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Shale-Derived Dissolved Organic Matter as a Substrate for Subsurface Methanogenic Communities in the Antrim Shale, Michigan Basin, USA

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SHALE-DERIVED DISSOLVED ORGANIC MATTER AS A SUBSTRATE FOR SUBSURFACE METHANOGENIC COMMUNITIES IN THE ANTRIM SHALE, MICHIGAN BASIN, USA

A Thesis Presented

by

ROGER HUANG

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Department of Geosciences

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ABSTRACT

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FEBRUARY 2008

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The microbial origin of methane produced from sedimentary basins is a subject of great interest, with implications for the global cycling of carbon as well as natural gas exploration. Despite the growing body of research in sedimentary basin methanogenesis, few studies have sought to understand the subsurface microbial communities that produce methane, the metabolic pathways involved in the decomposition of ancient organic matter, or the components of ancient organic matter that are consumed. This research examined shale-derived dissolved organic matter (DOM) as a potential substrate to support a subsurface methanogenic community in a known microbial shale gas reserve, the Antrim Shale in the Michigan Basin, USA.

Experiments were conducted that enriched fermentative and sulfate-reducing microbial communities from Antrim Shale formation waters. Additionally, ^{1}H NMR spectroscopy was used to characterize shale-derived DOM solutions before and after they were used as growth media for fermentative and sulfate-reducing microbial communities, and to characterize the DOM of the Antrim Shale formation waters.

The results of the enrichment studies demonstrate that both fermentative and sulfate-reducing microbial communities from the Antrim Shale are capable of growth using shale-derived DOM as their only source of organic carbon; further, the production of methane in a fermentative enrichment demonstrates that methanogenesis can be supported by shale-derived DOM alone. The ^{1}H NMR characterization studies of the shale-derived DOM solutions before and after growth revealed subtle but detectable differences in DOM compositions, indicating the production and consumption of DOM components by the fermentative and sulfate-reducing microbial communities. Characterization analyses of Antrim Shale formation waters suggest that salinity and microbiological activity may influence the liberation of aliphatic and aromatic compounds from shale. The DOM characterization studies also suggest that carboxylic acids may be consumed by methanogenic communities in the Antrim Shale, and aromatic compounds may be produced by the enriched microbial communities and the communities present in the Antrim Shale.

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CHAPTER 1

ANAEROBIC BIODEGRADATION AND METHANOGENESIS USING COMPONENTS OF SHALE-DERIVED DISSOLVED ORGANIC MATTER

1.1 Methanogenic Communities Supported By Ancient Sedimentary Organic Matter

The microbial origin of methane produced from sedimentary basins is a subject of great interest, with implications for the global cycling of carbon as well as natural gas exploration. Subsurface methanogenic communities that consume ancient sedimentary organic matter occur in diverse environments including deep marine sediments and sapropels (Biddle et al., 2006; Wellsbury et al., 2002; Orphan et al., 2001; Coolen et al., 2002), petroleum reservoirs (Head et al., 2003; Grabowski et al., 2005), and subsurface shales and coals (Takai et al., 2003; Krumholz et al., 1997). Despite the growing body of research in sedimentary basin methanogenesis, few studies have sought to understand the subsurface microbial communities that produce methane, the metabolic pathways involved in the decomposition of ancient organic matter, or the components of ancient organic matter that are consumed. Methanogens have a limited number of substrates which they can consume (Zinder, 1993), and it is not likely that they can directly consume recalcitrant ancient organic matter. The anaerobic degradation of complex organic compounds into methane is thought to require several types of microorganisms working together in syntrophic relationships (Grabowski et al., 2005; Zengler et al., 1999); however, these microbial relationships and communities have not been well characterized, and it is not known which types of microorganisms are responsible for

consuming components of ancient organic matter and producing substrates that methanogens can utilize.

This research examined shale-derived dissolved organic matter (DOM) as a potential substrate to support a subsurface methanogenic community in a known microbial shale gas reserve, the Antrim Shale in the Michigan Basin, USA. Previous geochemical and stable isotope analyses of CH_4, CO_2, and H_2O have conclusively shown that gas from the Antrim Shale is dominantly microbial in origin (Martini et al. 1996; Martini et al. 1998; Martini et al. 2003). Several other shale and coal gas plays in the United States and the world have been shown to be partly or dominantly microbial in origin (Shurr and Ridgley 2002; Aravena et al. 2003; Ahmed and Smith 2001; Martini et al. 1996; McIntosh et al. 2002); however, the Antrim Shale is one of the largest and most productive shale gas reserves in the United States. Extensive drilling and exploration, along with a large network of active gas wells, allows for a high degree of access to the subsurface environment and makes the Antrim an ideal location to study microbial gas generation in a black shale.

1.2 Subsurface Methanogenic Communities

Although methanogenic subsurface environments are diverse and widely studied, few studies have investigated the metabolic pathways, trophic chains, and microbial communities that are involved in the decomposition of ancient sedimentary organic matter into methane. Methanogens, the Archaea directly responsible for the production of methane, are known to use a relatively small number of substrates and organic compound classes as a source of energy. These substrates are H_2 and CO_2, formate, acetate, some

short-chain alcohols, methylated amines, and methylated sulfides (Zinder, 1993). Table 1.1 shows some representative methanogenic reactions from various substrates. Because organic matter found in black shales is not likely to contain these compounds, methanogens in these environments must rely on other microorganisms to break down organic matter into substrates that methanogens can consume. This initial breakdown of sedimentary organic matter into methanogenic substrates is a critical step in a trophic chain leading to methanogenesis from shale organic matter. Thus, the microbial generation of methane in black shales requires a variety of microorganisms and metabolic roles to occur together, and is the result of a community of microorganisms rather than any single metabolic reaction or type of organism. Still, it is not clear which types of microorganisms and metabolic roles are present in methanogenic communities in black shales and in the Antrim Shale.

1.3 Ancient Sedimentary Organic Matter As A Source of Energy

Many subsurface methanogenic communities are dependent on ancient sedimentary organic matter as their only source of organic carbon and energy (Biddle et al., 2006; Wellsbury et al., 2002; Orphan et al., 2001; Coolen et al., 2002; Head et al., 2003; Grabowski et al., 2005; Takai et al., 2003; Krumholz et al.,1997). In most cases this organic matter is thought to be recalcitrant and therefore survived the process of burial and diagenesis, and many questions remain as to how ancient sedimentary organic matter is biodegraded despite its age. In the case of methanogenic communities in black shales, questions also remain as to what portion of the shale organic matter these microbial communities utilize as a source of energy and organic carbon. It is not known if

the microbial communities in these environments select specific components of shale organic matter, or if particular types of organic matter are favored over others.

1.4 Metabolic Pathways for Anaerobic Shale Decomposition

Two likely metabolic pathways for the anaerobic consumption of shale organic matter are illustrated in Figure 1.1: anaerobic respiration that utilizes terminal electron acceptors (Fig 1.1A), and fermentation that requires no external supply of electron acceptors (Fig 1.1B).

In the electron acceptor pathway of anaerobic shale biodegradation, bacteria utilizing a supply of terminal electron acceptors such as Fe^{3+}, NO^{3-}, or SO_4^{2-} consume organic components of shale. The metabolic products of these bacteria, or the biomass of the bacteria themselves, support a community of fermenting bacteria. The fermenting bacteria then produce substrates, such as H_2-CO_2 or acetate, that can be consumed by methanogens. A substantial amount of research has been dedicated to the characterization of anaerobic heterotrophs capable of degrading hydrocarbons by utilizing electron acceptors (Widdel and Rabus 2001; So and Young 1999; Chakraborty and Coates 2004). These bacteria have been found in many environments and have been shown to be capable of degrading a wide variety of hydrocarbons (Anderson et al. 1998; Röling et al. 2003; Magot et al. 2000). One mechanism of anaerobic hydrocarbon degradation may occur through a substituted succinate metabolism. Laboratory experiments demonstrate that some sulfate and nitrate-reducing bacteria are capable of degrading a wide variety of hydrocarbons by the enzymatic addition of fumarate to a hydrocarbon, producing a substituted succinate of the hydrocarbon (Figure 1.2) (Wilkes

et al., 2000; Elshahed et al., 2001; Widdel and Rabus, 2001; Geig and Suflita, 2002; Röling et al., 2003; Aitken et al., 2004). Given an adequate supply of electron acceptors, hydrocarbon-degrading anaerobes should be capable of consuming components of black shales (Krumholz et al. 1997; Krumholz et al. 1999).

The second hypothesized pathway of methane formation from shale does not require an external source of terminal electron acceptors and may occur through a mechanism similar to that proposed by Zengler et al. (1999) for methane formation from long-chain alkanes (Figure 1.3), who showed that acetogenesis from the decomposition of hexadecane could be coupled to methanogenesis in a three-step process in which, (1) acetogenic bacteria consume hexadecane and produce acetate, protons, and H_2, (2) one group of methanogens consume the acetate and protons to produce CH_4 and CO_2, and (3) a second group of methanogens use H_2 and CO_2 to produce methane. The acetogenic reaction (1) is thermodynamically unfavorable ($\Delta G^0 > 0$) at standard activities of acetate and H_2, so it can only proceed if the methanogenic reactions (2) and (3) keep acetate and H_2 at concentrations low enough to keep the reaction (1) exergonic. Thus, in this pathway of methane formation, the acetogens and methanogens have a syntrophic relationship. The acetogens rely on the methanogens to consume their metabolic products to keep their metabolic reaction energetically favorable, and the methanogens are dependent on the acetogens to produce compounds that they can consume.

Previous researchers have provided laboratory evidence that shows that anaerobic microbial growth on shale components alone is possible either with or without an additional electron acceptor. Krumholz and colleagues showed that both acetogenic and

sulfate-reducing strains of bacteria isolated from cored material from a shale-sandstone interface were capable of growth using shale components as the only electron donor (Krumholz et al. 1997; Krumholz et al. 1999; Krumholz et al. 2002). It should be noted that the acetogenic bacteria were capable of consuming shale components without the presence of methanogens, indicating that the consumption of shale organic matter by acetogens does not explicitly require syntrophy with methanogens.

Although Krumholz's laboratory growth experiments show that sulfate-reducing bacteria and fermentative acetogens can consume components of shale organic matter, his research does not provide direct evidence that these bacteria are part of a trophic chain that leads to methanogenesis. It is also not known if these two pathways of anaerobic shale biodegradation are mutually exclusive in natural environments, or if they can occur together.

1.5 Bioavailability of Shale Components

In addition to uncertainties about the metabolic pathways that lead from the consumption of shale to methanogenesis, it is not currently known which components of shale organic matter are being consumed. Krumholz et al. (2002) conducted growth experiments using acetogenic and sulfate reducing bacteria isolated from rock cores of the Cubero Sandstone in New Mexico. These experiments showed that growth rates for acetogenic and sulfate-reducing bacteria in growth media were not greatly affected when only the water-soluble components of shale rather than the whole ground shale was provided as the carbon source. These results suggest that the water-soluble components

of shale organic matter may be the most accessible or bioavailable components of shale and may serve as the primary substrate for subsurface methanogenic communities.

Although Krumholz provided compelling evidence that shale-derived DOM can support growth of both acetogenic and sulfate-reducing bacteria, his work did not investigate the composition of the shale-derived DOM, or which components of the shale-derived DOM were consumed. DOM in subsurface environments is composed principally of organic acids (Kotelnikova, 2002; Lundegard and Kharaka, 1994), and subsurface DOM has also been suggested as a possible substrate for subsurface microbial communities (Kotelnikova, 2002). Carboxylic acids are one possible class of compounds that may exist in shale-derived DOM that can serve as a substrate for shale-consuming microbial communities. Laboratory experiments conducted by Lewan and Fisher (1994) demonstrate that carboxylic acids can be released from shales by interaction with water. Lewan and Fisher (1994) suggested that these organic acids were bound to kerogen macromolecules in the shales by weak hydrogen bonds, thus preserving them during diagenesis, but allowing the acids to be easily released from the kerogen with water.

It is not known whether fermentative acetogens and sulfate-reducing bacteria consume the same components of shale-derived DOM, or if the different metabolic pathways consume different components of shale-derived DOM. Additionally, it is not clear what metabolic products either shale-consuming SRB or acetogens produce.

1.6 Thesis Research

This thesis research was guided by three principal hypotheses:

1) The methanogenic communities in the Antrim Shale utilize shale-derived dissolved organic matter as their primary source of energy and organic carbon.
2) The microorganisms are directly consuming this shale-derived DOM, and are utilizing either a sulfate-reducing or fermentative metabolism.
3) Either the sulfate-reducing or fermentative bacteria leave an identifiable signature on the DOM composition, which can be identified in the formation waters of the Antrim Shale.

The experiments and analyses described in this thesis were designed to test these hypotheses. Data from these experiments can also provide insight into questions regarding the sources of energy and organic carbon that feed methanogenic communities in the Antrim Shale. In particular, which components of shale-derived DOM are consumed and how selectively they are consumed can be addressed by these experiments.

Three experiments were conducted to test the proposed hypotheses. The first experiment used laboratory generated shale-derived DOM as a medium for the enrichment of fermentative and sulfate-reducing microorganisms using Antrim formation water as an inoculum. Positive growth in these enrichments demonstrated that members of the Antrim microbial community are capable of utilizing the water soluble components of shale as their only source of organic carbon and energy.

The second experiment created sulfate-reducing and fermentative microcosms using a laboratory generated Shale-Derived DOM Solution (SDDS) containing shale-derived DOM as the only organic carbon and energy source. These microcosms were inoculated with the microorganisms enriched from the Antrim formation waters in the

first experiment. After microbial growth occurred, the DOM was characterized to determine which components of the DOM were consumed and produced by the microbial community. Characterization analyses of the DOM were conducted using Solid Phase Extraction (SPE) to isolate and concentrate the DOM for ^{1}H NMR spectroscopy. The composition of the post-growth microcosm DOM was compared with the DOM composition of the SDDS to assess which components of the DOM were consumed and produced by the sulfate-reducing and fermentative microbial communities.

In the third experiment, formation waters were collected from three wells producing gas and water from the Antrim formation in the Northern Producing Trend (NPT) of the Michigan Basin. The DOM from these waters was extracted using SPE and characterized using ^{1}H NMR spectroscopy. The three wells were distributed across a geochemical and microbiological gradient allowing for changes in the DOM composition along these gradients to be observed and compared with the DOM composition of the microcosms and SDDS.

1.6.1 Evidence of Microbial Methanogenesis in the Antrim

Multiple lines of evidence point to a microbial origin of the methane produced in the Antrim. High concentrations of dissolved inorganic carbon (DIC) in the formation waters are an important indicator of microbial activity in the Antrim (Martini et al., 1998). Figure 1.4 shows the range of DIC concentrations for the Antrim formation waters and surrounding glacial drift waters that recharge the Antrim. The ground waters in the overlying glacial drift of the Antrim have DIC concentrations in equilibrium with surrounding carbonates ranging from 0 to 5 meq/L. However, formation waters in the

methane producing margins of the basin have DIC concentrations significantly greater than 10 meq/L. These high DIC concentrations require a source of CO_2 other than the carbonates, and are likely the result of shale organic matter oxidation by microorganisms. Figure 1.4 also shows the relationship between DIC concentrations and $\delta^{13}C$ ratios in the Antrim formation waters. The highly enriched $\delta^{13}C$ ratios of the formation water DIC are also indicative of microbiological activity (Martini et al., 1998). Methanogens utilizing DIC to produce methane will preferentially consume ^{12}C over ^{13}C, thus leaving the residual DIC in the formation waters highly enriched in ^{13}C.

Additional evidence for a microbial origin of the produced methane in the Antrim is found in the $\delta^{13}C$ ratios of the methane. The relationship between the carbon isotope ratios of CO_2 and CH_4 are expressed as:

$\alpha_c = (\delta^{13}C_{CO2} + 1000)/(\delta^{13}C_{CH4} + 1000) \approx 1.070$ (Martini et al., 1998; Whiticar, 1999)

Methanogenesis results in a $\delta^{13}C$ value of methane that is approximately 70‰ depleted relative to $\delta^{13}C$ of the source of CO_2. The observed $\delta^{13}C$ values of the methane and formation water DIC produced from Antrim wells are consistent with this relationship and therefore indicative of methanogenesis (Figure 1.5).

There is also an exchange of hydrogen isotopes during microbial methanogenesis, resulting in a direct relationship between the D/H ratios of microbially produced methane and those of the formation waters in the Antrim. The D/H ratio relationship between CH_4 and H_2O from methanogenesis is:

$$\delta D_{CH4} = \delta D_{H2O} + 160\ (\pm\ 10‰)\ \text{(Schoell, 1980)}$$

A H-isotope gradient in the Antrim formation waters allows chemical tracing of hydrogen from H_2O and allows for the tracing of CH_4 produced from the Antrim to the H_2O of the Antrim formation waters. Figure 1.6 shows the range of δD_{CH4} and δD_{H2O} of methane and water produced from wells in the Antrim.

1.6.2 The Antrim Shale As A Study Site

The late Devonian Antrim Shale in the Michigan Basin provides an excellent setting for this study on subsurface methanogenic communities and the sources of organic matter that feed them. Figure 1.7 shows the subcrop of the Antrim Shale in the Michigan basin, and Figure 1.8 shows a cross section of the Michigan Basin. This work focuses on the northern margin of the Michigan Basin where there is strong geochemical and isotopic evidence of active microbial methane production (Martini et al., 1996; Martini et al., 1998; Martini et al., 2003). Additionally, a strong salinity gradient in the region caused by the mixing of meteoric waters and basinal brines has been shown to have strong controls on the production of methane as well as on microbiological diversity (Martini et al., 1998; Waldron et al., 2007). These geochemical and microbiological gradients, combined with access to the subsurface environment provided by the extensive network of gas and water producing wells in the region, yield a natural laboratory for the exploration of the relationships between DOM composition and microbiological activity.

Three wells spread along an approximate North to South transect of the northern margin of the Michigan Basin were selected as a focus for this study. Figure 1.9 shows the locations of the wells along the salinity gradient of the Antrim formation waters. Well D2-26 was the northernmost and least saline of the three selected wells, microbiological

surveys of the Antrim have shown that this well has an active microbial community (Waldron et al., 2007). Well B1-36 was the southernmost and most saline well (3490 mM Cl^-), no evidence of microbiological activity was found from this well (Waldron et al., 2007). Well B4-18 is located between wells D2-26 and B1-36 and has a salinity of 200 mM Cl^-. Formation water from well A1-18 was used as inoculum for the enrichment experiments of this study, this well is located several hundred feet from well B4-18 and draws water and gas from the same depth as well B4-18. Both A1-18 and B4-18 have ample evidence of microbiological activity from previous microbial diversity surveys (Waldron et al., 2007; Stout and Gomez-Alvarez, unpublished data).

1.6.3 Solid Phase Extraction of DOM

The low concentrations of DOM typical of most natural waters makes direct analysis difficult, therefore researchers have used methods such as ultrafiltration, XAD extraction, and freeze drying to extract, desalt, and concentrate DOM for more effective analyses. C_{18} resin Solid Phase Extraction (SPE) improves on these methods with simpler procedures, better recovery efficiency, and improved desalting characteristics (Kim et al. 2003; Louchouarn et al. 2000). For these reasons, SPE is well suited for analysis of DOM in natural waters; however, some researchers have reported that SPE selects for more aliphatic DOM components than ultrafiltration or XAD extraction (Kaiser et al. 2003; Schwede-Thomas et al. 2005). This study used C_{18} Solid Phase Extraction methods to isolate and concentrate Antrim formation water DOM for characterization analyses with ^1H NMR spectroscopy.

