4	2.2	7.4	15.3
294.0	5.6	5.1	1.8	1.1											
296.0							3.6			1.7	2.4	5.1	1.2	1.8	1.9
296.9			1.9	0.9		1.4	5.1			2.8	1.3	1.4	2.3		0.8
326.3	4.9	6.5	3.5	2.4					3.6	1.0			0.7		2.0
335.2	6.1	6.5	3.0	1.7					2.8	0.6					
349.3							0.9			1.9	4.5	5.3		1.4	3.9
360.7			1.0				0.7			1.0	5.0	7.4		0.8	4.2
362.7					6.4	4.4									
379.2			9.3							1.8		0.7		0.9	1.2
381.6			5.6				1.0			0.6					
382.6				1.0	4.4	6.1					1.2	1.8			1.3
469.9		0.7	1.8							7.0	5.9		1.6	4.0	
575.0								8.6							
578.8										10.5			7.3		
579.7								1.5		9.5			6.5		
594.9				7.3			4.8								
598.6					1.7	1.1						5.4		0.7	9.1
606.2									2.9	0.9			5.7		
617.5				0.7								0.9		3.3	6.0
647.0												1.6		1.3	5.3
660.7					5.5			3.5							
689.8					34.7			24.2							
707.4									6.7						
724.6											16.5			29.3	
725.6						17.2									
778.4									10.3						
781.7					13.6										
782.6					20.2	38.1		48.5							

Table E5. Fragment sizes and their relative abundance (% of total) for the triplicate microcosms amended with fracing fluid E. Shown are fragment sizes which exceed 5% of the total area in at least 1 sample.

Fragment Size	Day 0			Day 7			Day 13			Day 42	
91.0				1.9	1.5	15.6					
223.0				1.4	1.1	0.8				5.2	
230.1	0.8	1.4		6.1	4.1	2.1					
234.8	1.2	0.7	0.9	15.4	11.2	13.7					
253.2		1.4		6.8	7.3	4.9					
280.2				1.2	5.0	0.8					
286.3	2.3	3.7	5.5							0.8	
289.4	1.2	5.1	1.6			0.6				0.7	
291.8	15.0	2.1	32.7	0.9	1.1	0.7					
296.0	3.9	4.3	6.1								
298.5		9.0			0.8	1.6					
309.6	3.5	2.9	5.7		1.8						
335.2				2.3	6.0	3.3					
349.3	2.1	0.8	5.9								
360.7	2.2	1.4	6.1								
362.7			0.9				25.4	25.4	3.2	13.9	
425.4											19.6
459.2		11.2									
547.4							1.5	1.5	6.2	3.9	
559.6									0.7		45.9
571.9	4.9						5.0	5.0		1.7	
581.8	23.1										
582.4	19.1								1.3	0.9	
593.9			7.9								
605.2		5.9									
710.9							32.8	32.8	21.3	13.1	
719.3									5.6	3.0	1.1
782.6							22.6	22.6	34.1	43.0	22.7

Table E6. Fragment sizes and their relative abundance (% of total) for the triplicate microcosms amended with fracing fluid F. Shown are fragment sizes which exceed 5% of the total area in at least 1 sample

Fragment Size	Day 0			Day 7			Day 13		Day 30		
223.0				2.2	0.9	1.0	3.4	2.5	18.8		0.7
234.8	1.5	2.8	0.6	13.2	14.4	13.6	6.6	11.7	3.1		
253.2				2.6	7.9	6.4	12.8	11.0	1.5		
291.8	20.8	25.5	24.7						1.1		
294.0				3.6	4.6	4.6	6.6	5.1	4.5		
296.0	9.7	9.5	4.3					1.1	2.5		
296.9	3.4	5.5	2.2	1.2	0.8	1.8	0.8	1.7	2.0		
300.4				2.4	3.4	3.1	5.4	6.8	3.1		
309.6	5.6	7.5	6.1	1.2			3.8	1.8	2.6	1.2	
326.3		0.6		4.7	5.7	5.3	4.4	5.5	1.1		
360.7	4.8	3.8	5.1								
363.6				0.7						33.9	
428.5									1.0	9.4	1.2
432.1										20.4	
579.7	3.1	4.0	12.2								
597.0		1.7								8.4	
667.8											13.0
686.9										3.1	12.1

Table E7. Fragment sizes and their relative abundance (% of total) for the triplicate microcosms amended with mineral media only (control). Shown are fragment sizes which exceed 5% of the total area in at least 1 sample

Fragment Size	Day 0			Day 7			Day 13			Day 30		
223.0				0.7	1.1	1.2				0.6	16.7	40.2
234.8	1.1		1.6	12.6	13.5	9.2	0.6	1.1	1.9			
253.2	0.7		0.9	3.6	5.9	4.0		1.0	0.8			
283.4							9.5					0.7
285.3	3.3	2.5	5.7					0.8				
291.8	13.9	33.0	6.0									
294.0				4.0	5.6	1.0						
296.9	4.6	4.4	10.0	1.9	0.9							
298.5	3.5	1.1	9.7	0.7	1.6							
307.0				0.8	1.5	0.9	3.5	11.0	13.2	4.0	2.5	1.1
309.6	5.9	6.3	2.0				3.0	0.7	0.8			
310.7			0.9	0.8	1.0	1.0		6.6				
326.3	0.9		0.7	5.1	5.6	5.1	0.7	1.0	1.4			
335.2				4.6	5.9	2.9	0.9	1.0	1.8		0.8	
349.3	2.2	5.9										
360.7	2.6	5.9	1.2		1.8	1.2						
363.6							25.4		3.3			
398.0				1.6		7.0					5.0	11.8
402.8				2.3			3.6	3.3	10.7	3.0	1.5	
408.5											4.7	11.0
428.5				1.3			12.7	0.7	7.6	12.6	8.8	
429.6						0.9				11.1	7.0	
465.8							2.9	5.0	17.9	5.0	4.1	
547.4							0.6	1.6	6.0	2.8	1.6	
555.2								2.4	5.4	0.8		
579.7		2.1	10.3									
589.7							3.4	6.1	0.6			
597.0							5.5					2.1
598.6	5.4	2.3	4.3									
601.3	2.9						10.1	25.5	2.7	0.7	1.0	1.3
610.6											3.8	9.7
697.5										29.4	17.4	
709.7									1.4	5.5	3.5	