1.6.4 ^{1}H NMR Spectrometry

Characterization analyses of DOM from both the laboratory microcosms and from Antrim formation waters were performed using ^{1}H NMR spectroscopy, a non-destructive analytical technique that can be used to determine the molecular structure of analyzed compounds. The use of ^{1}H NMR spectroscopy is widespread in analytical chemistry, and has more recently been used in studies that seek to characterize DOM in natural waters (Kim et al, 2003; Kaiser et al., 2003; Repeta et al., 2003).

Some atomic nuclei, such as those in ^{1}H and ^{13}C, posses a quantum property known as spin, which causes the nuclei to act like a magnet with a north and south pole. When these nuclei are exposed to a strong magnetic field, they will align with that magnetic field. An NMR spectrometer works by using a constant magnetic field to align the spins of the ^{1}H nuclei in one orientation. A second orthogonally oriented magnetic field pulses on, which causes the spins of the ^{1}H nuclei to realign with this second field. When this second field pulses off, the nuclei return to alignment with the constant magnetic field. The alignment of ^{1}H nuclei to the constant and pulsing magnetic fields can be thought of as high and low energy states. Energy is required to switch from one state to another . The energy required by a ^{1}H nucleus to switch from high and low energy states can be related to an absorption on the electromagnetic spectrum and is known as its resonance frequency.

The resonance frequency of all ^{1}H nuclei in a molecule are not equal. Magnetic interactions between a ^{1}H nuclei and its close neighbors will affect the resonance frequency of that ^{1}H nuclei. Different configurations of atoms in a molecule will thus

affect the resonance frequencies of ^{1}H nuclei in that molecule. As a result of this phenomenon, known as chemical shift, ^{1}H nuclei in some functional groups and arrangements of atoms have characteristic resonance frequencies.

NMR spectroscopy reports the differences in the resonances in a sample by comparing the observed resonance with that of a standard. Differences in the observed resonance and the standard resonance are reported in units of parts per million (ppm). A NMR spectrum shows the different resonance frequencies in ppm of the ^{1}H nuclei in a particular sample.

A NMR spectrum that shows the resonance frequencies of the ^{1}H nuclei in a sample can then be interpreted by an experienced user. The resonance frequencies, and the functional groups or atom arrangements that they are indicative of, can then be interpreted by an experienced user to reconstruct the molecular structure of the analyzed compound.

A ^{1}H NMR spectrum can be divided into broad functional group regions. This study breaks up the NMR spectrum into aliphatic (0.1 to 1.8 ppm), carbonyl (1.8 to 2.2 ppm), substituted-aromatic (2.2 to 3.0 ppm), carbohydrate (3.0 to 5.5 ppm), and aromatic (6.5 to 8.0 ppm) regions. Figure 1.10 shows some important functional group resonances as well as the broad regions of this study.

Figure 1.11 shows the ^{1}H NMR spectrum and chemical structure of propylbenzene. The large peak, or resonance, from 7 to 7.4 ppm (a) is characteristic of ^{1}H nuclei bonded to an aromatic ring. The resonance at 2.5 ppm (b) is indicative of ^{1}H nuclei in methylene groups alpha to an aromatic ring. The resonance at 1.6 ppm (c) is

typical of ^{1}H nuclei in aliphatic mid-chain methylene groups, and the resonance at 1 ppm (d) is indicative of ^{1}H nuclei in a terminal methyl group. These resonances, and the molecular relationships they are indicative of, can be used to determine the structure of the compound.

The ^{1}H NMR spectra produced in this study are obtained from complex mixtures of compounds, rather than of a pure compound as in the previous example. Because of the complexity of the resulting spectra, ^{1}H NMR analyses of DOM cannot provide specific information on the exact composition of the DOM. Still, knowledge of the functional groups present in a complex sample can provide insight into the broad categories and compound classes present in the sample.

Table 1.1 Methanogenic substrate classes and representative reactions.

Representative Methanogenic Reactions
$H_2 + CO_2$
$4H_2 + CO_2 \rightarrow CH_4 + H_2O$
Acetate
$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$
Methylated Amines
$4(CH_3)_3NH^+ + 9H_2O \rightarrow 9CH_4 + 3HCO_3^- + 4NH_4^+ + 3H^+$
Alcohols
$4CH_3OH \rightarrow 3CH_4 + HCO_3^- + H_2O + H^+$

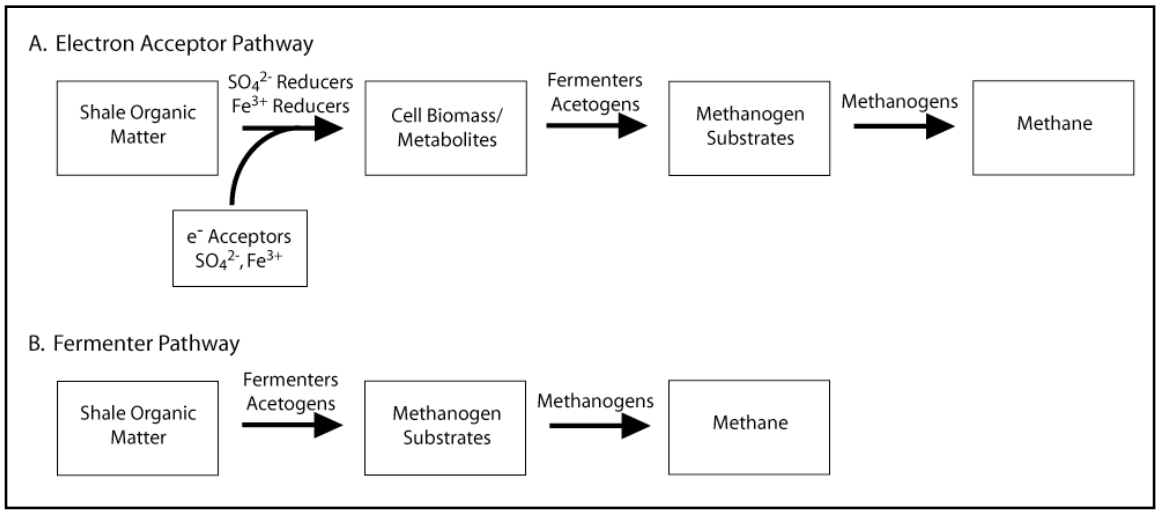

Figure 1.1. Schematic diagrams of likely pathways for microbial gas consumption from shale components.

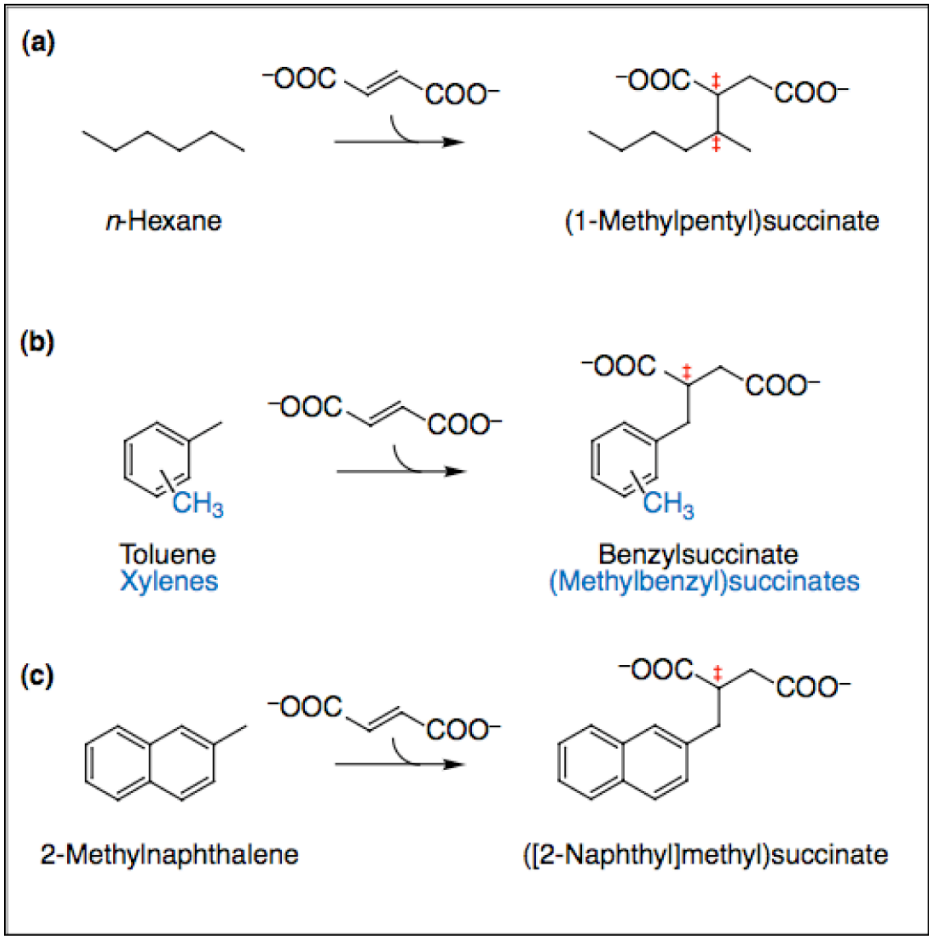

Figure 1.2. Production of substituted succinates by the enzymatic addition of fumerate to representative hydrocarbons (Modified from Widdel and Rabus, 2001).

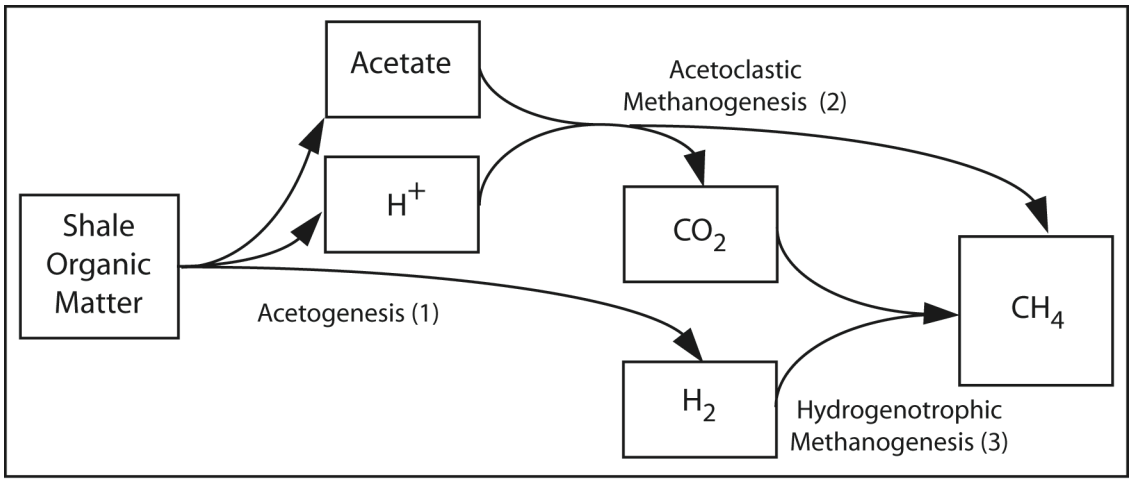

Figure 1.3. Schematic diagram of a syntrophic mechanism for shale consumption and microbial gas generation. Acetogens consume shale components and produce acetate, H^+, and H_2 (1), acetoclastic methanogens consume the acetate and H^+, producing methane and CO_2 (2), hydrogenotrophic methanogens produce methane after consuming CO_2 and H_2 (3). After Zengler et al., 1999.

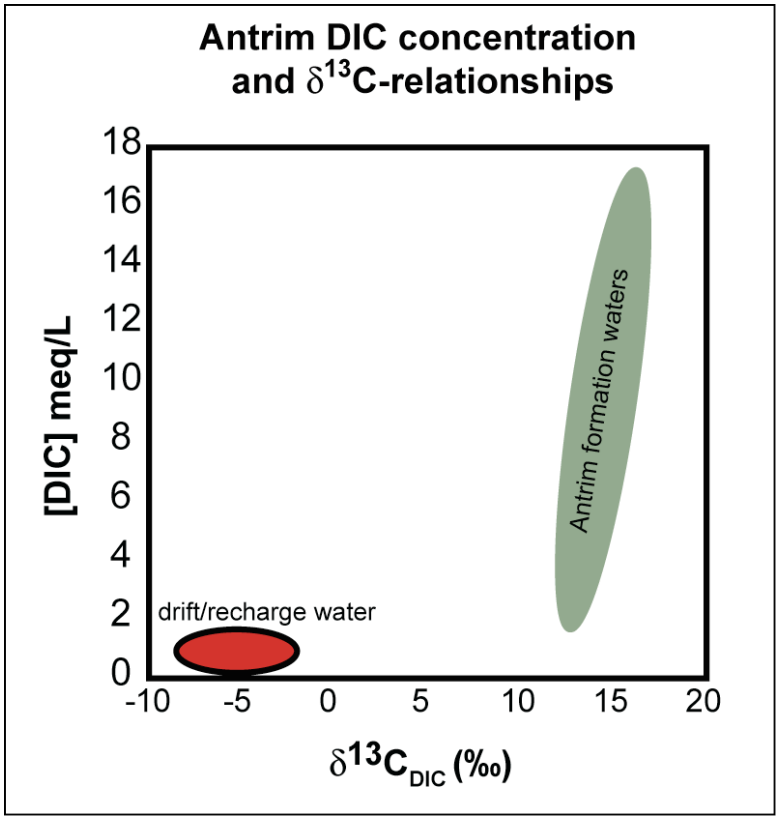

Figure 1.4. DIC concentration and $\delta^{13}C$ ratio relationships for Antrim Shale formation waters and glacial drift recharge waters (Modified from Petsch et al.2004).

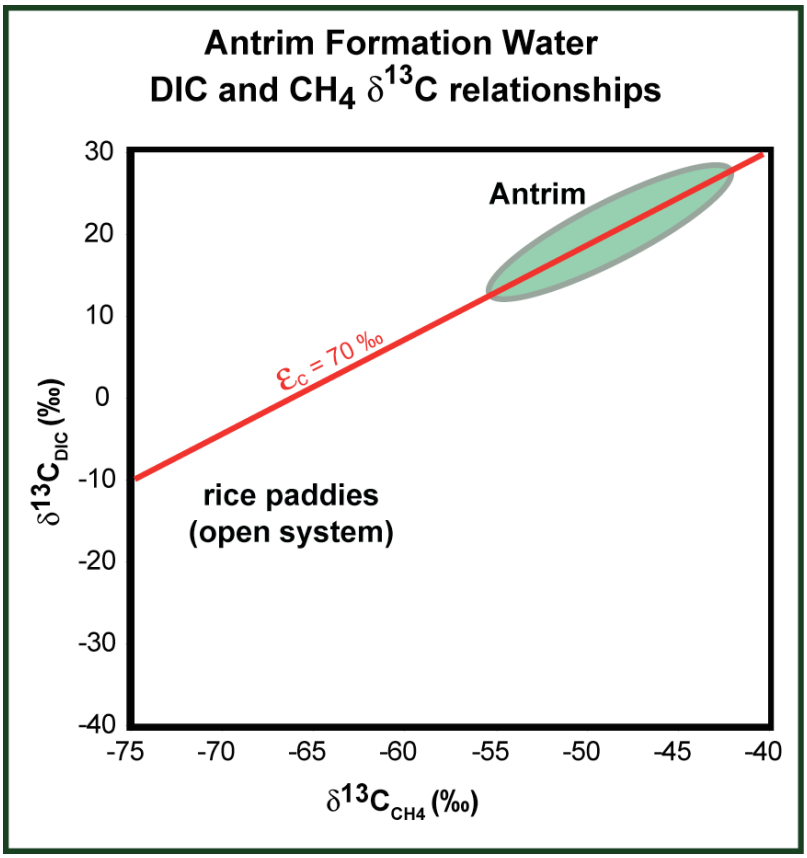

Figure 1.5. Antrim formation water DIC and CH_4 $\delta^{13}C$ ratio relationships (Modified from Petsch et al., 2004).

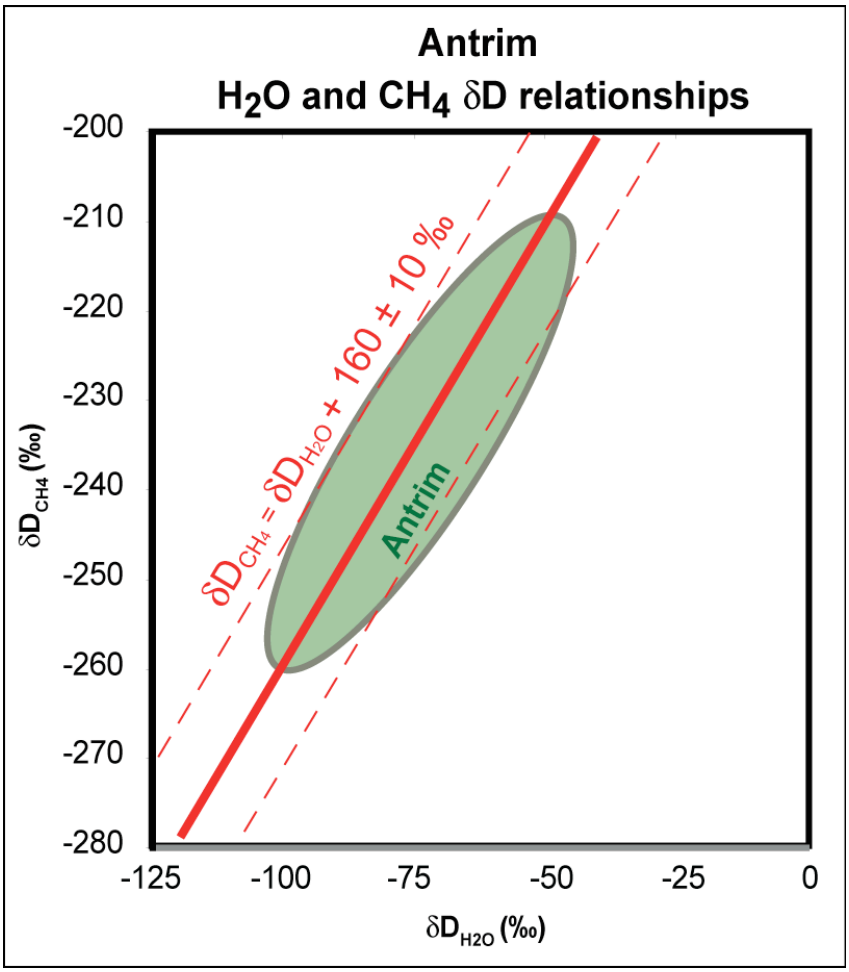

Figure 1.6. Relationship between δD ratios in Antrim formation waters and produced methane (Modified from Petsch et al., 2004).

Figure 1.7. Location of the Antrim Shale subcrop (Black) in the Michigan Basin (Modified from Martini et al., 1998).