Appendix F: Gas chromatography

Table F1. Gas chromatography peak areas for methanogenesis toxicity test

Sample	3.22 hrs Area	average	7.02 hrs Area	average	10.08 hrs Area	average	20.7 hrs Area	average	22.95 hrs Area	average
A1	3.81	3.97	6.55	5.51	6.55	5.51	5.17	6.09	10.16	8.005
	4.13		4.47		4.47		7.01		5.85	
A2	3.68	4.24	4.15	4.94	3.51	3.83	6.78	14.025	5.99	6.365
	4.8		5.73		4.15		21.27		6.74	
A3	3.9	4.125	5.27	4.6	5.73	5.49	4.79	5.78	5.8	6.93
	4.35		3.93		5.25		6.77		8.06	
A4	3.79	3.64	5.25	4.615	3.61	3.7	6.93	6.755	6.56	5.885
	3.49		3.98		3.79		6.58		5.21	
A5	3.17	3.64	3.69	3.74	3.7	3.585	9.62	9.8	6.16	5.895
	4.11		3.79		3.47		9.98		5.63	
A6	3.7	3.925	3.7	3.685	9.04	8.965	8.07	6.965	4.88	5.21
	4.15		3.67		8.89		5.86		5.54	
A7	3.43	4.59	4.49	4.315	6.94	7.525	3.81	3.705	6.51	5.84
	5.75		4.14		8.11		3.6		5.17	
A8	3.57	3.38	6.92	6.41	11.11	9.76	6.49	5.87	4.38	4.45
	3.19		5.9		8.41		5.25		4.52	
A9	3.4	6.515	4.27	6.34	8.26	7.565	8.36	9.21	3.99	4.33
	9.63		8.41		6.87		10.06		4.67	
A10	6.3	6.475	5.04	4.36	7.13	9.93	2.19	2.71	7.84	6.815
	6.65		3.68		12.73		3.23		5.79	
A11	10.7	12.11	7.13	7.28	10.71	11.98	1.61	3.74	7.67	8.62
	13.52		7.43		13.25		5.87		9.57	
A12	10.36	12.92	5.44	9.345	19.45	14.885	5.68	6.465	4.8	6.505
	15.48		13.25		10.32		7.25		8.21	
B1	8.14	8.95	19.45	12.205	14.39	14	13.5	11.08	5.14	5.93
	9.76		4.96		13.61		8.66		6.72	
B2	5.24	12.32	14.39	9.05	14.84	17.01	16.07	13.86	5.17	4.775
	19.4		3.71		19.18		11.65		4.38	
B3	4.17	6.49	7.91	11.415	13.92	17.31	11.38	9.71	4.52	4.255
	8.81		14.92		20.7		8.04		3.99	
B4	8.55	10.96	19.18	16.55	21.43	16.345	11.59	8.07	4.67	6.255
	13.37		13.92		11.26		4.55		7.84	
B5	17.69	10.655	20.7	21.065	10.65	12.77	6.03	8.65	5.79	6.73
	3.62		21.43		14.89		11.27		7.67	
B6	10.08	7.5	11.26	10.955	12.22	7.66	4.3	5.46	8.71	9.685
	4.92		10.65		3.1		6.62		10.66	
B7	8.09	6.435	11.89	12.055	3.07	3.59	12.66	14.485	10.11	12.43
	4.78		12.22		4.11		16.31		14.75	
B8	3.99	7.895	1.54	2.825	3.96	4.99	18.63	17.675	11.76	10.285
	11.8		4.11		6.02		16.72		8.81	
B9	13.4	8.52	3.96	4.99	6.82	6.2	20.65	11.95	10.52	9.965
	3.64		6.02		5.58		3.25		9.41	
B10	7.17	5.22	6.82	6.355	7.15	8.04	13.5	14.49	8.01	12.515
	3.27		5.89		8.93		15.48		17.02	
B11	3.62	4.97	3.44	3.65	12.01	10.975	14.02	8.85	14.16	13.59
	6.32		3.86		9.94		3.68		13.02	