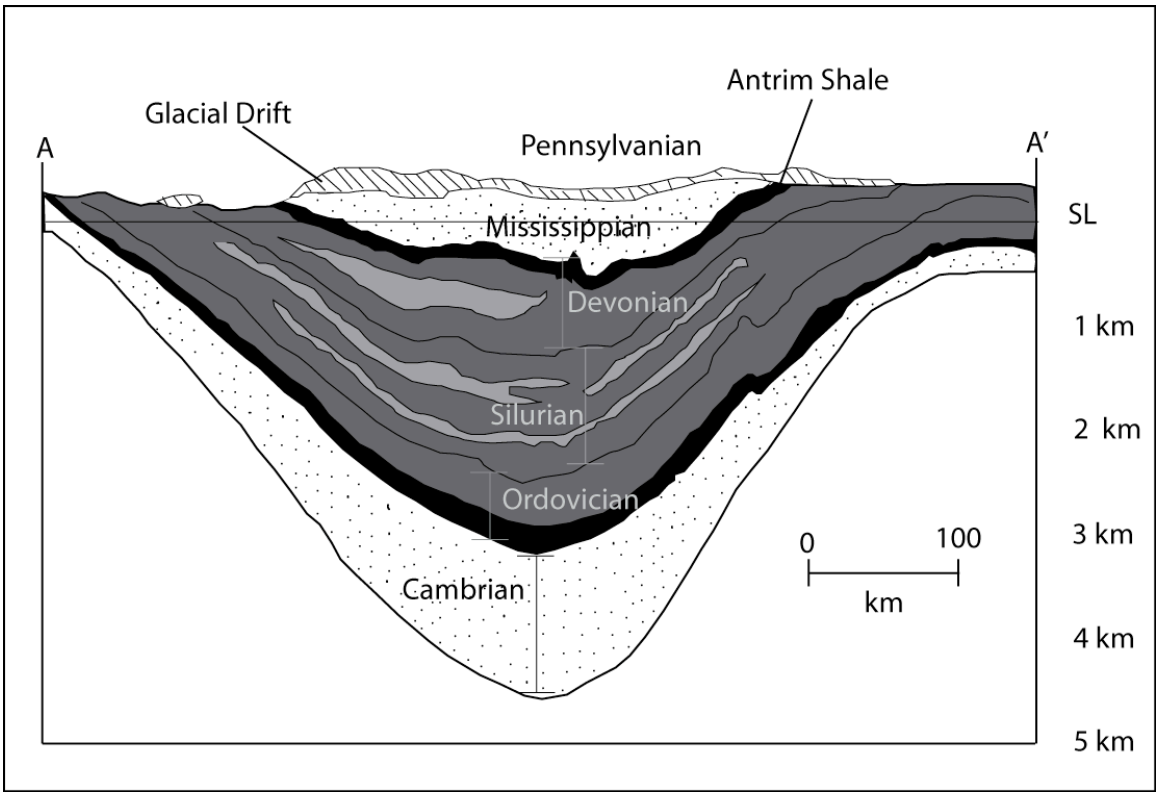

Figure 1.8. Cross section of the Michigan Basin (Modified from Martini et al., 1998).

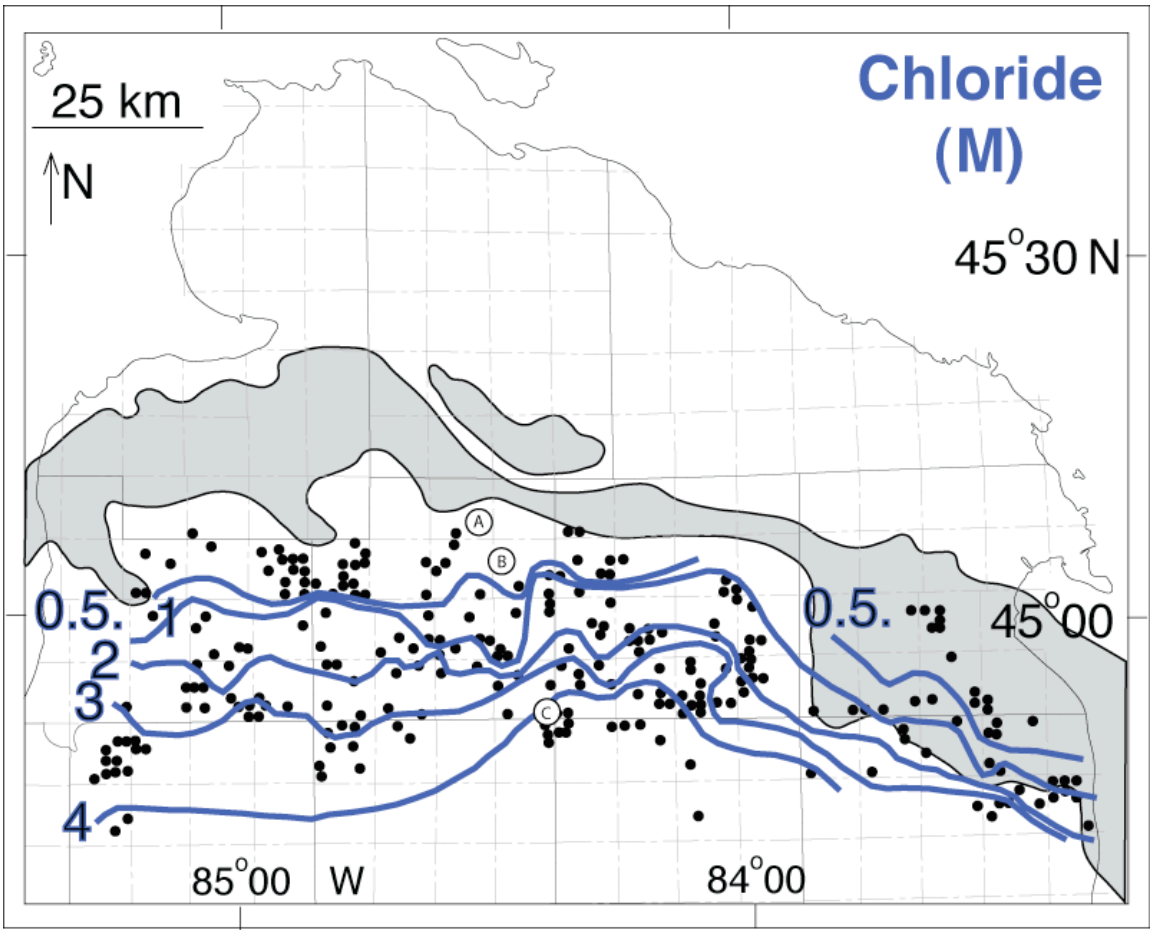

Figure 1.9. Map of study area and well locations in the northern margin of the Michigan Basin. Site A is well D2-26, site B is well B4-18/A1-18, and site C is well B1-36. The grey region marks the subcrop of the Antrim Formation. The contour lines show changes in Antrim formation water salinity in M Cl^-. The black dots mark well locations from previous studies. Modified from Petsch et al., 2004.

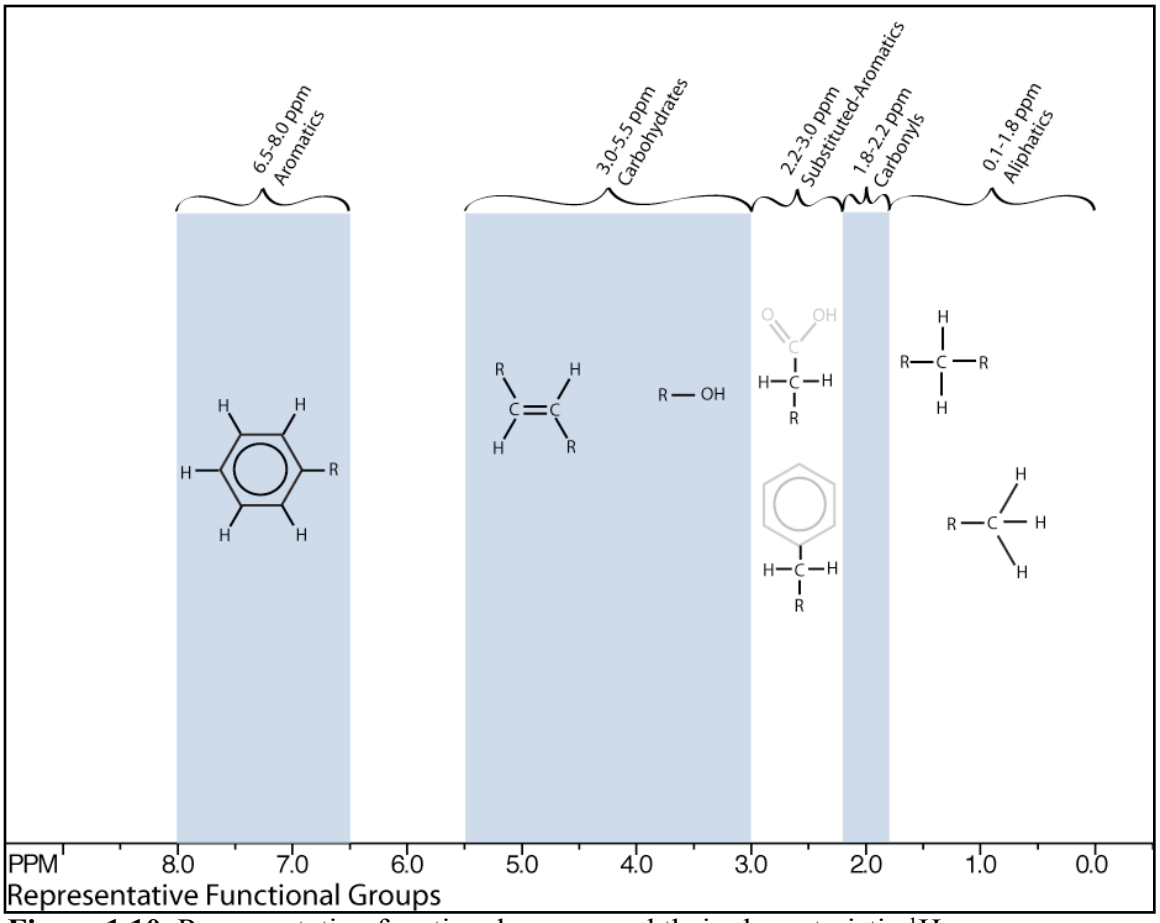

Figure 1.10. Representative functional groups and their characteristic ^{1}H resonances. The ^{1}H atoms indicated in these functional groups have characteristic resonances in the approximate area of the spectrum where the group is located. The ^{1}H NMR spectrum is also divided into the functional group regions used in this study.

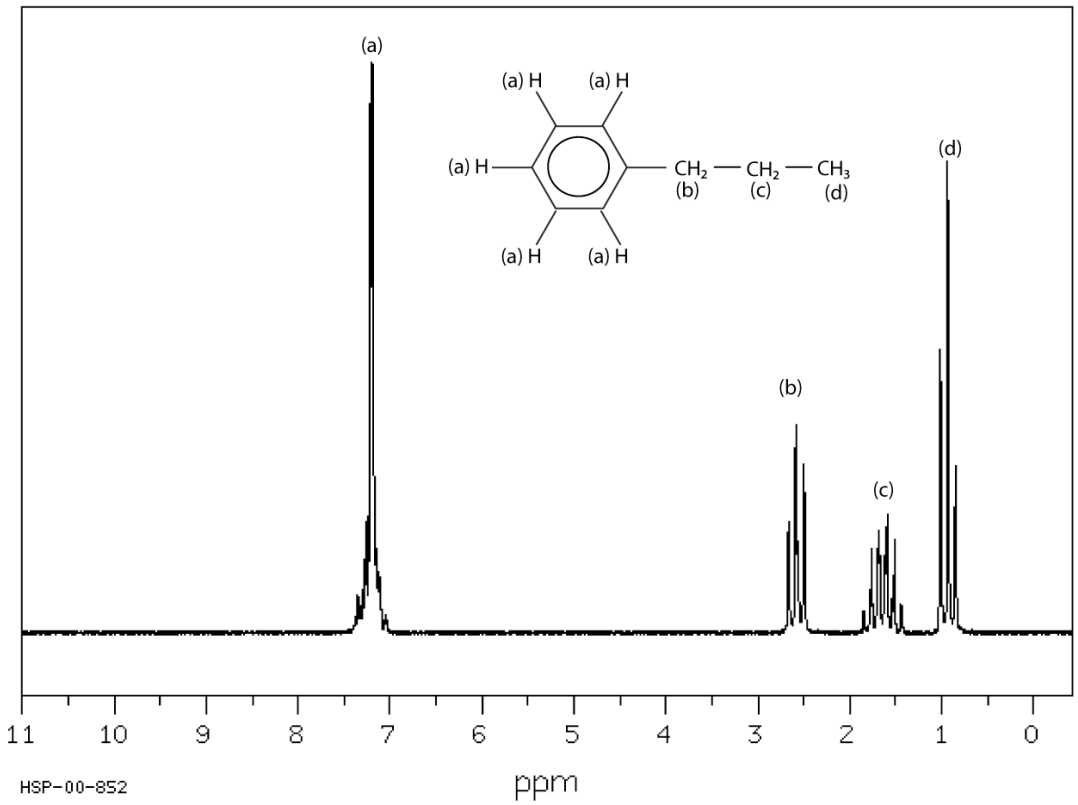

Figure 1.11. ^{1}H NMR spectrum and chemical structure of propylbenzene with peak assignments. The large peak, or resonance, from 7 to 7.4 ppm (a) is characteristic of ^{1}H nuclei bonded to an aromatic ring. The resonance at 2.5 ppm (b) is indicative of ^{1}H nuclei in methylene groups alpha to an aromatic ring. The resonance at 1.6 ppm (c) is typical of ^{1}H nuclei in aliphatic mid-chain methylene groups, and the resonance at 1 ppm (d) is indicative of ^{1}H nuclei in a terminal methyl group. These resonances, and the molecular relationships they are indicative of, can be used to determine the structure of the compound. Spectrum and peak assignments obtained from SDBS Web : http://riodb01.ibase.aist.go.jp/sdbs/ (National Institute of Advanced Industrial Science and Technology, 2007).

CHAPTER 2

ENRICHMENT OF ANTRIM SHALE MICROORGANISMS IN A SHALE-DERIVED DOM MEDIUM

2.1 Shale-Derived DOM as a Growth Substrate for Methanogenic Communities.

Methanogens, the Archaea ultimately responsible for the generation of methane, have a limited number of substrates which they are able to metabolize (Zinder, 1993), and are unlikely to be able to directly consume the organic matter present in subsurface shale environments. Therefore, methanogens in these environments must rely on other types of microorganisms to break down the organic mattert into substrates that methanogens can consume. The organisms that perform this role and the metabolic processes that occur are not well understood. Questions also remain as to what portion of shale organic matter is consumed by these microbial communities. The water soluble components of shale organic matter are one possible class of shale-derived substrates that may be accessible to microbial communities in subsurface shales.

The objective of this research is to address the hypotheses that the microbial communities that breakdown shale organic matter into methanogenic substrates consume the water soluble components of shale organic matter and are utilizing either a sulfate-reducing or fermentative metabolism.

To test these hypotheses, a set of enrichment experiments was conducted using anaerobic growth media containing laboratory generated shale-derived Dissolved Organic Matter (DOM) inoculated with Antrim formation water collected from a microbially active water and gas producing well in the Northern Producing Trend of the Michigan

Basin. One set of media contained sulfate to allow growth of sulfate-reducing bacteria (SRB), and a second set contained no added source of terminal electron acceptor except for CO_2 to encourage growth of fermentative bacteria. Archaeal and Bacterial 16S rRNA gene amplification and sequencing analyses were also performed on these enrichments to provided additional data about the communities present.

Growth occurred in both the fermentative and sulfate-reducing enrichments, indicating that members of the Antrim microbial community are capable of growth on the dissolved components of shale as their only source of organic carbon and energy. These enrichments also show that members of the Antrim microbial community are capable of consuming shale-derived DOM using both fermentative and sulfate-reducing metabolisms. Additionally, methane detected in one fermentative enrichment demonstrates that a complete methanogenic community can be supported by using shale-derived DOM as the only source of energy and organic carbon.

2.2 Methods

2.2.1 Field Collection of Inoculum

Well water from well A1-18 in the Michigan basin was collected for enrichment of sulfate-reducing and fermentative bacteria. A length of Tygon tubing was connected to the well pump, and well water was allowed to run from the well for 2-3 minutes before sampling. The other end of the tubing was inserted into a sterile oxygen-impermeable PETC Nalgene bottle so that the end touched the bottom of the bottle and the bottle was filled from the bottom up. The bottle was filled and emptied twice, and the contents of the third filling were sealed without headspace and packed with frozen cold packs in a

cooler. The water was shipped overnight to the Biogeochemistry Lab at the University of Massachusetts-Amherst. Upon arrival at the lab, the waters were immediately transferred into autoclaved 125 ml serum vials and sealed with autoclaved butyl rubber stoppers in an anaerobic chamber with an argon atmosphere.

2.2.2 Generation of a Shale-Derived DOM Solution

Cored material from the Antrim shale was used to produce shale-derived DOM solutions. Shale from the Middle Lachine member of the Antrim Shale Formation was obtained from the Western Michigan University core library from the core Joles C1-34 at 795 feet below surface level. This core site is located in southern Cheboygan county, MI, south of the Northern Producing Trend of the Michigan Basin. The shale used for the shale-derived DOM solutions had 6.9 wt. % TOC (M. Formolo, personal communication). Shale chips from the core were rinsed in Nanopure water and placed in an ultrasonic bath repeatedly to remove traces of drilling fluid. The chips were then air-dried at 50°C overnight. The dried chips were then ground into powder in a stainless steel ball mill. 10 g of powdered shale was combined with 1 L Nanopure water in combusted Corning media bottles. The bottle was then autoclaved for 1 hour. After autoclaving, the bottle was sealed with a gas-permeable membrane and stirred in darkness for at least two months.

The powdered shale was removed from the solution by staged filtration through combusted glass wool, 0.7 μm glass fiber filter (Whatman Inc., Florham Park, NJ), and 0.2 μm Durapore membrane filters (Millipore, Billerica, MA). The resulting filter-

sterilized shale-derived DOM solution had a total organic carbon (TOC) concentration of approximately 2 to 10 mg/L. These solutions were used for the enrichment experiments.

2.2.3 Enrichment in Shale-Derived DOM Medium

Enrichment experiments were conducted in 120 mL serum vials filled with 50 ml of anaerobic growth media using shale-derived DOM as the only source of organic carbon and optimized for either SRB or fermentative bacteria. The media were made to approximate the salinity and alkalinity of the formation water produced from well A1-18. Table 2.1 shows the dissolved ion concentrations in fermentative and sulfate reducing media and in the formation water from well A1-18. The fermentative medium recipe was modified from Waldron et al. (2007) and contained $CaCl_2$-$2H_2O$ (0.53 g), $MgCl_2$-$6H_2O$ (0.33 g), NH_4Cl (0.10 g), KH_2PO_4 (0.10 g), NaBr (0.024 g), $NaHCO_3$ (2.10 g), NaCl (10.96 g), and $NaMoO_4$ (2.42 g) dissolved in 1 L of shale-derived DOM solution. The sulfate-reducing medium recipe was modified from Waldron et al. (2007) and Postgate (1984) and contained $CaCl_2$-$2H_2O$ (0.53 g), $MgCl_2$-$6H_2O$ (0.33 g), NH_4Cl (0.90 g), KH_2PO_4 (0.394 g), NaBr (0.024 g), $NaSO_4$ (4.50 g), $FeSO_4$•$7H_2O$ (0.40 g), $NaHCO_3$ (2.10 g), NaCl (8.76 g) dissolved in 1 L of the shale-derived DOM solution. Both types of media were degassed with 80%:20% N_2:CO_2 and filter sterilized with 0.2 μm Durapore membrane filters (Millipore, Billerica, MA) into autoclaved and degassed serum vials. These vials were then inoculated with the previously described well water at a ratio of 1:100 and incubated at room temperature (approximately 20°C). Duplicate enrichment cultures were maintained for the fermentative enrichments (FERM 3 and FERM 4), and for the sulfate-reducing enrichments (SRB 3 and SRB 4). The

enrichments were transferred to new medium approximately every two to three months by 1:100 dilution into fresh media (Table 2.2).

2.2.4 Cell Enumeration

Cell growth in the enrichments was confirmed with direct cell counts using a Petroff-Hauser counting chamber.

2.2.5 Total Organic Carbon Analyses

Total organic carbon analyses were performed using a Shimadzu TOC-V. A calibration curve was determined using standards at 0, 1.16, 5.29, and 17.13 mg/L.

2.2.6 Methane Measurement

Methane detection analyses were conducted using an Agilent 3000A micro Gas Chromatograph. Only the presence or absence of methane was detected, a calibration curve was not established for these analyses. The retention time of the methane peak was determined by injecting a methane standard. A 1 ml gas sample was drawn directly from the headspace of the enrichment vial and injected into the GC.

2.2.7 DNA Extraction and Amplification

DNA amplification analyses were performed on cells from enrichments FERM 3, FERM 4, and SRB 3. Cells from the enrichments were concentrated for DNA extraction by centrifugation. One ml of enrichment media was centrifuged at 13,000 x g in a sterile eppendorf tube for two minutes. The supernatant was discarded and another 1 ml of enrichment media was added and centrifuged in the same manner as above. This process was repeated 20 times to concentrate a sufficient number of cells for DNA extraction. DNA was extracted from the resulting pellet using a Wizard Genomic DNA Purification

kit (Promega, Madison, WI) and by following the protocol provided by Promega for gram-positive bacteria. The resulting DNA product was used undiluted for PCR amplification.

Amplification of the Bacterial 16S rRNA gene from each sample was done using PCR with the forward primer 8F and universal reverse primer 1492R (Lane et al., 198) . The PCR reaction mixture was composed of: 0.50 ng/µL of purified DNA, 0.50 µM each of primers 8F and 1492R, 0.25 µM of each dNTP (Sigma-Aldrich Co., St. Louis, MO), 2.00 $MgCl_2$ (Sigma-Aldrich Co., St. Louis, MO), and 0.08 U/µL of Taq DNA polymerase (Promega Corp., Madison WI) in 10x PCR Buffer (Promega Corp., Madison WI). The reactions were performed on a MJresearch PTC-200 Peltier Thermal Cycler using the following program: 95˚C for 3 minutes, followed by 30 cycles at 94˚C for 30 seconds, 55˚C for 30 seconds, and 72˚C for 30 seconds, and a final step of 72˚C for 5 minutes. Amplification of a 1500 bp PCR product was confirmed by comparison to a DNA ladder (New England BioLabs, Ipswich, MA) by agarose gel electrophoresis. Experimental control for the amplification of Bacterial 16S rRNA was provided using extracted DNA from cultures of *Bacillus cereus*. Negative controls contained no added DNA.

Amplification of the Archaeal 16S rRNA gene was performed using nested PCR. The first reaction used the Archaea-specific primer 21F (DeLong, 1992) and reverse primer 1492R (Lane, 1985). In the second reaction, 1µL of PCR product from the first reaction was used as the DNA template for a second PCR reaction using the Archaea-specific primers 25F (Dojka et al., 1998) and 958R (DeLong, 1992). The reaction mixtures for these nested reactions were the same as that used for the Bacterial PCR

reactions and were performed on a MJresearch PTC-200 Peltier Thermal Cycler using the following program: 95˚C for 5 minutes, 95˚C for 45 seconds, 55˚C for 45 seconds, 72˚C for 1 minute, and a final step of 72˚C for 5 minutes. Amplification of a 1000 bp PCR product was confirmed by comparison to a DNA ladder (New England BioLabs, Ipswich, MA) by agarose gel electrophoresis. DNA from cultures of *Thermococcus* sp, *Methanosarcina barkeri*, and *Halobacterium* sp. were used as positive controls for the amplification of Archaeal 16S rRNA genes. Negative controls contained no added DNA.

2.2.8 Phylogenic Analysis

The products of the PCR reactions for FERM 3, FERM 4, and SRB 3 were were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) prior to sequencing. The resulting purified but otherwise raw PCR products were sequenced using a model 3730xl DNA Analyzer (Applied Biosystems, Inc., Foster City, CA). As the raw PCR product likely contained the amplified 16S rRNA of multiple Bacterial and Archaeal species, this sequencing method likely only sequenced the most abundant sequences or sequences most susceptible to this particular sequencing method. The sequencing reactions for the Bacterial 16S gene was carried out with the primer 8F (Lane et al., 198), while reactions for the Archaeal 16S gene used primer 25F (Dojka et al., 1998). Only sequences with more than 800 bp were used for further analyses. All sequences were manually edited using the software BioEdit v7.0.4 (Hall, 1999) and aligned using ClustalX v1.83 (Thompson *et al.,* 1997). The computer program Mallard v1.02 (Ashelford *et al.,* 2006) was used to check for chimera artifacts. No chimeric sequences were found in any sequences.

A phylogenetic tree was constructed from the alignments based on the Minimum Evolution method and was calculated with the software MEGA v. 3.1 (Kumar *et al.,* 2004) using the Tamura-Nei algorithm (Tamura and Nei, 1993).

2.3 Results

2.3.1 Enrichment of Fermentative and Sulfate-Reducing Bacteria

Cell growth in the fermentative and sulfate-reducing enrichments was demonstrated by qualitative turbidity assessments and direct cell counts. After two months of growth the sulfate reducing enrichment media contained black precipitate and a cell concentration of 2×10^8 cells/mL. The fermentative enrichment media was somewhat turbid and had cell concentrations ranging from 5×10^6 to 2×10^7 cells/mL after the same growth period. Initial cell concentrations were not quantifiable using the Petroff-Hauser chamber method, but cell concentrations in Antrim well waters were estimated to be approximately 6×10^3 (Waldron et al., 2007) and the initial inoculation was at a ratio of 1:100. Subsequent dilutions of both enrichments resulted in similar turbidity assessments and cell concentrations after equivalent periods of growth. Methane was detected in the fermentative enrichment FERM 4, but not in any sulfate-reducing enrichments or sterile controls.

2.3.2 DNA Extraction and Amplification

DNA extraction and amplification experiments were performed on three samples; a fermentative enrichment that did not produce methane (FERM 3), a fermentative enrichment that did produce methane (FERM 4), and a sulfate-reducing enrichment that did not produce methane (SRB 3). The results of the gel electrophoresis of the PCR

products are shown in Figure 2.1, and Table 2.3 shows the presence or absence of Archaeal and Bacterial 16S rRNA genes in each enrichment. Bacterial 16S rRNA genes were amplified in all three samples, while the Archaeal 16S rRNA genes were amplified only in the methane-producing FERM 4 and the sulfate-reducing SRB 3. There was no amplification of Bacterial 16S rRNA in the *Thermococcus* sp (sample H), *Methanosarcina barkeri* (sample C), or *Halobacterium* sp. (sample N), or the negative controls (sample (-)), indicating that the positive results for the experimental samples were not the result of contamination. Similarly there was no amplification of Archaeal 16S rRNA in the *Bacillus cereus* or negative controls, indicating that there was no contamination by Archaeal rRNA.

2.3.3 Sequencing and Phylogenetic Analysis

As the raw PCR product likely contained the amplified 16S rRNA of multiple Bacterial and Archaeal species, the sequencing method used was likely only to produce sequences for the most abundant 16S rRNA sequences or the 16S rRNA sequences most susceptible to the particular primers used. This method also did not successfully produce sequences for all of the successfully amplified Archaeal and Bacterial 16S rRNA genes. For example, Archaeal 16S rRNA genes were successfully amplified in enrichment FERM 4, but no Archaeal sequences were successfully obtained in this enrichment. Similarly, no Bacterial sequences were obtained in enrichment SRB 3, despite the successful amplification of Bacterial 16S rRNA genes in that enrichment.

One Bacterial 16S rRNA sequence was obtained from each of FERM 3 and FERM 4, and a single Archaeal 16S rRNA sequence was obtained for SRB 3.

Phylogenetic analyses show that both Bacterial sequences from the fermenting enrichments are placed within the class *Epsilon-Proteobacteria/Campylobacterace*. The phylogenetic analyses show that the sequence obtained from FERM 3 clusters with the genus *Arcobacter*. The Bacterial sequence obtained from FERM 4 clusters with the genus *Sulfurospirillum*. There is an 88% sequence similarity between the two sequences. No Archaeal sequences were obtained from either fermenter enrichment. Phylogenetic analysis of the Archaeal 16S rRNA sequence obtained from the SRB 3 places it within the class *Methanomicrobia/Methanosarcinaceae* and clusters with the genus *Methanolobus*. Figure 2.2 shows the sequences obtained from the enrichements ploted on a phylogenetic tree.

2.4 Discussion

The successful enrichment of both Archaea and Bacteria in the fermentative shale-derived DOM growth medium demonstrates that the enriched microorganisms are capable of growth on shale-derived DOM without the use of an electron acceptor other than CO_2 or any additional source of electron donor. Similarly the enrichment of Archaea and Bacteria in the sulfate-reducing growth medium shows that the enriched strains are capable of growth when shale-derived DOM is the only source of organic carbon and sulfate is provided as an electron acceptor. These results are consistent with those of Krumholz et al. (1997; 1999; 2002), who demonstrated that acetogenic and sulfate-reducing bacteria isolated from the Cubero Sandstone in New Mexico were capable of growth on the dissolved components of a shale. This data effectively addresses the

hypotheses that fermentative or sulfate-reducing bacteria enriched from the Antrim can consume the water-soluble organic components of a shale.

The successful enrichment of sulfate-reducing bacteria is significant given that there is little or no sulfate in most Antrim well waters (McIntosh and Walter, 2005), and genetic sequences of SRB have not been detected in Antrim formation waters (Stout and Gomez-Alvarez unpublished data) This indicates that SRB may be present and viable but inactive, or that the genetic sequencing techniques failed to detect the SRB. It is possible that some mechanism allows episodic pulses of sulfate-bearing water to infiltrate the Antrim, thereby allowing the inactive SRB present in the Antrim to grow using shale components. Such an episodic pulse of sulfate may originate from the underlying and sulfate-bearing Traverse Limestone formation (Martini et al., 2003). When the sulfate is exhausted, the SRB return to their inactive state, and fermenting and hydrolytic bacteria may then consume their metabolic products or biomass and produce methanogenic substrates.

The presence of methane in FERM 4 demonstrates that the enriched community is capable of methane generation when shale-derived DOM is the only source of organic carbon and no source of electron acceptor such as SO_4^{2-} is present. Because methanogens are limited to utilizing only a few specific substrates (Zinder, 1993), and are thus not likely to be capable of directly accessing the shale-derived DOM directly, other members of the enriched community are likely consuming the organic matter present and producing substrates that the methanogens can then utilize to produce methane. Since both Archaeal and Bacterial 16S rRNA were successfully amplified in this enrichment, it

is possible that this occurs through a mechanism similar to that proposed by Zengler et al. (2003) for the anaerobic breakdown of hexadecane into methane by syntrophic Bacteria and methanogens. In such a syntrophic mechanism, fermentative bacteria consume the shale-derived DOM and produce acetate, protons, CO_2, and H_2 which are then consumed by hydrogenotrophic and acetoclastic methanogens (Figure 1.2). This mechanism is syntrophic in nature because the bacterial production of acetate, protons, CO_2, and H_2 would be thermodynamically unfavorable without the concurrent consumption of these products by methanogens. However, Krumholz et al. (2002) showed that pure cultures of acetogenic bacteria were capable of producing acetate from dissolved shale components without the presence of methanogens; thus it is likely that the fermentative breakdown of shale-derived DOM does not require syntrophic relationships with methanogens. Similarly, growth was observed in FERM 3 but methane was not detected in the headspace and Archaeal 16S rRNA was not amplified, suggesting that in this enrichment fermentative bacteria were consuming shale-DOM without the presence of methanogens.

The absence of the Archaeal 16S rRNA genes in FERM 3 is consistent with the failure to detect methane in this sample. Because all methanogens are Archaea, the lack of Archaeal 16S rRNA genes confirms that no methanogens are present in FERM 3. The presence of methanogens in FERM 4, but absence in FERM 3 may indicate that the microorganisms in FERM 3 were exposed to conditions unfavorable for methanogens. Methanogens are very sensitive to oxygen exposure, and may be killed by even low concentrations of oxygen (Zinder, 1993). Oxygen exposure may have occured during the

transfer of cells into new media, killing the methanogens and resulting in their absence in subsequent transfers.

The presence of Archaeal 16S rRNA genes in the sulfate-reducing enrichment does not directly imply the presence of methanogens, although the sequence analyses of the amplified genes show that Archaea closely related to the methanogenic genus *Methanolobus* are present in the enrichment. The Archaea present in SRB 3 may be methanogens that have not produced enough methane to be detected by the GC analyses. Alternatively, anaerobic oxidation of methane may be occuring in the enrichments, consuming the methane produced by the methanogens. Anaerobic oxidation of methane is known to occur under sulfate-reducing conditions by syntrophic consortia of methanogenic Archaea that reverse their metabolism (producing H_2 and CO_2 from methane) and hydrogenotrophic sulfate-reducing bacteria (Orphan et al., 2001).

Figure 2.3 is a phylogenetic tree that includes sequences previously obtained directly from Antrim well waters as well the sequences obtained from the enrichment experiments (Stout and Gomez-Alvarez, Unpublished Data.). The Bacterial sequences from the fermenter enrichments have greater than 90% sequence similarity with Bacterial sequences previously obtained directly from Antrim well waters. The Archaeal sequence from the sulfate-reducing enrichment has between 92% to 95% sequence similarity with Archaeal sequences previously obtained from the Antrim. This level of sequence similarity is consistent with genus level similarity and suggests that the enriched strains are similar to strains active in the Antrim.

A study of microbial diversity in a biodegraded low-temperature and low-salinity petroleum reservior in the Western Canadian Sedimentary Basin by Grabowski et al. (2005) identified similiar sequeces of Bacteria and Archaea. Sequences closely related to *Arcobacter*, *Sulfurospirillum*, and *Methanolobus* species were obtained from enrichment cultures that used production waters from operating oil wells as inoculum. The *Arcobacter* sequences were 93% identical to that of a denitrifying *Arcobacter* strain isolated from cyanobacterial mats The *Sulfurospirillum* sequence has a 99% sequence similarity with the dehalogenating bacterium *Sulfurospirillum multivorans.* The *Methanolobus* sequence was found in media for denitrifying bacteria containing acetate and in media for denitrifying bacteria containing monaromatic compounds.

Grabowski et al. (2005) identified several other sequences closely related to fermentative bacteria, denitrifying bacteria, and methanogens. The method used to obtain sequences from shale-derived DOM enrichment experiments in this thesis research, applying sequencing techniques to raw PCR products, likely produced sequences for only the most abundant sequences present in the raw PCR product, or for the sequences most susceptible to amplification by the sequencing method applied. Thus it is likely that the sequences obtained only describe a subset of the total diversity present in the enrichments. The fermentative and sulfate-reducing enrichments may contain other species of Bacteria and Archaea for which sequences were not obtained, and these may also have close relatives in the enrichments conducted by Grabowski et al. (2005). The similarities between the the sequences obtained by Grabowski et al. (2005) and those

obtained in this study suggest that similiar metabolic roles and pathways occur in the biodegradation of petroleum reserviors as do in subsurface shale biodegradation.

2.5 Conclusions

These enrichment experiments show that fermentative and sulfate-reducing members of the Antrim microbial community are capable of consuming shale-derived DOM. Further, the presence of methane in FERM 4 demonstrates that a complete methanogenic community can be sustained on shale-derived DOM as the only source of organic carbon and energy. These data confirm the hypotheses that microbial communities in the Antrim can consume the water soluble components of a shale, and that they can utilize fermentative and sulfate-reducing metabolisms to consume these water soluble components of a shale. The successful enrichment of sulfate-reducing bacteria indicates that bacteria capable of utilizing sulfate as an electron acceptor are present in the Antrim despite the current absence of sulfate in the Antrim formation waters.

Table 2.1. Dissolved ion concentrations (in mM) of the Antrim formation water from well A1-18, fermenter enrichment medium, and sulfate-reducer enrichment medium.

	Antrim Formation Water Well A1-18	Fermenter Enrichment Medium	Sulfate-Reducer Enrichment Medium
Na	216	268.73	240.07
Mg	3.4	1.6	1.6
K	0.75	0.73	2.9
Ca	3.6	3.6	3.6
Fe	0.15	0	2.9
Cl	200	228.57	199.07
Br	0.23	0.23	0.23
SO_4^{2-}	0	0	39.24
PO_4^{3-}	0	0	2.9
HCO_3^-	31.9	25	25

Table 2.2. Schedule of transfers into new media for each of the fermentative and sulfate-reducing enrichments.

	FERM 3	FERM 4	SRB 3	SRB 4
May 24, 2006	INITIAL INOCULATION FROM A1-18			
	1	**1**	**1**	**1**
	2	**2**	**2**	**2**
	3	**3**	**3**	**3**
	4	**4**	**4**	**4**
	5	**5**	**5**	**5**
	6	**6**	**6**	**6**
	7	**7**	**7**	**7**
	8	**8**	**8**	**8**
	9	**9**	**9**	**9**
Aug 9, 2006	TRANSFER	TRANSFER	TRANSFER	TRANSFER
	1	**1**	**1**	**1**
	2	**2**	**2**	**2**
	3	**3**	**3**	**3**
	4	**4**	**4**	**4**
Sept 13, 2006	TRANSFER	TRANSFER	TRANSFER	TRANSFER
	1	**1**	**1**	**1**
	2	**2**	**2**	**2**
	3	**3**	**3**	**3**
	4	**4**	**4**	**4**
	5	**5**	**5**	**5**
	6	**6**	**6**	**6**
	7	**7**	**7**	**7**
	8	**8**	**8**	**8**
	9	**9**	**9**	**9**
Nov 22, 2006	**10**	**10**	TRANSFER	TRANSFER
	11	**11**	**1**	**1**
	12	**12**	**2**	**2**
Dec 13, 2006	TRANSFER	TRANSFER	**3**	**3**
	1	**1**	**4**	**4**
	2	**2**	**5**	**5**
	3	**3**	**6**	**6**
	4	**4**	**7**	**7**
	5	**5**	**8**	**8**
	6	**6**	**9**	**9**
	7	**7**	**10**	**10**
	8	**8**	**11**	**11**
	9	**9**	**12**	**12**
Feb 21, 2007	TRANSFER	TRANSFER	TRANSFER	TRANSFER

Table 2.3. DNA yield upon extraction. Bacterial 16S rRNA genes were successfully extracted and amplified from all three enrichments, indicating the presence of Bacterial biomass in all three enrichments. Archaeal 16S rRNA genes were successfully extracted and amplified from the fermentative enrichment FERM 4 and the sulfate-reducing enrichment SRB 3, indicating that Archaeal biomass was only present in these two enrichments.