B12	10.67	8.9	8.93	7.705	14.02	13.82	8.13	9.74	14.56	10.84
	7.13		6.48		13.62		11.35		7.12	
C1	3.54	5.065	3.23	3.135	14.08	16.915	4.62	8.625	5.78	6.505
	6.59		3.04		19.75		12.63		7.23	
C2	2.34	3.04	4.74	5.72	29.1	25.09	8.95	12.075	8.12	8.16
	3.74		6.7		21.08		15.2		8.2	
C3	4.28	5.52	13.62	13.835	22.62	21.27	8.28	9.68	12.27	8.57
	6.76		14.05		19.92		11.08		4.87	
C4	3.12	4.095	19.75	24.425	20.04	18.4	11.28	12.865	5.01	4.6
	5.07		29.1		16.76		14.45		4.19	
C5	3.17	3.575	21	21.81	13.2	18.165	26.15	19.695	4.62	3.805
	3.98		22.62		23.13		13.24		2.99	
C6	22.55	17.76	19.92	19.98	25.19	19.27	6.9	6.585	9.52	8.035
	12.97		20.04		13.35		6.27		6.55	
C7	2.94	12.79	16.76	10.365	8.86	13.92	6.07	10.885	3.9	3.845
	22.64		3.97		18.98		15.7		3.79	
C8	3.87	10.07	13.85	10.36	10.63	7.33	14.63	15.115	9.1	10.075
	16.27		6.87		4.03		15.6		11.05	
C9	17.03	12.375	22.75	24.115	11.88	8.305	12.56	18.02	4.26	4.545
	7.72		25.48		4.73		23.48		4.83	
C10	4.96	13.05	13.35	11.105	4.52	9.48	30.23	21.525	5.02	5.945
	21.14		8.86		14.44		12.82		6.87	
C11	23.1	23.095	18.98	14.575	15.06	13.48	12.51	8.82	7.91	9.65
	23.09		10.17		11.9		5.13		11.39	
C12	4.38	13.3	11.85	8.185	5.35	5.245	3.06	4.245	12.36	11.355
	22.22		4.52		5.14		5.43		10.35	
D1	15.51	15.825	14.44	13.17	5.37	5.095	6.57	6.08	10.39	11.88
	16.14		11.9		4.82		5.59		13.37	
D2	5.03	14.62	5.14	5.255	11.99	8.83	10.07	11.13	13.66	13.485
	24.21		5.37		5.67		12.19		13.31	
D3	22.9	27.275	4.82	8.405	5.2	5.205	11.98	13.035	14.9	10.925
	31.65		11.99		5.21		14.09		6.95	
D4	24.07	14.345	5.67	5.435	8.08	10.48	13.39	13.485	7.01	4.165
	4.62		5.2		12.88		13.58		1.32	
D5	13.93	11.94	5.21	6.63	7.12	5.625	13.9	10.095	7.76	8.855
	9.95		8.05		4.13		6.29		9.95	
D6	12.99	8.77	12.88	10	7.24	7.745	11.85	14.925	8.51	5.365
	4.55		7.12		8.25		18		2.22	
D7	5.56	8.005	4.13	5.685	14.69	12.985	12	9.34	4.91	4.65
	10.45		7.24		11.28		6.68		4.39	
D8	5.72	5.85	8.25	11.47	11.41	11.955	5.45	7.915	10.26	11.26
	5.98		14.69		12.5		10.38		12.26	
D9	4.98	4.66	11.28	11.345	6.17	5.595	13.43	10.085	4.01	4.265
	4.34		11.41		5.02		6.74		4.52	
D10	10.95	9.25	12.5	9.335	5.24	4.6	4.93	5.535	5.54	6.4
	7.55		6.17		3.96		6.14		7.26	
D11	9.18	7.52	5.02	5.13	3.67	6.085	6.71	5.925	4.07	4.185
	5.86		5.24		8.5		5.14		4.3	
D12	8.35	11.43	3.96	3.815	8.79	7.61	5.97	4.73	3.11	3.6
	14.51		3.67		6.43		3.49		4.09	