	FERM 3	FERM 4	SRB 3
Bacteria	yes	yes	yes
Archaea	no	yes	yes

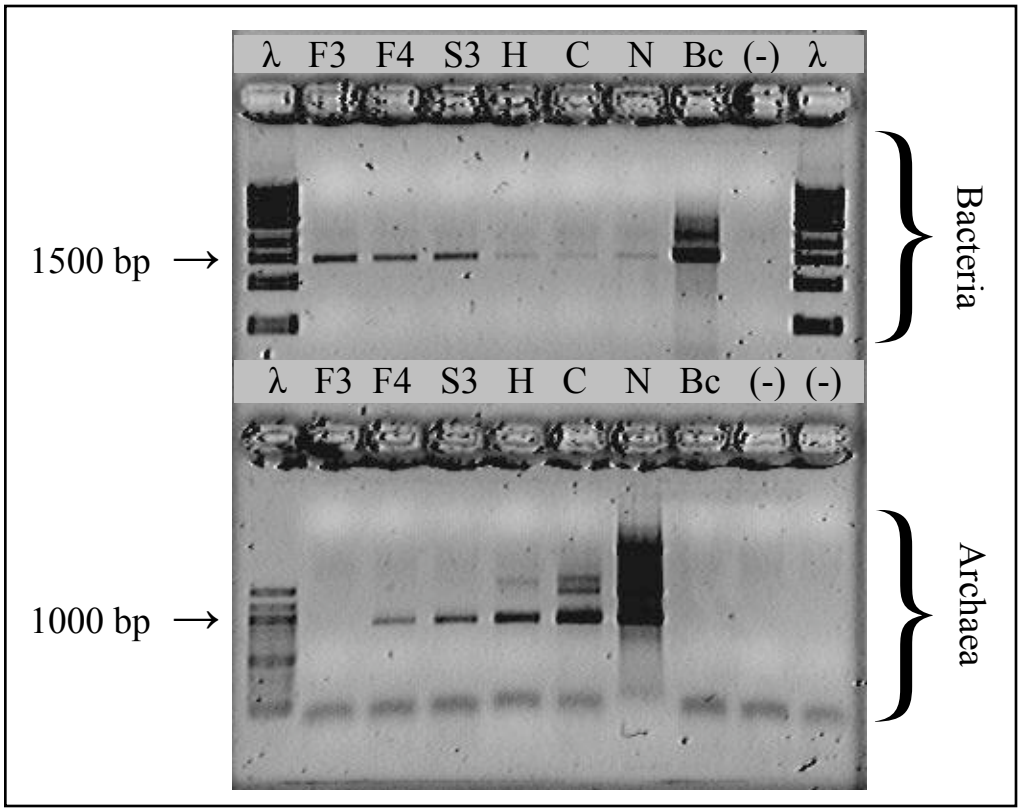

Figure 2.1. Electrophoresis gel of PCR products from FERM 3 (**F3**), FERM 4 (**F4**), and SRB 3 (**S3**). The dark bands at 1500 bp in the **F3**, **F4**, and **S3** columns in the upper gel indicate positive amplification of the Bacterial 16S rRNA gene in all three samples. Dark bands at 1000 bp in the **F4** and **S3** columns in the lower gel indicate positive amplification of the Archaeal 16S rRNA gene in the FERM 4 and SRB 3 enrichments. Columns **H**, **C**, and **N** were Archaeal controls with 16S rRNA from pure cultures of *Thermococcus* sp, *Methanosarcinia barkeri*, and *Halobacterium* sp respectively. Column **Bc** was a Bacterial control with 16S rRNA amplified from pure culture of *Bacillus cereus*. Column **(-)** was a negative control. Column **λ** contains the DNA ladder.

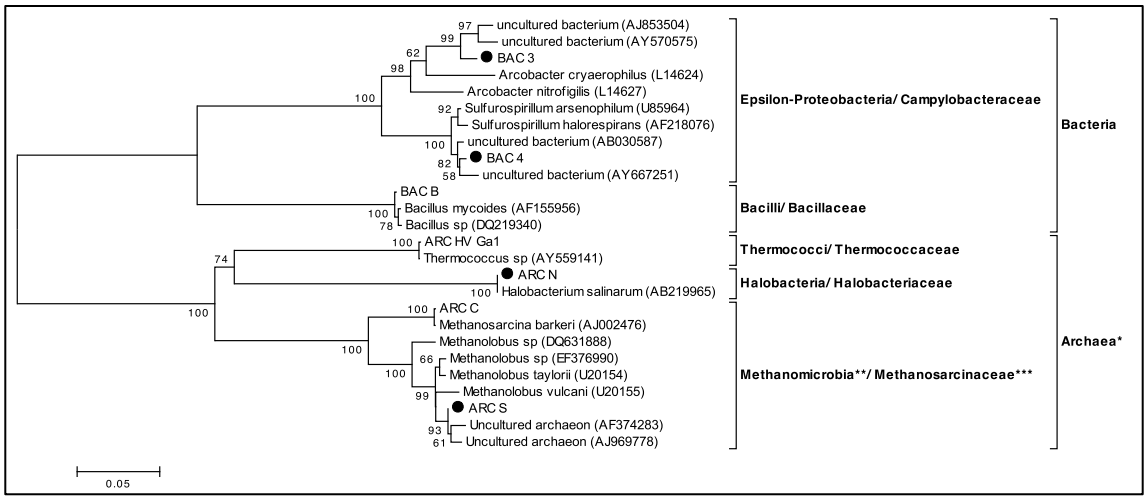

Figure 2.2. Phylogenetic tree of 16S rRNA gene sequences obtained from enrichments FERM 3, FERM 4, and SRB 3 compared with closely related species and reference strains. BAC 3 and BAC 4 were the Bacterial 16S rRNA sequences obtained from FERM 3 and FERM 4 respectively. ARC S was the Archaeal 16S rRNA sequence obtained from SRB 3. ARC N was the 16S rRNA sequence obtained from a control culture of the Archaea *Halobacterium* sp. The scale bar represents 5% estimated sequence divergence.

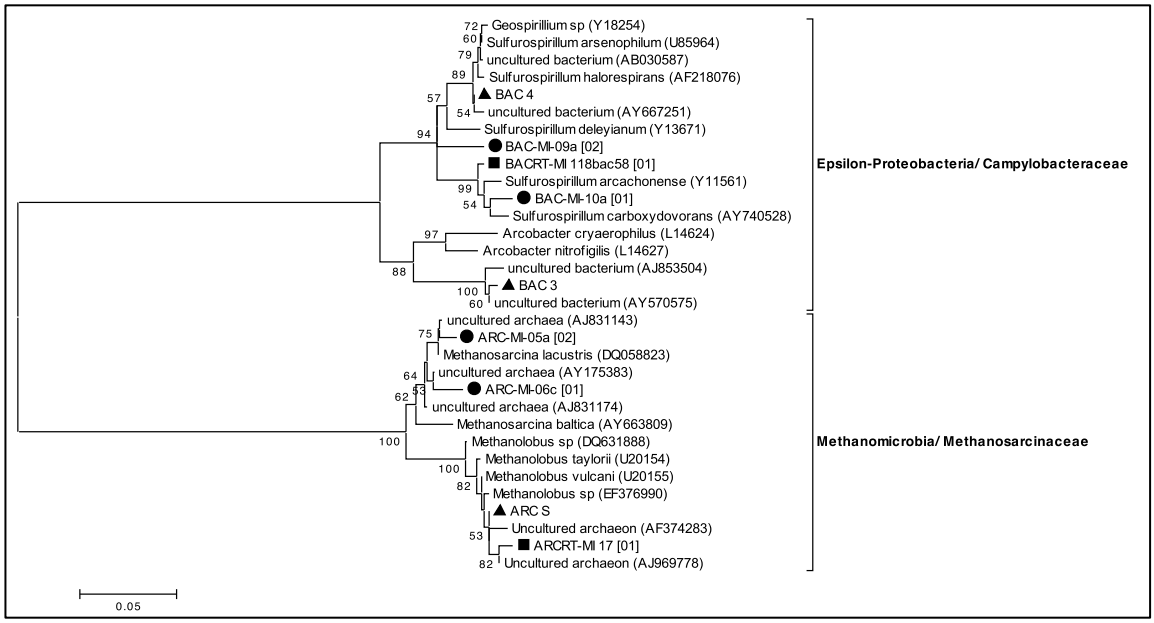

Figure 2.3. Phylogenetic tree of 16S rRNA gene sequences obtained from the enrichment experiments and previous surveys of microbiological diversity in the Antrim as well as closely related sequences and reference strains. ▲ denotes sequences obtained from the enrichment experiments. ■ and • denote sequences obtained from the Antrim by Stout and Gomez-Alvarez (unpublished data). Sequences BAC 3 and BAC 4 were obtained from enrichments FERM 3 and FERM 4 respectively. These Bacterial sequences have greater than 90% sequence similarity with the previously obtained Bacterial sequences obtained from the Antrim. Archaeal sequence ARC S was obtained from enrichment SRB 3, and has between 92% to 95% sequence similarity with other Archaeal sequences previously obtained from the Antrim. The scale bar represents 5% estimated sequence divergence.

CHAPTER 3

CHARACTERIZATION OF SHALE-DERIVED DISSOLVED ORGANIC MATTER USED AS A GROWTH SUBSTRATE IN ANAEROBIC MICROCOSMS

3.1 Shale-Derived DOM as a Substrate in Subsurface Methanogenic Communities

The ancient organic matter present in shales and other sedimentary basins was once assumed to be resistant to anaerobic microbial degradation (Fredrickson and Balkwill, 2006). Recent work, however, demonstrates that ancient organic matter is susceptible to anaerobic biodegradation in many environments (eg. Biddle et al., 2006; Wellsbury et al., 2002; Orphan et al., 2001; Coolen et al., 2002; Head et al., 2003; Grabowski et al., 2005; Takai et al., 2003; Krumholz et al., 1997; Fredrickson and Balkwill, 2006). In the Antrim Shale, questions remain as to what portion of shale organic matter the microbial communities utilize as a source of energy and organic carbon. In addition, the metabolic pathways that are used to consume the shale organic matter are not well understood. It is not known if shale-consuming microbial communities select specific components of shale organic matter or if particular types of organic matter are favored over others. In growth experiments using fermentative acetogens and sulfate-reducing bacteria (SRB) isolated from a subsurface shale, Krumholz et al. (2002) showed that growth on the water-soluble components of a shale was nearly equal to growth on ground shale. These results suggest that the water-soluble components of shale organic matter may be the most accessible or bioavailable components of a shale and may serve as the primary substrate for subsurface methanogenic communities.

Although Krumholz et al. (2002) showed that shale-derived dissolved organic matter (DOM) can support growth of both acetogenic bacteria and SRB, their work did not investigate the composition of the DOM or which components of DOM were consumed. It is also not known whether the fermentative and sulfate-reducing bacteria consume the same components of shale-derived DOM, or if the different metabolic pathways consume different components of shale-derived DOM. Additionally, it is not clear what metabolic products either shale-consuming SRB or fermentative acetogens produce.

The composition of DOM in subsurface waters has not been well characterized, although previous reseachers have identified organic acids as the principal components (Kotelnikova, 2002; Lundegard and Kharaka, 1994). Carboxylic acids are one component of shale-derived DOM that may be likely substrates for anaerobic microorganisms (Madigan et al., 2003). Lewan and Fisher (1994) have shown in laboratory experiments that carboxylic acids are weakly hydrogen-bonded to kerogen macromolecules in shales. One mechanism of anaerobic hydrocarbon respiration produces substituted succinates as metabolic byproducts (Wilkes et al., 2000; Elshahed et al., 2001; Widdel and Rabus, 2001; Geig and Suflita, 2002; Röling et al., 2003; Aitken et al., 2004). This mechanism of hydrocarbon degradation may be employed by microorganisms utilizing terminal electron-accepting metabolisms to consume components of shale-derived DOM.

The microcosm experiments and analyses described in this chapter were designed to test the hypothesis that the growth of fermentative and sulfate-reducing

microorganisms isolated from Antrim shale formation water (see previous chapter) alter the composition of shale-derived DOM when it is provided as the only organic substrate in a growth medium. These experiments and analyses may shed light as to which components of shale-derived DOM are consumed by the fermentative and sulfate-reducing bacteria enriched from the Antrim, and show how selectively these bacteria consume the components of shale-derived DOM.

The microcosm experiments were set up using sterile shale-derived DOM generated in the laboratory using cored material from the Antrim Shale. The shale-derived DOM solution was combined with salts to create microcosms replicating the salinity and alkalinity of well A1-18. These microcosms were inoculated with the sulfate-reducing and fermentative bacteria enriched from Antrim formation waters (see previous chapter). After sufficient growth was observed, the DOM in these microcosms was isolated using Solid Phase Extraction (SPE) and characterized using ^{1}H NMR spectroscopy (see section 1.6.4 for a brief review of this analytical method). The composition of the post-growth DOM was compared with un-inoculated control shale-derived DOM to assess which components of the DOM were consumed by the sulfate-reducing and fermentative bacteria.

Subtle differences were apparent in the NMR spectra of the fermentative and sulfate-reducing microcosms, indicating that the microbial communities in each of these microcosms were selectively consuming different components of the shale-derived DOM. The NMR analyses indicate that carboxylic acids may be produced by the interaction of

ground shale and water, and that substituted succinates may be present in the sulfate-reducing microcosm.

3.2 Methods

3.2.1 Generation of a Shale-Derived DOM Solution

Cored material from the Antrim Shale was used to produce a shale-derived DOM solution (SDSS). Shale from the Middle Lachine member of the Antrim Shale Formation was obtained from the Western Michigan University core library from the core Joles C1-34 at 795 feet below surface level. This core site is located in southern Cheboygan county, MI, south of the Northern Producing Trend of the Michigan Basin. The shale used for the shale-derived DOM solutions had a wt. % TOC of 6.9 (M. Formolo, personal communication). Shale chips from the core were rinsed in Nanopure water and placed in an ultrasonic bath repeatedly to remove traces of drilling fluid. The chips were then air-dried at 50°C overnight. The dried chips were then ground into powder in a stainless steel ball mill. One hundred grams of powdered shale was combined with 10 L of Nanopure water in a 10 L Corning media bottle and autoclaved for 1 hour. After autoclaving, the bottle was sealed with a gas-permeable membrane and stirred in darkness for two months.

Because a large volume of shale-derived DOM solution was required for the microcosm experiment, three 10 L batches were generated simultaneously and then homogenized. The powdered shale was removed from the solution by staged filtration through combusted glass wool, 0.7 μm glass fiber filter (Whatman Inc., Florham Park, NJ), and 0.2 μm membrane filters (Millipore, Billerica, MA). The resulting filter-

sterilized shale-derived DOM solution had measured total organic carbon (TOC) concentration of 4.08 mg/L. This SDSS solution was used for the microcosm experiments.

3.2.2 Large Volume Microcosm Experiment

The shale-derived DOM solution was combined with salts to create growth media that approximated the salinity and alkalinity of Antrim formation waters produced from well A1-18, a mid-range salinity well with an active microbial community (Waldron et al., 2007). Table 3.1 shows dissolved ion concentrations in each of the microcosm media and in the formation water from well A1-18. Two types of growth media were made: a sulfate-reducing medium containing sulfate as an electron acceptor, and a fermentative medium containing no external electron acceptors except CO_2. In both media the DOM from the aqueous shale extract was the only source of organic carbon. The sulfate-reducing microcosm media was modified from recipes described by Waldron et al. (2007) and Postgate (1984), and was composed of $CaCl_2$-$2H_2O$ (5.3 g), $MgCl_2$-$6H_2O$ (3.3 g), NH_4Cl (9.0 g), KH_2PO_4 (3.94 g), NaBr (0.24 g), $NaSO_4$ (45 g), $FeSO_4 \bullet 7H_2O$ (4.0 g), $NaHCO_3$ (21 g), NaCl (87.6 g) dissolved in 10 L of the shale-derived DOM solution. The fermentative microcosm media recipe was modified from Waldron et al. (2007), and contained $CaCl_2$-$2H_2O$ (5.3 g), $MgCl_2$-$6H_2O$ (3.3 g), NH_4Cl (1.0 g), KH_2PO_4 (1.0 g), NaBr (0.24 g), $NaHCO_3$ (21 g), NaCl (109.6 g), and $NaMoO_4$ (24.2 g) dissolved in 10 L of the shale-derived DOM solution. The salts for each type of media were dissolved into the filtered shale-derived DOM solution and then filter sterilized into four combusted 2 L media bottles each. Each bottle was then degassed by bubbling with sterile 80%:20%

N_2:CO_2 for 12 hours and then sealed with a gas-impermeable stopper with an 80%:20% N_2:CO_2 headspace.

The fermentative microcosm bottles were inoculated with 100 ml of enrichment media from FERM 4 (see previous chapter). The sulfate-reducing microcosm bottles were inoculated with 50 ml of enrichment media from SRB 3 (see previous chapter). The sulfate-reducing microcosms (SRM 1-4) were incubated at room temperature (approximately 22˚C) in darkness for 10 weeks. The fermentative microcosms (FM 5-6) were incubated at room temperature in darkness for 11 weeks. Growth was assessed by direct cell counts with a Petroff-Hauser counter and by turbidity assessments.

3.2.3 Solid Phase Extraction of DOM

Solid Phase Extraction of microcosm DOM was performed using Varian Bond Elut LRC cartridges (Varian Inc., Palo Alto, CA). Each cartridge was packed with 1 g of a hydrophobic C_{18} resin that adsorbs dissolved organic components present in water. The cartridges were attached to side-arm flasks, and fluid was drawn through the cartridges using vacuum pressure. The C_{18} resin was conditioned by first drawing 5 mL Optima grade Methanol (Fisher Scientific, Hampton, NH) through the cartridge followed by an 80 mL rinse of pH=2 nanopure water. Prior to Solid Phase Extraction of the microcosm DOM, the cells were separated from the microcosm media by filtration through a 0.2 μm Durapore polyvinylidene flouride membrane filter (Millipore, Billerica, MA). The filtered microcosm DOM was then acidified to pH=2 with HCl (Fisher Scientific, Hampton, NH). 2 L of filtered and acidified microcosm medium was run through each cartridge. The organic compounds adsorbed onto the resin were released by elution with

10 mL Optima grade methanol (Fisher Scientific, Hampton, NH). This extract was then evaporated in a combusted glass vial by gently blowing N_2 gas into the warmed vial (approximately 40˚C). The resulting dried DOM extract was then stored at 4˚C.

3.2.4 Total Organic Carbon Analyses

Total organic carbon analyses were performed using a Shimadzu TOC-V. A calibration curve was determined using standards at 0, 1.16, 5.29, and 17.13 mg/L.

3.2.5 ^{1}H NMR Analyses

Characterization of the extracted DOM from the laboratory microcosms was performed using ^{1}H NMR. The dried DOM extract of each sample was re-dissolved in 1 ml of CD_3OD methanol (Sigma-Aldrich, St. Louis, MO). The solution was then centrifuged at 1,000 rpm for 10 minutes to remove undissolved particulates (most likely C_{18} resin particles) before it was dispensed into a NMR sample tube. NMR data was acquired using a Bruker Avance 400 MHz NMR spectrometer. ^{1}H NMR spectra were conducted using pre-saturation water suppression, a 2 s recycle delay time and 1 Hz line broadening. 600 scans were performed for each sample.

Integrations of the spectra were manually calculated using the software program Spinworks Version 2.5.5 (Marat, 2006) by establishing the area from 0.1 to 9.0 ppm (excluding the large methanol peak) and then calculating the area of each functional group region as a percentage of the total area. Functional group regions were defined as aliphatic (0.1 to 1.8 ppm), carbonyl (1.8 ppm to 2.2 ppm), substituted-aromatic (2.2 ppm to 3.0 ppm), carbohydrate (3.0 ppm to 5.5 ppm), and aromatic (6.5 ppm to 8.0 ppm).

3.3 Results

Growth in the microcosms was assessed by direct cell counts and by qualitiative assessment of turbidity. Direct cell count data from the microcosms show that growth occurred in all of the microcosms (Figures 3.1 and 3.2). Cell counts in the sulfate-reducing microcosm (SRM) plot along a roughly log-linear trend ($y=8 \times 10^6 e^{0.4267x}$, $R^2=0.7115$) indicating an exponential growth rate. The fermentative microcosms (FM) do not demonstrate the same exponential growth trend. Instead, cell concentrations seem to increase in the first 4 weeks of growth and then level off. The final cell count for the SRM ranged from 2.4×10^8 to 3.5×10^8 cells/mL, the final cell counts for the FM ranged from 2.65×10^6 to 3.25×10^7 cells/mL.

Table 3.2 shows data from the TOC analyses of the microcosms. The sulfate-reducing microcosms have final TOC concentrations ranging from 4.22 to 4.60 mg/L, while the fermentative microcosms have final TOC concentrations that range from 6.2 to 6.81 mg/L. The TOC of the shale-derived DOM solution was 4.08 mg/L, which is less than any of the final microcosm TOC concentations. A blank nanopure water sample from the same source as that used to generate the shale extract had a TOC of 0.86 mg/L.

Figures 3.3, 3.4, and 3.5 show the ^{1}H NMR spectra for the shale-derived DOM solution (SDDS), fermentative microcosm (FM), and sulfate-reducer microcosm (SRM) respectively. Significant resonances are numbered for reference across the spectra of the three lab generated DOM samples and the three spectra of Antrim formation water DOM (see next chapter). Figure 3.6 and Table 3.3 show integration data for each of the three samples.

Peaks 1-7 are within the aliphatic region. Peak 1 is characteristic of protons in methane. Peak 2 and 3 are typical of a terminal methyl group. Peaks 4-7 are indicative of aliphatic mid-chain methylene groups. Peaks 8 and 9 are in the carbonyl region, resonances in this area are characteristic of ^{1}H nuclei associated with carbonyl groups. Peaks 10-14 are in the substituted aromatic region. These peaks are indicative of ^{1}H nuclei bonded to carbons alpha to carboxyl or aromatic functional groups. Peaks 15-27 are in the carbohydrate region. Resonances in this region are characteristic of ^{1}H nuclei associated with alcohol functional groups and carbohydrates. Peaks 28-35 are in the aromatic region and are diagnostic resonances for aromatic protons. These peak associations are broad generalizations, characteristic resonance frequencies for protons in functional groups can overlap and can be shifted due to magnetic interaction between neighboring atoms and functional groups.

The strongest resonances in all three spectra are in the aliphatic region (0 to 1.8 ppm), which account for 51 to 55% of the total integrated area. Carbohydrate resonances (3.0 to 5.5 ppm) are also a dominant feature in all three spectra, accounting for 18 to 25% of the total integrated area. Carbonyl (1.8 to 2.2 ppm) and substituted-aromatic (2.2 to 3.0) resonances are also significant in all three spectra, accounting for 9 to 14% of the total area. Minor resonances are also present in the aromatic region (6.5 to 8.0 ppm) in both of the microcosm spectra. Some differences between the three spectra are readily apparent. The aliphatic resonances in all three samples are similar in shape and intensity. Large resonances are present at approximately 1.25 ppm in all three spectra as peaks 4 and 5. In the SDDS spectrum peaks 4 and 5 are approximately equal in intensity,

accounting for 19.5% and 15.3% of the total integrated area respectively. In both microcosm samples peak 4 has a much stronger resonance than peak 5; peak 4 in the FM and SRM spectra account for 20.6% and 26.4% of the total integrated area, while peak 5 accounts for only 7.7% and 6.5% of the total integrated area. Peaks 6 and 7 at approximately 1.8 ppm are also present in all three spectra. In the SDDS and FM spectra both peaks 6 and 7 are roughly equal in intensity, although these peaks are much larger in the SDDS spectrum than in the FM spectrum. In the SRM spectrum, peaks 6 and 7 have significantly unequal intensities, with peak 6 accounting for 6.0% of the total integrated area, while peak 7 accounts for 9.7% of the total integrated area. Peak 8 at approximately 2.0 ppm in all three spectra is smaller in the FM spectrum and larger in the SRM spectrum when compared to the same peak in the SDDS spectrum. The resonances in the substituted aromatic region are roughly equal in distribution and intensity in the SDDS and FM samples, but have greater intensity in the SRM sample. The distribution of resonances in the carbohydrate region are similar in the SDDS and SRM spectra, but the intensities of peaks 15, 17, and 22 are greater in the SRM spectrum. Compared to the SDDS and SRM spectra, the FM spectrum has a greater diversity of resonances in the carbohydrate region.

3.4 Discussion

The lower concentrations of DOM in the SRM relative to the FM suggest that the higher cell concentrations in the sulfate-reducing microcosm are correlated with increased microbial consumption of DOM. The lower DOM concentration in the SDDS than in any of the microcosm samples is likely a result of a loss of DOM in the SDDS

sample used for TOC analysis. This may have occurred through contamination of the sample by aerobic microorganisms that then consumed the DOM, or by loss of volatile DOM components during storage. The alternative explanation for the discrepancy in DOM concentrations between the SDDS and microcosms is the production of DOM in the microcosms by anaerobic carbon fixation. However, if this were the case in the fermentative microcosm, a nearly 60% increase in DOM would have to be produced by anaerobic carbon fixation to account for the observed DOM concentrations; further, the newly fixed organic matter would have to have nearly the same composition as the shale-derived DOM to not dramatically alter the ^{1}H NMR spectra of the FM DOM compared to the SDDS DOM. The loss of DOM in the SDDS TOC sample is therefore a more plausible explanation for the lower DOM concentration in the SDDS compared to the FM and SRM.

The greater DOM consumption and growth rates in the SRM compared to the FM are consistent with the more favorable thermodynamics of electron-accepting metabolisms over fermentative metabolisms. The exponential growth rate and high cell concentrations also indicate that the microorganisms in the sulfate-reducing microcosm find the conditions of the microcosm favorable for growth. In contrast, the initial rise in cell concentrations in the fermentative microcosm followed by a lack of increase in cell concentrations suggest may indicate that the microorganisms in the fermentative microcosm exhaust the available supply of substrates that they can consume. This may indicate that the fermentative microorganisms are more selective of substrates than the sulfate-reducing microorganisms.

A phenomenon of NMR spectroscopy known as metal-bridging may explain why the NMR spectrum from the SDDS sample has broader and smoother peaks than the spectra from the microcosms. Metal-bridging occurs when the metal species that are commonly associated with DOM cause rapid relaxation of ^{1}H resonances during NMR analyses, resulting in a smoothing and broadening of peaks in the NMR spectra (Simpson et al., 2001; Kim et al., 2003). Low-molecular weight molecules such as volatile fatty acids (VFAs) and simple sugars are less likely to contain these metals, resulting in NMR spectra with sharper and better defined peaks (Kim et al., 2003). VFAs are often produced as byproducts during the degradation of hydrocarbons by anaerobic microorganisms (de Bok et al. 2001), and therefore may be likely metabolic products of the microbial community in the microcosms. Thus, the production of VFAs by the metabolic communities present in the microcosms may explain the increase in sharp peaks and narrow peaks superimposed on broader peaks in the NMR spectra of the microcosms relative to the spectrum from the SDDS.

Some differences are apparent when the SDDS, FM, and SRM spectra are compared to other published ^{1}H NMR spectra of solid-phase extracted DOM from surface waters, such as the spectra shown in Figure 3.7 obtained from Kaiser et al. from the River Tagliamento, Italy (Kaiser et al., 2003). The ^{1}H NMR spectrum of the River Tagliamento DOM is more dominantly composed of aliphatic proton resonances than spectra of shale-derived and microcosm DOM of this study, which have more resonances in the carbonyl, substituted aromatic, and carbohydrate regions. Aromatic resonances are also present in the riverine DOM spectrum that are virtually absent in the shale-derived

and microcosm DOM. Kaiser et al.(2003) suggested that the solid-phase extracted DOM from the river was primarily comprosed of terrestrial plant-derived cellular material and microbially-derived aliphatic esters, thus this source of terrestrial DOM in the SDDS, FM, and SRM samples is highly unlikely as ground shale was the only source for the DOM to originate.

The differences in the spectra of the SRM and FM samples suggests that the two microcosms have generated detectable differences in consumed or produced compounds despite having access to the same pool of DOM extracted from shale. Evidence of produced compounds may lie in the increased intensity of peaks 7, 8, and 12 in the SRM spectrum relative to the same peak in the SDDS spectrum (Figures 3.3 and 3.5). The increased intensity of peak 12 in the SRM spectrum in this region may be evidence of a substituted succinate mechanism of anaerobic biodegradation of DOM. The protons bonded to the carbons alpha to the carboxyl groups in alkyl-substituted succinates would produce resonances in the substituted-aromatic region of a ^{1}H NMR spectrum. A substituted succinate is produced by the enzymatic addition of fumarate to a hydrocarbon (Figure 1.2) (Widdel and Rabus, 2001; Aitken et al., 2004). This mechanism of anaerobic hydrocarbon degradation has been shown in laboratory experiments with both sulfate and nitrate-reducing bacteria (Wilkes et al., 2000; Elshahed et al., 2001; Widdel and Rabus, 2001; Geig and Suflita, 2002; Röling et al., 2003; Aitken et al., 2004). Although substituted succinates have not been detected in Antrim formation waters (M. Formolo, Personal Communication), members of the microbial community in the sulfate-reducing microcosm may be ultilizing a substituted succinate mechanism to consume the shale-

derived hydrocarbons present in the DOM, resulting in the increased resonances in the substituted-aromatic region of the SRM spectrum. Substituted succinates have been identified as the initial metabolites in the anaerobic degradation of several hydrocarbon classes including n-alkanes, benzenes, and naphthalenes (Wilkes et al., 2000; Elshahed et al., 2001; Widdel and Rabus, 2001; Aitken et al., 2004) Consumption of aromatic compounds, such as benzenes and napthalenes, in the microcosms is unlikely, as aromatic resonances are very minor in the SDDS. However, the consumption of n-alkanes in the microcosms is possible, as these compounds would produce resonances in the aliphatic region, which are abundant in the shale-derived DOM. Since there is already a substantial peak in the substituted-aromatic region in the NMR spectrum for the SDDS, substituted succinates produced by anaerobic hydrocarbon degradation cannot account for all of the resonances in the substituted-aromatic region of the SRM NMR spectrum. Further, the closest matches for alkyl-substituted succinates in an online repository of ^{1}H NMR spectra have resonances in the 2.4 to 3.0 ppm area of the substituted-aromatic region (SDBS Web : http://riodb01.ibase.aist.go.jp/sdbs/ (National Institute of Advanced Industrial Science and Technology, 2007)), while the most of peak 12 in the SRM DOM spectrum lies between 2.2 to 2.5 ppm. Although the data available from the SDBS are not exact matches for alkyl substituted succinates, they do cast doubt on the substituted-succinate interpretation for the increased intensity of peak 12 in the SRM DOM spectrum compared to the SDDS DOM spectrum.

The presence of resonances in the substituted-aromatic region of the SDDS and both the FM and SRM indicate that some compound class is present in all three of these

samples and is producing these resonances. Carboxylic acids are a class of organic compounds that are common in subsurface waters (Lundegard and Kharaka, 1994). In all carboxylic acids, the protons bonded to the C atom alpha to the carboxyl group resonate in the substituted-aromatic region; thus the presence of peak 12 in the SDDS, FM, and SRM NMR spectra may indicate the presence of these compounds. Lewan and Fisher (1994) have conducted laboratory experiments that suggested that carboxylic and other organic acids are bound to kerogen macromolecules by weak hydrogen bonds and thus preserved during diagenesis. These hydrogen-bonded organic acids are easily released from the kerogen macromolecule by intereaction with water, and could thus make up a portion of the water soluble components of shale organic matter.

3.5 Conclusions

The results of the microcosm experiments show that the growth of organisms enriched from the Antrim on shale-derived DOM will produce detectable differences in DOM composition when examined using ^{1}H NMR analyses. Although the exact nature of the consumed and produced compounds cannot be determined, the evidence gathered suggests that the communities in the fermentative and sulfate-reducing microcosms produce a different suite of metabolic products and may be selectively consuming different components of the available DOM. Resonances in the substituted-aromatic region of the NMR spectra of the SDDS may support findings by Lewan and Fisher (1994) that carboxylic acids can be produce from by interaction with water shales.

Table 3.1. Dissolved ion concentration (in mM) in well A1-18, the fermenter microcosm medium, and the sulfate-reducer microcosm medium.

	Antrim Formation Water Well A1-18	Fermenter Microcosm Media	Sulfate-Reducer Microcosm Media
Na	216	268.73	240.07
Mg	3.4	1.6	1.6
K	0.75	0.73	2.9
Ca	3.6	3.6	3.6
Fe	0.15	0	2.9
Cl	200	228.57	199.07
Br	0.23	0.23	0.23
SO_4^{2-}	0	0	39.24
PO_4^{3-}	0	0	2.9
HCO_3^-	31.9	25	25

Table 3.2. Total organic carbon concentrations for the Shale-Derived DOM Solution (SDDS), Sulfate-Reducer Microcosm (SRM), and Fermenter Microcosm (FM).

	Total Organic Carbon (mg/L)
Nanopure Water	0.86
SDDS	4.08
SRM 1	4.35
SRM 2	4.60
SRM 3	4.48
SRM 4	4.22
SRM Average TOC	4.41
Standard Deviation	0.16
FM 1	6.47
FM 2	6.20
FM 3	6.81
FM 4	6.22
FM Average TOC	6.43
Standard Deviation	0.28

Table 3.3. Peak areas as percentage of total integrated area for the SDDS, FM, and SRM NMR spectra.

Peak Number	SDDS	FM	SRM
1	NP[a]	0.1	0.2
2	8.8	7.8	5.0
3	NP	NP	NP
4	19.5	20.6	26.4
5	15.3	7.7	6.5
6	7.6	5.5	6.0
7	7.8	7.5	9.7
8	4.2	3.5	4.7
9	NP	NP	0.9
10	4.2	NP	NP
11	NP	NP	NP
12	7.8	9.4	11.3
13	NP	NP	NP
14	NP	NP	NP
15	0.5	0.4	0.6
16	1.0	2.9	0.6
17	2.8	4.6	3.8
18	3.6	NP	3.7
19	NP	1.9	NP
20	NP	1.4	NP
21	NP	1.4	NP
22	9.6	8.8	15.2
23	NP	NP	NP
24	0.5	NP	NP
25	NP	0.5	0.7
26	NP	0.7	NP
34	NP	0.6	0.4
35	NP	0.5	0.2

[a] NP, no peak at this position.

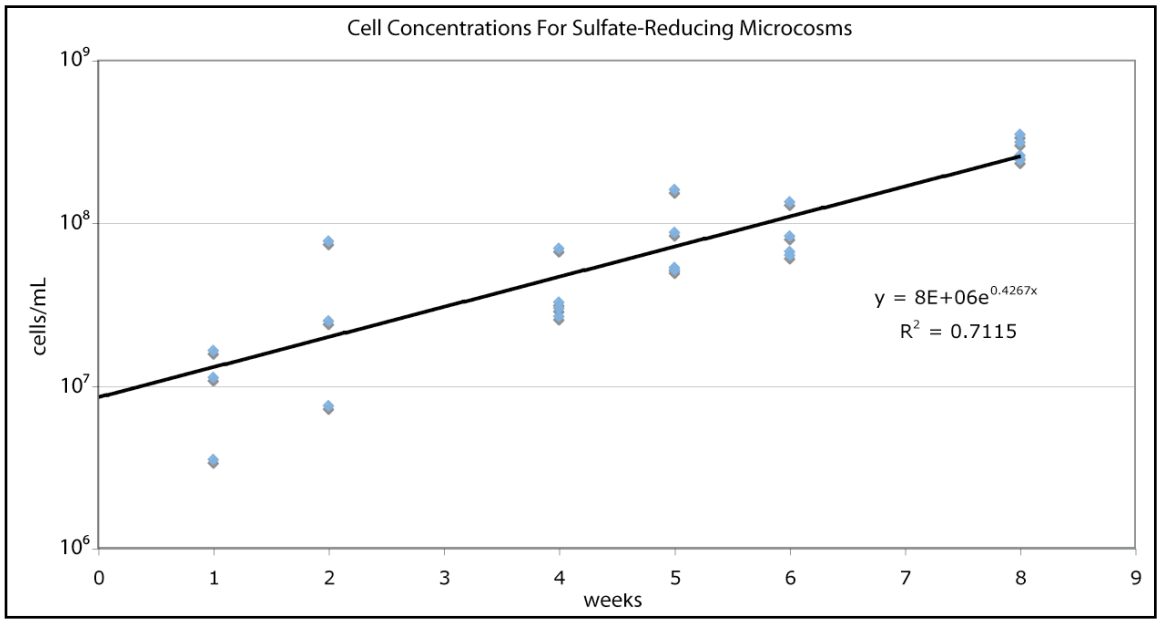

Figure 3.1. Cell concentrations obtained by Petroff-Hauser counts over time for the sulfate-reducer microcosms.