E1	7.69	8.125	8.5	8.645	8.42	7.6	3.45	7.695	12.29	13.325
	8.56		8.79		6.78		11.94		14.36	
E2	5.03	6.945	6.43	7.425	7.1	5.545	13.39	13.45	12.44	12.945
	8.86		8.42		3.99		13.51		13.45	
E3	5.96	5.44	6.78	6.94	5.08	4.545	14.49	14.85	15.54	16.025
	4.92		7.1		4.01		15.21		16.51	
E4	4.86	5.86	3.99	4.535	4.77	5.195	17.06	13.63	10.34	10.735
	6.86		5.08		5.62		10.2		11.13	
E5	10.53		4.77	5.195	7.53	7.08	11.92	11.16	13.39	12.56
	8.02	9.275	5.62		6.63		10.4		11.73	
E7	7.68		6.63	7.67	8.71	6.87	12.22	12.71	13.13	13.2
	5.96	6.82	8.71		5.03		13.2		13.27	
E8	1.84		5.03	4.7	4.37	5.75	13.86	14.03	14.95	14.455
	4.17	3.005	4.37		7.13		14.2		13.96	
E9	7.94		7.19	8.855	10.52	8.185	10.75	10.91	11.53	11.62
	8.16	8.05	10.52		5.85		11.07		11.71	
E10	4.92		5.85	6.655	7.46	5.595	17.47	17.04	16.52	16.64
	4.05	4.485	7.46		3.73		16.61		16.76	
E11	5.8		8.29	7.65	8.29	7.65	17.44	16.89	16.84	17.09
	1.22	3.51	7.01		7.01		16.34		17.34	
E12	7.1		7.6	7.52	7.6	7.52	16.18	15.995	16.14	16.545
	4.83	5.965	7.44		7.44		15.81		16.95	
F1	5.15		4.41	4.785	4.41	4.785	16.99	16.805	17.24	16.765
	4.47	4.81	5.16		5.16		16.62		16.29	
F2	3.75		5.6	5.485	5.6	5.485	9.74	9.565	9.91	9.74
	5.11	4.43	5.37		5.37		9.39		9.57	
F3	2.23		5.52	5.725	5.52	5.725	11.74	12.205	12.33	11.87
	1.07	1.65	5.93		5.93		12.67		11.41	
F4	5.21		6.59	7.09	6.59	7.09	11.58	11.645	11.44	11.14
	6.11	5.66	7.59		7.59		11.71		10.84	
F5	5.32		9.48	7.08	9.48	6.78	10.42	10.595	10.68	10.54
	5.08	5.2	4.68		4.08		10.77		10.4	
F6	2.41		7.89	12.385	7.89	12.385	8.02	7.86	7.8	7.69
	5.98	4.195	16.88		16.88		7.7		7.58	
F7	5.93		5.53	5.195	5.53	5.195	6.09	6.51	6.02	5.275
	5.04	5.485	4.86		4.86		6.93		4.53	
F8	3.61		5.04	3.84	5.04	3.84	5.97	6.2	4.59	4.67
	4.24	3.925	2.64		2.64		6.43		4.75	
F9	4.47		8.33	7.94	8.33	7.94	7.08	7.22	5.69	5.93
	5.8	5.135	7.55		7.55		7.36		6.17	
F10	7.09		5.92	7.135	5.92	7.135	7.23	7.46	6.79	6.955
	6.06	6.575	8.35		8.35		7.69		7.12	
F11	5.28		4.93	7.125	4.99	7.155	5.41	5.975	5.35	5.305
	4.66	4.97	9.32		9.32		6.54		5.26	
F12	6.96		7.11	6.94	7.11	6.94	6.09	6.48	5.74	5.75
	6.82	6.89	6.77		6.77		6.87		5.76	
Control 1	5.61		9.47	10.375	9.47	10.375	17.98	18.76	18.21	17.935
	5.24	5.425	11.28		11.28		19.54		17.66	
Control2	6.2		8.68	9.175	8.68	9.175	12.93	13.275	13.25	12.865
	6.38	6.29	9.67		9.67		13.62		12.48	