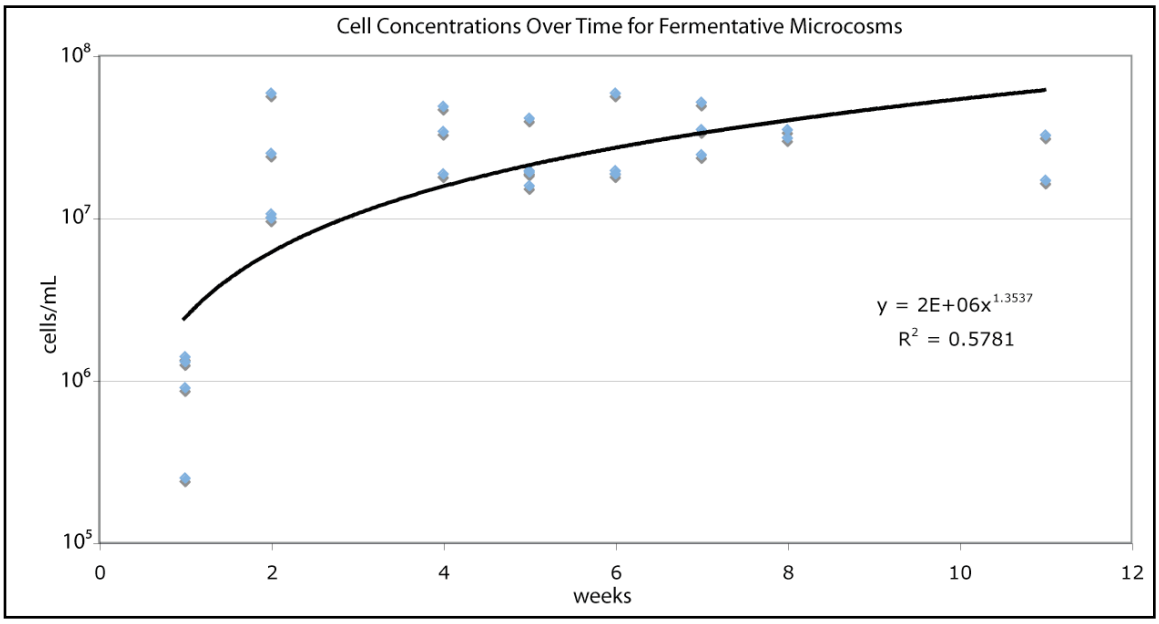

Figure 3.2. Cell concentrations over time obtained by the Petroff-Hauser counting method for the fermenter microcosms.

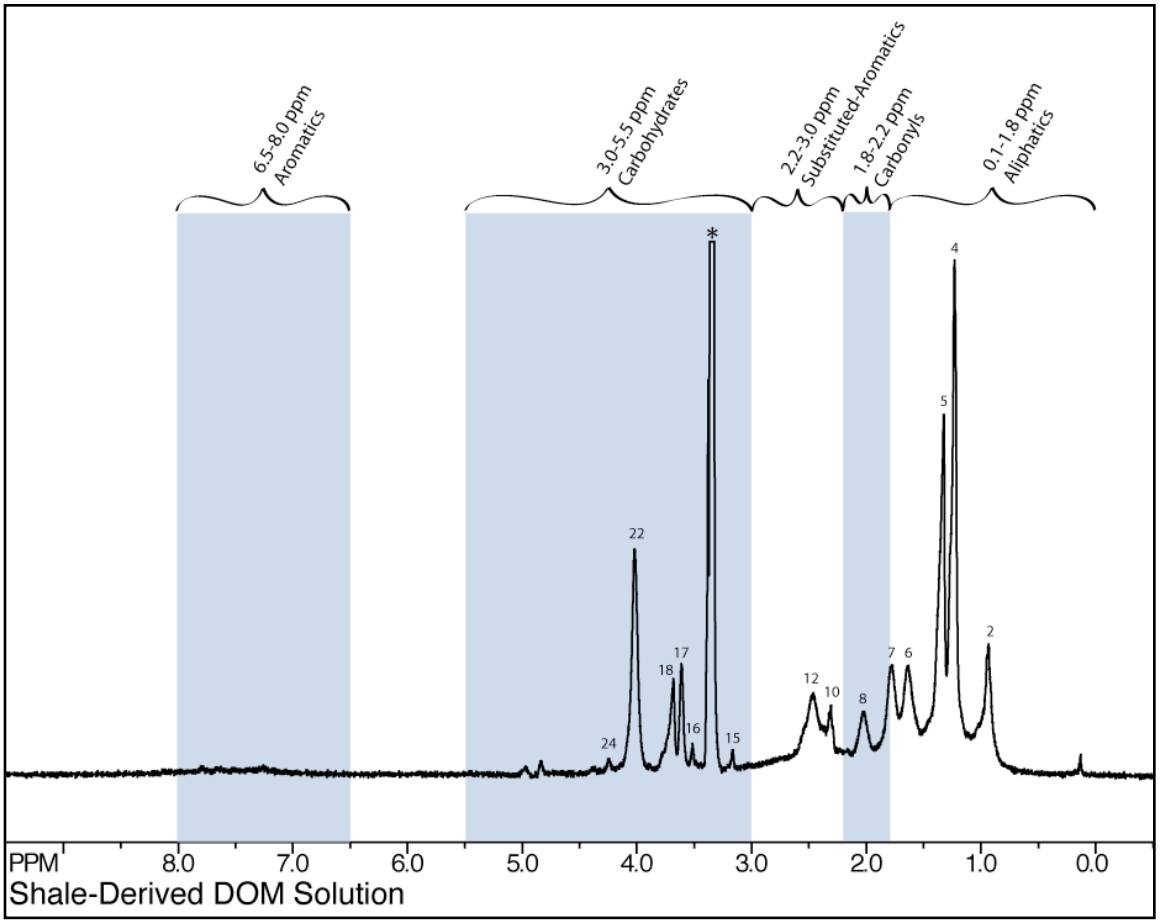

Figure 3.3. ^{1}H NMR spectrum for the Shale-Derived DOM Solution. ✻ denotes methanol solvent peak

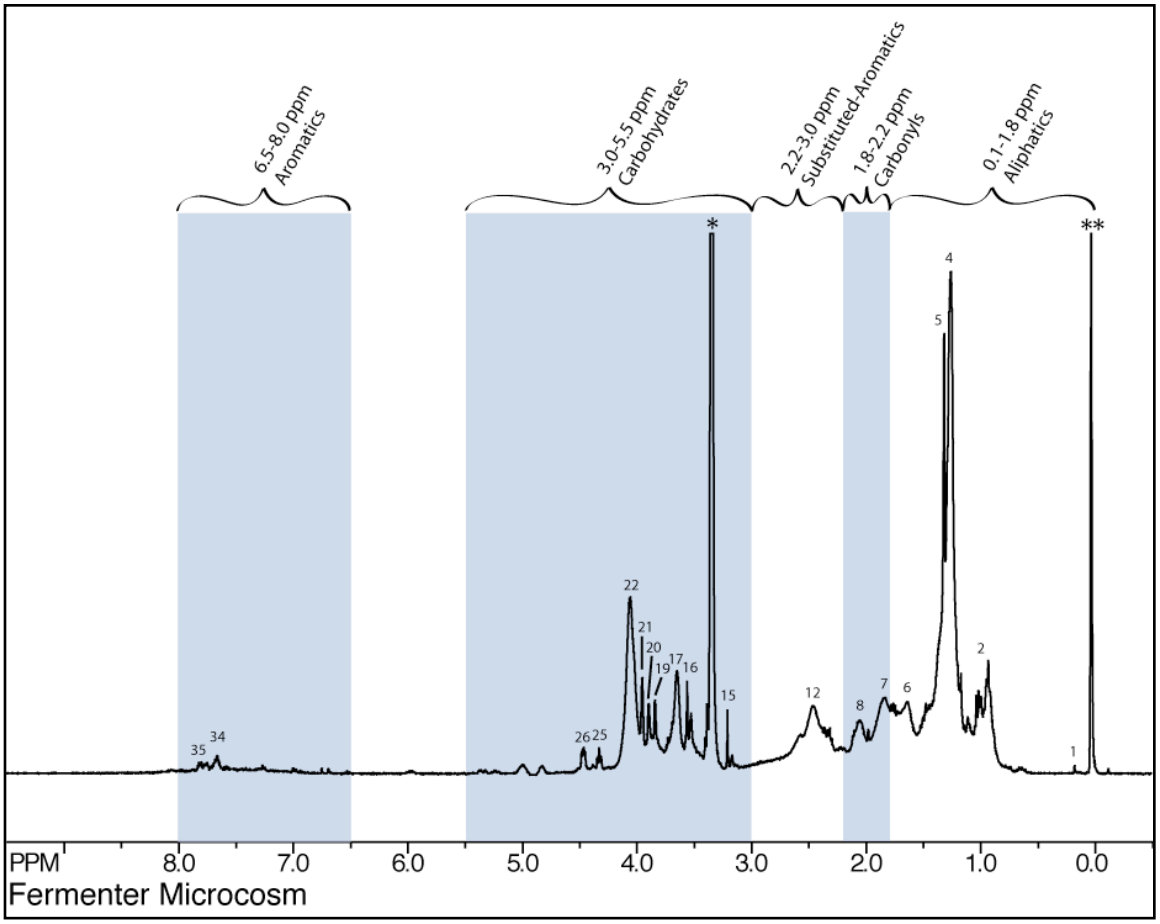

Figure 3.4. ^{1}H NMR spectrum for the Fermenter Microcosm. ✻ denotes methanol solvent peak ✻✻ denotes TMS peak

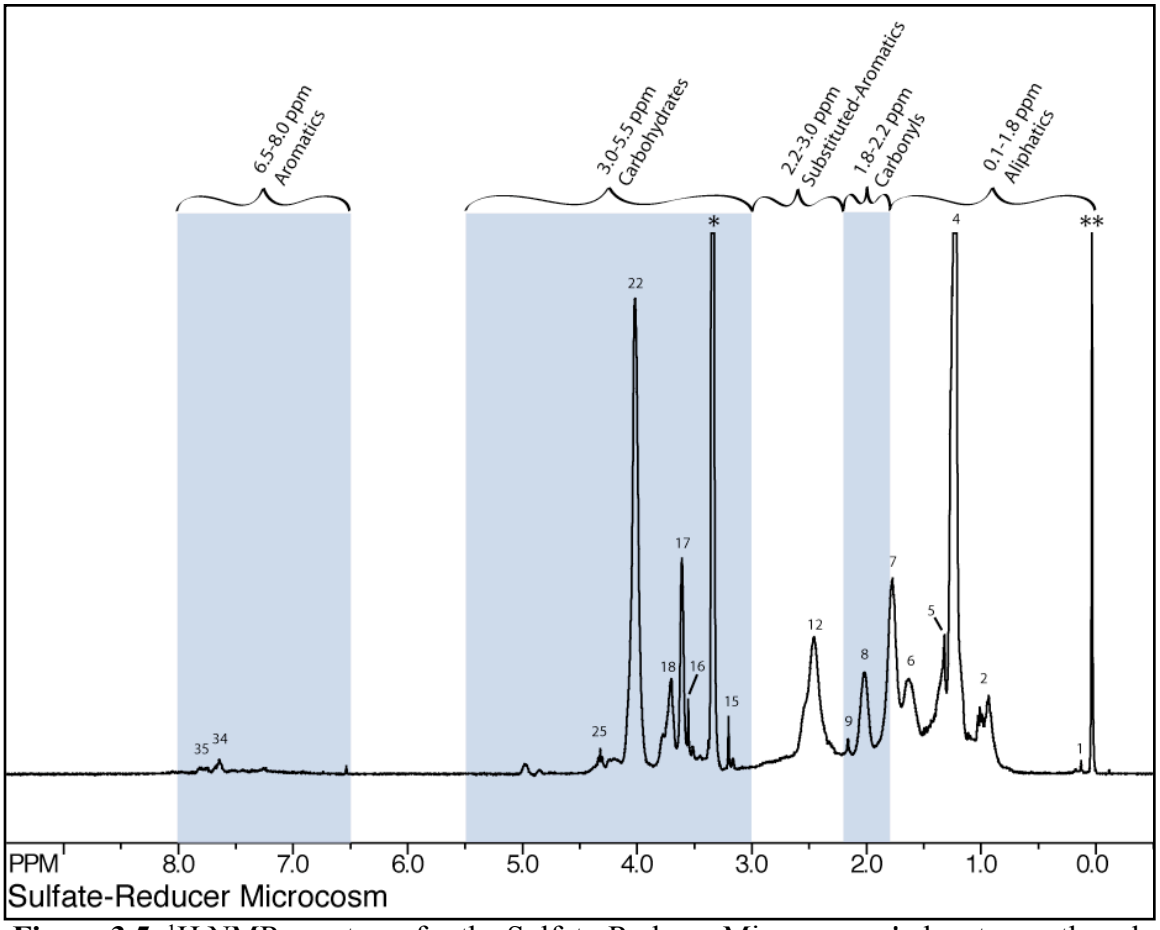

Figure 3.5. ^{1}H NMR spectrum for the Sulfate-Reducer Microcosm. ✻ denotes methanol solvent peak ✻✻ denotes TMS peak

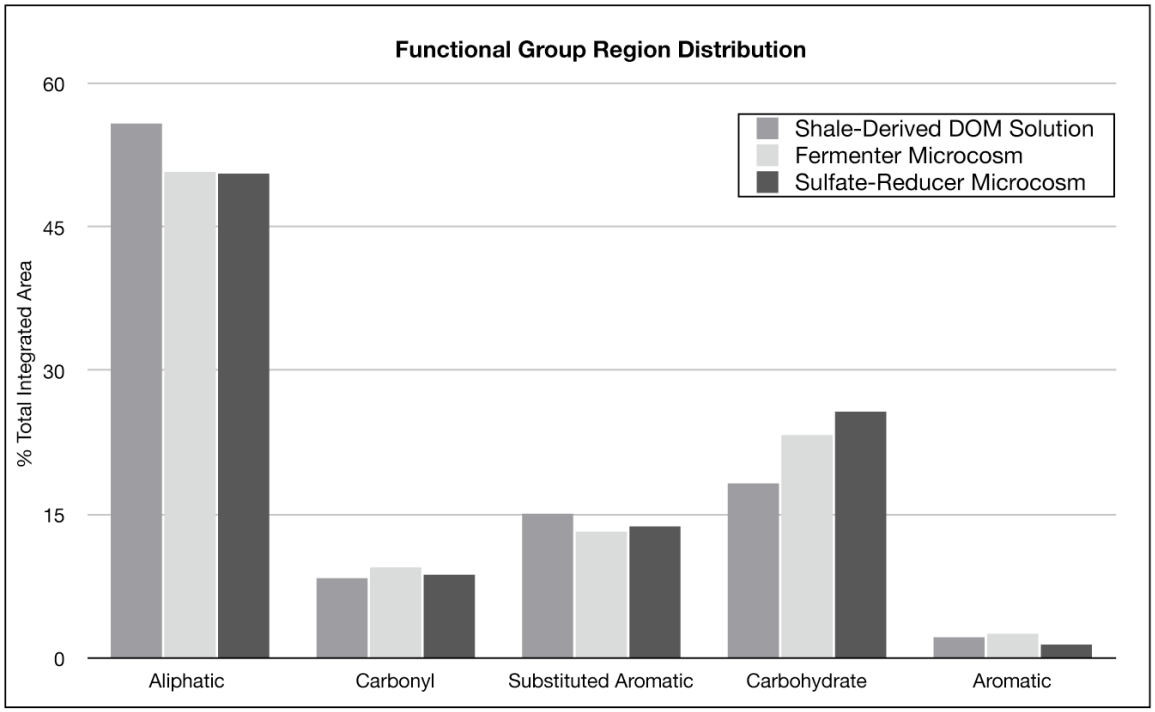

Figure 3.6. Distribution of functional group regions as percentage of total integrated area for the shale-derived DOM solution, fermenter microcosm, and sulfate-reducer microcosm. Fraction of total integrated area for each of the functional group regions: aliphatic (0.1 to 1.8 ppm), carbonyl (1.8 to 2.2 ppm), substituted aromatic (2.2 to 3.0 ppm), carbohydrate (3.0 to 4.5 ppm), and aromatic (6.5 to 8.0 ppm).

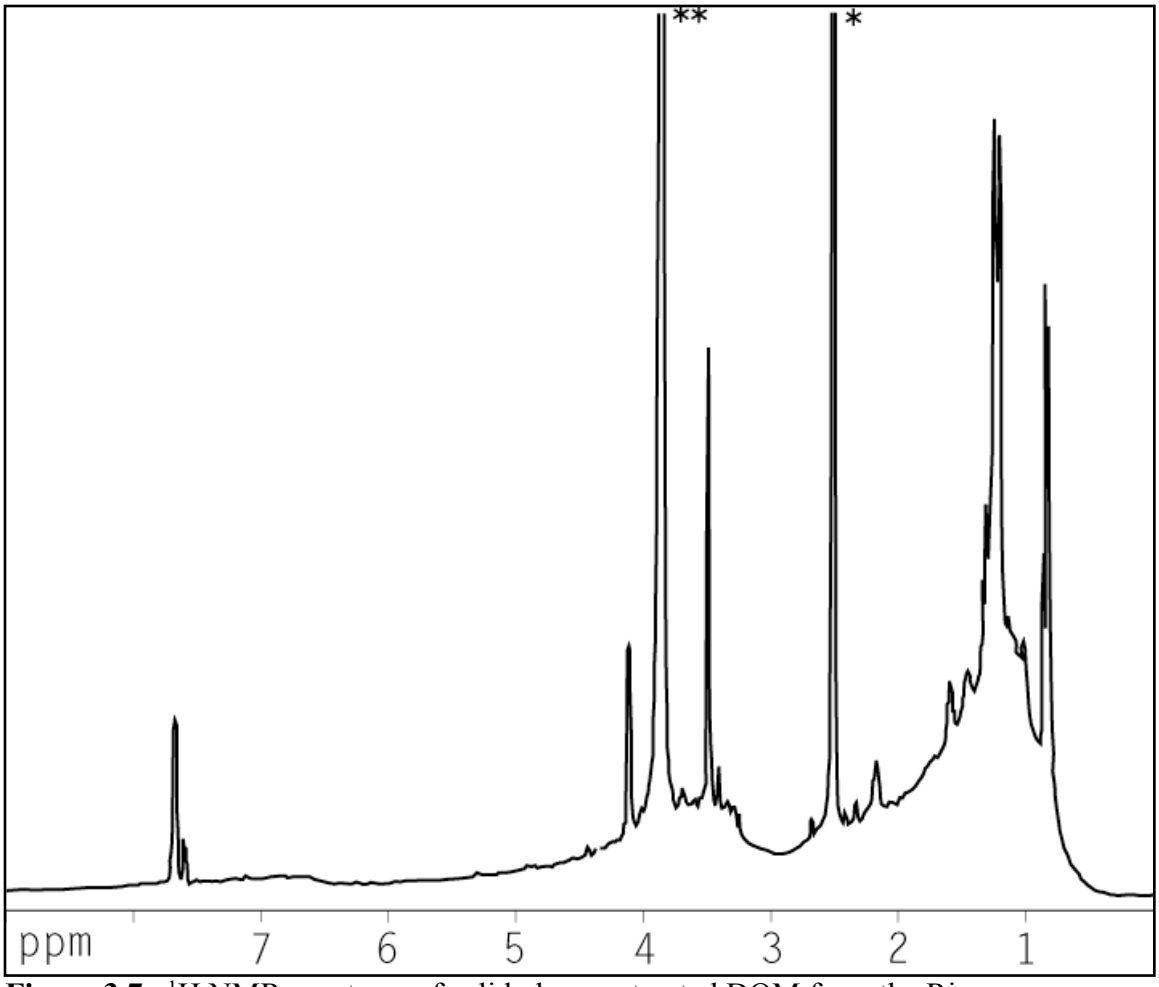

Figure 3.7. ^{1}H NMR spectrum of solid phase extracted DOM from the River Tagliamento, Italy. * DMSO-d_6 solvent peak, ** water peak. Modified from Kaiser et al., 2003.