Sample	28.28 hrs Area	average	31.41 hrs Area	average	48.43 hrs Area	average	53.75 hrs Area	average	58.07 hrs Area	average
A1	12.23	10.565	8.08	13.87	12.31	13.93	14.43	12.53	14.69	15.505
	8.9		19.66		15.55		10.63		16.32	
A2	6.21	6.95	8.73	8.725	7.31	10.945	9.79	11.71	9.25	12.515
	7.69		8.72		14.58		13.63		15.78	
A3	6.74	6.91	9.17	8.58	8.15	5.73	11.93	13.005	11.23	12.16
	7.08		7.99		3.31		14.08		13.09	
A4	8.2	7.815	10.36	11.475	5.04	8.795	11.43	10.72	11.01	10.325
	7.43		12.59		12.55		10.01		9.64	
A5	8.49	6.885	8.32	6.885	7.86	5.155	8.49	9.78	8.98	8.96
	5.28		5.45		2.45		11.07		8.94	
A6	6.3	6.785	10.26	11.675	8.34	5.885	10.24	9.085	10.47	10.055
	7.27		13.09		3.43		7.93		9.64	
A7	7.78	8.15	6.77	8.865	7.86	9.57	7.44	6.75	8.09	9.7
	8.52		10.96		11.28		6.06		11.31	
A8	11.16	8.79	7.22	7.99	13.64	11.095	14.38	10.555	16.98	12.46
	6.42		8.76		8.55		6.73		7.94	
A9	5.24	4.705	9.64	9.255	13.2	10.335	4.77	5.24	6.59	6.7
	4.17		8.87		7.47		5.71		6.81	
A10	3.99	5.065	8.71	8.755	9.74	10.095	4.82	5.42	9.68	10.015
	6.14		8.8		10.45		6.02		10.35	
A11	6.88	6.755	7.92	10.31	2.43	5.35	4.35	4.08	5.69	7.325
	6.63		12.7		8.27		3.81		8.96	
A12	7.22	9.35	10.24	10.015	3.19	3.175	3.17	3.955	5.32	6.05
	11.48		9.79		3.16		4.74		6.78	
B1	5.05	6.88	3.86	3.9	5.01	6.535	3.76	6.23	6.29	5.6
	8.71		3.94		8.06		8.7		4.91	
B2	15.96	12.9	6.9	5.91	10.4	8.105	12.42	9	16.14	9.82
	9.84		4.92		5.81		5.58		3.5	
B3	6.58	6.39	4.21	7.57	8.27	10.21	10.25	9.905	3.7	5.49
	6.2		10.93		12.15		9.56		7.28	
B4	6.97	6.865	10.87	11.56	10.4	9.22	4.77	6.275	12.87	11.2
	6.76		12.25		8.04		7.78		9.53	
B5	7.39	10.725	9.41	9.055	17.61	15.295	12.53	13.195	6.51	5.525
	14.06		8.7		12.98		13.86		4.54	
B6	16.18	15.75	4.89	8.12	16.77	19.875	24.36	23.6	3.54	7.73
	15.32		11.35		22.98		22.84		11.92	
B7	5.29	9.15	9.71	11.14	18.9	14.325	19.88	20.52	11.62	10.495
	13.01		12.57		9.75		21.16		9.37	
B8	16.15	16.715	8.99	8.37	13.39	13.94	2.95	4.745	11.16	10.485
	17.28		7.75		14.49		6.54		9.81	
B9	10.44	13.33	17.76	12.14	21.25	20.26	5.25	5.405	8.19	8.71
	16.22		6.52		19.27		5.56		9.23	
B10	16.76	16.07	6.97	6.045	10.39	6.755	6.1	6.605	8.52	7.7
	15.38		5.12		3.12		7.11		6.88	
B11	12.26	10.535	6.14	5.79	3.71	3.515	7.64	6.235	7.87	6.695
	8.81		5.44		3.32		4.83		5.52	
B12	7.09	7.485	15.52	14.445	2.74	3.27	15.83	10.235	7.05	7.365
	7.88		13.37		3.8		4.64		7.68	
C1	8.13	6.055	15.43	14.6	2.94	2.71	19.99	14.28	5.35	5.12
	3.98		13.77		2.48		8.57		4.89	
C2	11.67	15.08	8.13	10.47	4.73	5.285	16.42	16.455	4.49	4.05
	18.49		12.81		5.84		16.49		3.61	
C3	16.1	14.45	25.17	21.625	15.73	13.065	5.94	5.93	4.03	7.42
	12.8		18.08		10.4		5.92		10.81	
C4	9.46	9.175	12.43	9.76	5.01	5.41	6.6	4.805	8.66	9.205
	8.89		7.09		5.81		3.01		9.75	
C5	11.8	11.75	3.77	6.41	8.06	7.125	4.93	4.675	10.34	8.93
	11.7		9.05		6.19		4.42		7.52	
C6	7.71	6.565	6.65	8.7	7.84	14.57	4.07	3.935	10.57	9.835
	5.42		10.75		21.3		3.8		9.1	
C7	4.75	4.805	8.08	8.625	12.15	23.755	3.81	3.24	9.2	8.805
	4.86		9.17		35.36		2.67		8.41	
C8	16.69	12.735	12.74	8.835	11.45	14.185	1.13	3.77	10.51	11.225
	8.78		4.93		16.92		6.41		11.94	
C9	8.79	7.56	10.12	10.07	29.76	19.555	4.84	4.685	11.37	11.435
	6.33		10.02		9.35		4.53		11.5	
C10	10.87	9.275	12.01	11.83	11.75	17.785	3.4	4.08	11.55	12.19
	7.68		11.65		23.82		4.76		12.83	
C11	12.15	10.09	11.13	9.885	9.17	13.205	3.86	3.56	12.81	11.36
	8.03		8.64		17.24		3.26		9.91	
C12	7.94	11.395	3.46	9.55	8.85	11.77	2.35	11.465	5.73	6.565
	14.85		15.64		14.69		20.58		7.4	
D1	7.52	7.47	16.32	15.6	10.45	8.6	18.65	18.65	5.48	6.205
	7.42		14.88		6.75		18.65		6.93	
D2	6.69	9.395	12.65	15.615	8.35	10.665	16.86	21.565	8.27	8.52
	12.1		18.58		12.98		26.27		8.77	
D3	11.43	14.195	19.19	18.51	14.96	13.95	26.03	25.155	8.11	19.26
	16.96		17.83		12.94		24.28		30.41	
D4	16.39	16.555	18.77	13.16	18.56	18.46	24.48	18.795	29.27	27.16
	16.72		7.55		18.36		13.11		25.05	
D5	14.97	10.745	7.19	11.86	15.49	18.065	15.9	14.11	33.2	23.48
	6.52		16.53		20.64		12.32		13.76	
D6	6.27	8.11	12.58	9.82	22.87	27.525	5.24	9.4	18.52	17.57
	9.95		7.06		32.18		13.56		16.62	
D7	10.49	7.79	7.45	10.275	19.38	22.19	5.92	6.1	14.86	12.26
	5.09		13.1		25		6.28		9.66	
D8	3.66	7.045	12.85	10.175	17.47	20.66	17.05	17.79	7.32	5.575
	10.43		7.5		23.85		18.53		3.83	
D9	10.31	7.36	8.62	8.33	31.25	31.755	6.86	6.13	6.26	6.94
	4.41		8.04		32.26		5.4		7.62	
D10	3.53	4.31	5.08	5.355	20.07	19.545	9.03	7.625	9.53	7.765
	5.09		5.63		19.02		6.22		6	
D11	4.57	4.505	5.99	5.37	12.39	10.81	4.63	4.05	8.91	9.155
	4.44		4.75		9.23		3.47		9.4	
D12	4.39	3.815	4.95	11.44	18.51	17.925	26.17	26.12	11.14	11.895
	3.24		17.93		17.34		26.07		12.65	
E1	3.15	9.085	17.36	19.36	12.42	28.585	28.43	34.21	14.67	21.905
	15.02		21.36		44.75		39.99		29.14	
E2	15.55	15.51	19.01	22.345	18.17	24.705	32.7	34.405	24.68	19.985
	15.47		25.68		31.24		36.11		15.29	
E3	15.88	17.335	22.29	18.2	24.27	23.84	23.23	24.215	16.09	25.43
	18.79		14.11		23.41		25.2		34.77	
E4	19.56	16.845	18.67	17.78	15.94	12.6	25.78	26.325	34.41	28.62
	14.13		16.89		9.26		26.87		22.83	
E5	13.38	13.9	17.26	18.35	15.91	21.03	27.95	29.095	25.98	29.485
	14.42		19.44		26.15		30.24		32.99	
E7	16.44	15.745	20.25	19.8	26.78	29.85	32.76	31.98	29.96	27.505
	15.05		19.35		32.92		31.2		25.05	
E8	12.98	14.54	21.85	19.495	12.05	16.345	26.79	26.67	24.37	19.69
	16.1		17.14		20.64		26.55		15.01	
E9	16.65	15.22	14.96	18.895	32.39	29.82	35.94	36.24	9.88	14.33
	13.79		22.83		27.25		36.54		18.78	
E10	12.58	16.42	24.71	26.735	28.95	39.535	39.09	40.385	20.89	14.23
	20.26		28.76		50.12		41.68		7.57	
E11	21.64	21.69	31.36	27.68	19.16	17.04	38.1	37.725	12	14.41
	21.74		24		14.92		37.35		16.82	
E12	20.44	20.13	24.6	24.925	13.85	16.775	32.85	32.56	18.43	11.76
	19.82		25.25		19.7		32.27		5.09	
F1	19.39	19.33	25.39	19.41	8.55	10.285	31.08	25.365	3.9	7.91
	19.27		13.43		12.02		19.65		11.92	
F2	12.07	11.785	12.4	13.31	19.88	27.47	18.21	20.71	6.37	6.285
	11.5		14.22		35.06		23.21		6.2	
F3	15.11	14.925	17	16.57	7.24	8.03	24.61	23.2	7.31	7.12
	14.74		16.14		8.82		21.79		6.93	
F4	14.59	14.99	13.78	18.545	7.33	6.3	20.83	17.865	3.84	17.25
	15.39		23.31		5.27		14.9		30.66	
F5	12.06	12.33	12.31	13.6	8.53	6.905	13.92	11.355	28.41	29.66
	12.6		14.89		5.28		8.79		30.91	
F6	7.99	7.87	8.51	8.735	20.4	12.78	7.63	6.79	54.26	43.575
	7.75		8.96		5.16		5.95		32.89	
F7	6.25	6.34	5.72	5.975	25.42	29.3	5.51	5.985	42.9	38.955
	6.43		6.23		33.18		6.46		35.01	
F8	6.4	7.045	6.4	6.215	28.5	30.16	5.34	5.875	28.62	27.635
	7.69		6.03		31.82		6.41		26.65	
F9	6.95	6.56	7.14	7.36	43.69	36.76	6.03	5.32	33.55	30.395
	6.17		7.58		29.83		4.61		27.24	
F10	6.94	7.055	10.09	8.345	23.71	22.895	8.28	7.81	44.17	39.065
	7.17		6.6		22.08		7.34		33.96	
F11	5.75	5.655	5.26	5.82	54.85	44.65	5.63	5.315	30.06	28.99
	5.56		6.38		34.45		5		27.92	
F12	5.93	5.975	9.54	9.915	31.74	38.585	5.68	20.685	32.01	34.305
	6.02		10.29		45.43		35.69		36.6	
Control 1	24.34	24.82	27.52	32.615	61.5	50.76	36.29	30.975	42.76	41.435
	25.3		37.71		40.02		25.66		40.11	
Control2	16.44	16.28	17.55	15.755	36.72	49.55	20.42	21.63	39.9	49.225
	16.12		13.96		62.38		22.84		58.55	

Appendix G: Methanogenesis Toxicity Test

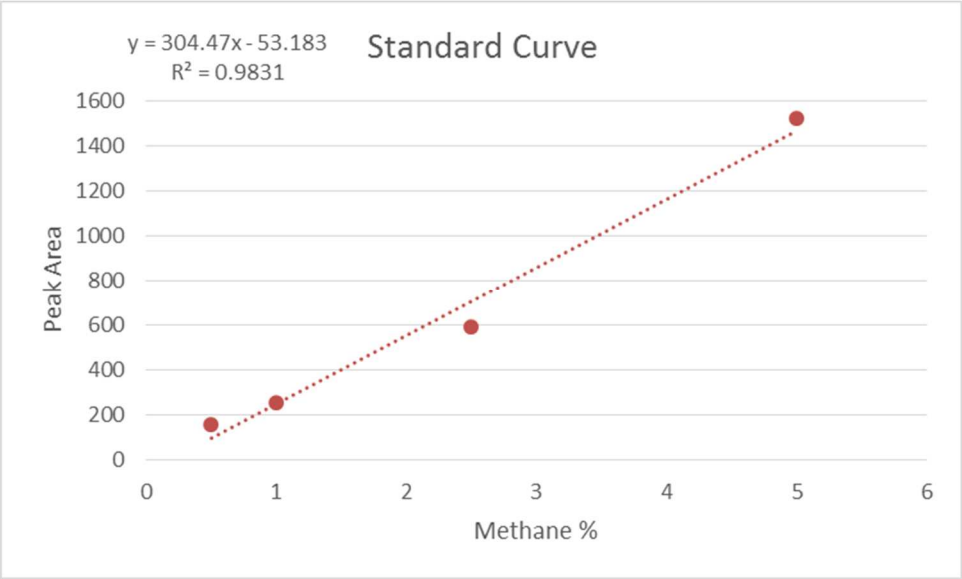

Figure G1. Standard curve for methanogenesis toxicity test

VITA

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