CHAPTER 4

CHARACTERIZATION OF DISSOLVED ORGANIC MATTER IN ANTRIM SHALE FORMATION WATERS

4.1 Dissolved Organic Matter in the Formation Waters of the Antrim Shale

The composition and origin of Dissolved Organic Matter (DOM) in methanogenic environments in subsurface shales has not been closely investigated. We do not yet understand what controls the composition of DOM in such environments or how the composition of DOM influences or reflects the activity of microbiological communities involved in sedimentary basin methanogenesis. Characterization of the DOM in the formation water of such environments may provide insight into the types of organic compounds that the microbial communities present consume and produce. Currently, little is known about the shale-derived organic compounds that serve as the substrates for methanogenic communities in subsurface shales. Questions also remain as to what portion of the shale organic matter these microbial communities utilize as a source of energy and organic carbon. The experiments described in this chapter aimed to address these questions by characterizing the DOM in the formation water pumped from three wells in the Northern Producing Trend of the Antrim Shale in the Michigan Basin. The DOM from these wells was extracted using C_{18} Solid Phase Extraction and characterized with ^{1}H NMR analyses (see section 1.6.4 for a brief review of this analytical method). The results of these experiments demonstrated that the DOM composition in the formation waters produced from the three wells vary considerably. It is possible that these differences are the result of differences in the microbial consumption and

production of DOM in each of these wells. These differences may be caused by differences in the level of microbiological activity and variations in the types of microorganisms and metabolisms in each of the sampled wells.

The Antrim Shale in the Michigan Basin provides an excellent setting to investigate the controls on DOM composition in a subsurface methanogenic environment. Previous studies in the Northern Producing Trend of the Antrim Shale have produced geochemical and isotopic evidence that have conclusively shown that the methane produced from this region is dominantly microbial in origin (Martini et al. 1996; Martini et al. 1998; Martini et al. 2003). Further, a sharp gradient of increasing salinity from north to south in the Northern Producing Trend of this basin has been shown to have strong controls on microbiological activity and diversity (Waldron et al., 2007), and thus provides an excellent setting for the exploration of the relationships between microbiological activity and the DOM composition of formation waters.

The low concentrations of DOM typical in most natural waters makes direct analysis difficult, therefore researchers have used methods such as ultrafiltration, XAD extraction, and freeze drying to extract, desalt, and concentrate DOM for more effective analyses. C_{18} resin Solid Phase Extraction (SPE) improves on these methods with simpler procedures, better recovery efficiency, and improved desalting characteristics (Kim et al. 2003; Louchouarn et al. 2000). For these reasons, SPE is a method well suited for analysis of DOM in natural waters; however, some researchers have reported that SPE selects for more aliphatic DOM components than ultrafiltration or XAD extraction (Kaiser et al. 2003; Schwede-Thomas et al. 2005). This study used C_{18} Solid

Phase Extraction methods to isolate and concentrate Antrim formation water DOM for characterization analyses with ^{1}H NMR spectroscopy.

4.2 Methods

4.2.1 Collection of Antrim Formation Water

In the summer of 2005, water samples were collected from three wells (D2-26, B4-18, and B1-36) that produce gas and water from the Antrim Shale Formation in the Michigan Basin for characterization of the DOM present in the formation water. The three wells were chosen to cover the range of salinities in the formation water of the Northern Producing Trend. Well D2-26 is the northernmost well and produced low salinity water, B4-18 drew mid-range salinity water from the Antrim, and B1-36, the southernmost well, produced highly saline formation water. Table 4.1 provides salinity and depth information for each sampled well. One hundred liters of water was collected directly from each well in five 20 L HDPE carboys. The water was acidified immediately with 12.1 M HCl to pH≈2 (Fisher Scientific, Hampton, NH). The samples were then transported to the lab and filtered within seven days using 0.7 μm glass fiber filters (Whatman Inc., Florham Park, NJ).

4.2.2 Isolation and Concentration of Antrim Formation Water DOM

DOM from each of the well water samples was isolated and concentrated using Varian Mega Bond Elut SPE cartridges (Varian Inc., Palo Alto, CA). These cartridges are packed with 10 g of a hydrophobic C_{18} resin, which adsorbs the dissolved organic components present in the water. Fluid was drawn through the cartridge by placing a peristaltic pump downstream of the cartridge so that fluid would be drawn through the

cartridge by negative pressure. All tubing used was platinum silicon tubing (Cole Parmer, Vernon Hills, IL). The C_{18} resin was conditioned by elution of 100 mL of GC Resolve methanol (Fisher Scientific, Hampton, NH) followed by 2 L of nanopure water acidified to pH = 2. Twenty L of pH=2 sample was then passed through the cartridge. A final rise of 1 L of acidified nanopure water was run through the cartridge to wash out any salts that had built up on the resin. The resin was eluted with 50 mL GC resolve methanol (Fisher Scientific, Hampton, NH). This process was repeated until all 100 L collected from each well was extracted. Cartridges were reused for water from the same well, but no cartridges were used for more than 60 L or sample.

The eluted methanol from the Solid Phase Extraction (approximately 250 mL from each well sample) was evaporated using rotary evaporation to evaporate the bulk of the methanol. The remaining methanol was then evaporated using N_2 air-stream evaporation into a heated (to approximately 40˚C), pre-weighed, combusted glass vial. The dried extract then refrigerated at 4°C.

The mass of DOM extract recovered by the SPE procedure was determined by weighing a glass vial before and after the DOM extract was dried from the eluted methanol in the vial. 15.6 mg of dried extract was recovered from well D2-26, and 16.5 mg of dried DOM extract was recovered from well B4-18. The mass of DOM extract recovered from well B1-36 could not be determined because the balance used to pre-weigh the glass vial for this sample was re-calibrated before the vial could be re-weighed with the extract.

4.2.3 Dissolved Organic Carbon Analyses

Dissolved Organic Carbon (DOC) concentrations were determined by filtering a water sample through a 0.2 μm membrane filter and measuring the Total Organic Carbon (TOC) of the filtrate using a Shimadzu TOC-V Total Organic Carbon Analyzer. Calibration standards for the instrument were made by dissolving sodium acetate (Sigma-Aldrich, St. Louis, MO) into nanopure water. A calibration curve was created with 0, 1, 2, 5, 10, and 20 mg/L standards.

4.2.4 ^{1}H NMR Analyses

Characterization of DOM extracted from each of the three wells were performed using ^{1}H NMR. The dried DOM extract of each sample was re-dissolved in 1 ml of CD_3OD methanol (Sigma-Aldrich, St. Louis, MO). The solution was then centrifuged at 1,000 rpm for 10 minutes to remove undissolved particulates (most likely C_{18} resin particles) before it was dispensed into a NMR sample tube. NMR data were acquired using a Bruker Avance 400 MHz NMR spectrometer. ^{1}H NMR spectra were conducted using pre-saturation water suppression, a 2 s recycle delay time and 1 Hz line broadening. 600 scans were performed for each sample.

Integrations of the spectra were manually calculated using the software program Spinworks Version 2.5.5 (Marat, 2006) by establishing the area from 0.1 to 9.0 ppm (excluding the large methanol peak) and then calculating the area of each functional group region as a percentage of the total area. Functional group regions were defined as aliphatic (0.1 to 1.8 ppm), carbonyl (1.8 ppm to 2.2 ppm), substituted-aromatic (2.2 ppm to 3.0 ppm), carbohydrate (3.0 ppm to 5.5 ppm), and aromatic (6.5 ppm to 8.0 ppm).

4.3 Results

4.3.1 Dissolved Organic Carbon Analyses

Table 4.1 shows the DOC concentrations in each of the three wells. Well B4-18 had the lowest DOC concentration at 4.3 mg/L. Well D2-26 had the highest concentration of DOC at 12.75 mg/L. B1-36 had a DOC concentration of 7.3 mg/L. From these data and the mass of the recovered DOM extract from the SPE procedure, a recovery efficiency of DOM can be calculated. DOM was recovered from well D2-26 with a 1.2% efficiency and from well B4-18 with a 3.8% efficiency.

4.3.2 Characterization of Antrim DOM

Figures 4.1, 4.2, and 4.3 show the ^{1}H NMR spectra for each of the three well samples. ^{1}H NMR spectra of all three Antrim Well water SPE extracts have strong aliphatic (0 to 1.8 ppm) carbohydrate (3.42 to 4.5 ppm), and aromatic (6.5 to 8.5 ppm) resonances in all three wells. The aliphatic resonances are the most dominant feature of the spectra. Figure 4.4 and Table 4.2 show the relative percentage of area occupied by each functional group region as determined by integration of the spectral data. Aliphatic resonances account for roughly 50 to 70% of the total integrated area in the three wells. Carbohydrates are the next most dominant resonances, accounting for 10 to 25% of the total area. Aromatic resonances are also prominent features of the spectra, accounting for 8 to 20% of the area. Carbonyl and substituted-aromatic resonances are less significant parts of the spectra, comprising between 2 to 6% of the total integrated area. Well B1-36 has significantly fewer aliphatic resonances than either B4-18 and D2-26, but significantly greater contributions from carbohydrates and aromatics than the other wells.

Aliphatic Region

The aliphatic regions of all three spectra are dominated by peaks labeled 1-7 in figures 4.1, 4.2, and 4.3. Peak 1 is characteristic of protons in methane. Peaks 2 and 3 are typical resonances for protons in a terminal methyl group. Peaks 4-7 are indicative of protons in aliphatic mid-chain methylene groups. Peak 1 is only present in the D2-26 spectrum and is very sharp and well defined. Peaks 2,5,6, and 7 are present in all three spectra. Peaks 2 and 5 are composed of several peaks in all three spectra and are the most dominant peaks in B4-18 and D2-26, but in B1-36 peaks 2 and 5 are significantly less intense than peak 6. Table 4.3 shows the area that each peak in the aliphatic region occupies relative to the total aliphatic area for each sample. In both B4-18 and D2-26 the area of peaks 2 and 5 account for approximately 30% and 57% of the total area in the aliphatic region, while peaks 6 and 7 each account for less than 7% of the total aliphatic area. In contrast, peaks 2, 5, and 6 in the spectrum of B1-36 each account for approximately 30% of the total aliphatic area.

Carbonyl and Substituted-Aromatic Region

The carbonyl and substituted-aromatic regions of all three spectra have few major peaks compared to the other regions. Resonances in these regions are indicative of ^{1}H nuclei bonded to protons associated with carbonyl groups, protons bonded to carbons alpha to carboxyl groups, and protons bonded to carbons alpha to aromatic functional groups. The only notable resonance is peak 11, which appears in all three spectra with similar intensity.

Carbohydrate Region

Peaks 15-27 are in the carbohydrate region. Resonances in this region are characteristic of ^{1}H nuclei associated with alcohol functional groups and carbohydrates. D2-26, the low salinity well, and B1-36, the high salinity well, both have a large number of intense resonances in the carbohydrate region, while well B4-18, the mid-range salinity well, has notably fewer resonances in this region. The peak at 3.4 ppm in all spectra is a methanol solvent peak. Both wells D2-26 and B1-36 have strong resonances centered about 3.75 ppm (peaks 16-20). In D2-26 these resonances are clearly resolvable as distinct peaks but in B1-36 the peaks are much more difficult to distinguish from each other. Peaks are also present at positions 16 and 18 in B4-10, but these resonances are not as intense as those at the same frequency in D2-26 and B1-36. A very intense set of twin peaks are present at position 22 in B1-36 which are not found in either of the other samples. A large peak is also present at position 24 in all three spectra, although it is more intense in B4-18 and D2-26 than in B1-36. Peak 17, a small, sharp peak is present in the spectrum from well D2-26, but it is not present in either of the other two spectra.

Aromatic Region

Peaks 28-35 are in the aromatic region and are diagnostic resonances for aromatic protons. The aromatic regions in the three spectra are dominated by resonances at positions 28 and 30, 29 and 31, and 34 and 35. These resonances appear to be paired, as these sets of resonances occur together and at roughly the same intensity. Peaks at positions 32, 34, and 35 appear in all three spectra. Peaks at positions 28, 30, and 33 are present in D2-26 and B1-36, and peaks at positions 29 and 31 are present in B4-18 and

B1-36. Table 4.4 shows the relative area that each peak in the aromatic region occupies as a percentage of the total aromatic area. In B4-18 peaks at 34 and 35 are the largest in the aromatic region and account for 57% of the total aromatic area. In D2-26 the peaks at positions 34 and 35 account for 32% of the total aromatic area and peaks at positions 28 and 30 account for 50% of the total aromatic area. B1-36 has the greatest diversity of aromatic resonances, with peaks at all positions between 28 and 35. The peaks at positions 29 and 31 are by far the largest in the aromatic region of B1-36, accounting for 82% of the total aromatic area.

4.4 Discussion

The NMR spectra of the three Antrim formation water DOM samples show that there are detectable differences in the composition of the DOM in the formation waters collected from each of the three Antrim wells. The TOC concentrations in each of the well waters are also distributed across a wide range, suggesting significant differences in the quantity of DOM present in the formation waters.

The Antrim DOM spectra have some features in common with the proton NMR spectra of other natural waters published by other workers. The proton NMR spectrum of solid-phase extracted DOM from the River Tagliamento, Italy published by Kaiser et al. (2003) is dominated by resonances in the aliphatic and carbohydrate regions with smaller resonances in the aromatic, carbonyl, and substituted aromatic regions (Figure 3.7). The overall shape of the River Tagliamento spectrum is roughly similar to that of the Antrim formation water spectra, indicating that the DOM in these waters are similarly composed of mixtures of compounds with aliphatic and carbohydrate functional groups. Kaiser et

al. (2003) suggested that the DOM from the River Tagliamento was dominated by aliphatic esters derived from bacteria and microalgae as well as by terrestrial plant derived sugars and fatty acids. While it is possible that some component of the Antrim formation water DOM is derived from microbial components, it is unlikely plant derived sugars and fatty acids are present in the subsurface.

The Antrim DOM spectra contrast sharply with the proton NMR spectra of DOM from several fresh and marine waters (Repeta et al., 2002). Figure 4.5 shows four spectra obtained by Repeta et al. from Nobska Pond (Woods Hole, MA), the Delaware River near Philadephia, PA, West Neck Bay (Long Island, NY), and Woods Hole Seawater (Woods Hole, MA). All four of these spectra have a significantly greater proportion of resonances in the carbohydrate region than any of the Antrim DOM spectra. Repeta et al. (2002) reported that the DOM from these diverse samples was composed of a surprisingly compositionally uniform distribution of monsaccarides, acetate, and lipids. While it is possible that the Antrim DOM may contain similar compounds, as with the DOM from the River Tagliamento, the widely different environments from which these compounds are derived makes direct comparisons difficult.

Differences in the aromatic regions of the three spectra may be related to differences in geochemistry and microbiology. The intensities of the resonances at positions 34 and 35 decrease with increasing salinity in the three wells. Table 4.1 shows geochemical, cell count, and DNA extraction data collected by Waldron et al. (2007). Well D2-26 has the least saline waters of the three wells, and has the most intense peaks at positions 34 and 35. Conversely, well B1-36 was the most saline of the wells sampled,

and the peaks at positions 34 and 35 are the least intense of the sampled wells. Peaks at positions 6, 29, and 31 in sample B1-36 are far more intense than any other peaks (excluding the solvent peak) in any of the spectra. These peaks may be resonances from some contaminant compound, such as a solvent or other industrial organic compound introduced into the formation water through the well. Figure 4.6 and Table 4.5 show recalculated integrations for the three samples that exclude the suspected contaminant peaks. If the possible contaminant peaks in sample B1-36, 29 and 31, are excluded from integration calculations, then the total area of resonances in the aromatic region decrease with increasing salinity in each of the three samples. Cell counts from the three wells also decrease with increasing salinity in the three sampled wells (Waldron et al., 2007). These data may indicate that aromatic compounds may be produced or liberated from the shale kerogen by biological activity. The data from the ^{1}H NMR spectra alone are not sufficient to determine what types of aromatic compounds are present in the sampled Antrim wells. Aromatic compounds commonly found in other formation waters include phenols, benzenes, and cyclohexanones (Lundegard and Kharaka, 1994). Aromatic hydrocarbons have been shown to be microbiologically produced as degradation products in laboratory experiments, including under methanogenic conditions (Widdel and Rabus, 2001; Harder and Foss, 1999).

Aliphatic resonances also decrease with decreasing cell counts and increasing salinity(Figure 4.4 and Table 4.4). This trend is more pronounced when when the possible contaminant peak 6 in sample B1-36 is removed from integration calculations, as shown in Figure 4.6. This may indicate that aliphatic molecules are liberated from the

shale kerogen by microbiological activity, or that aliphatic compounds are produced by microbiological products.

Carbohydrate resonances do not follow the same salinity and cell count trends as the aliphatic and aromatic resonances. While the total area of carbohydrate resonances decreases from well D2-26 to well B4-18, they increase by a factor greater than two in well B1-36. The number of distinct resonances is greatest in well D2-26 and is lowest in well B4-18. Despite the lower cell counts in well B4-18 relative to D2-26, Waldron et al. (2007) extracted significantly more DNA from well B4-18 than D2-26. This may be an indication of a greater diversity of microbiological species and metabolic roles in well B4-18 relative to D2-26. This greater microbiological diversity may result in a more complete consumption of carbohydrate compounds present in the formation water of well B4-18. Waldron et al. (2007) did not recover any DNA from well B1-36 and cell counts were below detectable limits. In light of the high diversity and total area of carbohydrate resonances in well B1-36, this data may suggest that either shale-derived carbohydrates, or biologically produced carbohydrates, are not efficiently re-consumed by the extant microbial community in that well.

The relative lack of resonances in the substituted-aromatic region is notable in that it may exclude or limit a significant contribution of carboxylic acids to the total composition of the Antrim DOM. The protons bonded to the carbon alpha to the carboxyl group resonate in the substituted-aromatic region, thus the lack of significant resonances in this region rules out a significant presence of carboxylic acids in the Antrim DOM. Organic acids have been identified as important components in the overall

composition of DOM in subsurface waters, and moncarboxylic and dicarboxylic acids make up the majority of these acids (Lundegard and Kharaka, 1994). These acids may be generated directly from shale kerogen. Laboratory experiments conducted by Lewan and Fisher (1994) have suggested that organic acids are retained to kerogen macromolecules by weak hydrogen bonding during the early diagenesis of the sediment. These experiments have shown that C_2-C_5 moncarboxylic acids are the dominant acids generated. These acids are often referred to as Volatile Fatty Acids (VFAs), and they are likely substrates for hydrolytic and fermentative bacteria (Madigan et al. 2003), which can provide H_2 and other substrates for methanogens and are thus a potentially vital component in the trophic chain leading to methanogenesis in a black shale. The absence of significant resonances from carboxylic acids may be due to consumption of these compounds by these types of bacteria. An alternative explanation for the absence of a significant contribution of carboxylic acids to the composition of the Antrim DOM may be volatilization of the acids during the sampling procedure. Many of the carboxylic acids can be volatile at low pH conditions, and thus the acidification of the samples to approximately pH=2 may have allowed the acids to volatilize from the samples (Lundegard and Kharaka, 1993). However, acetate, the most common moncarboxylic acid in subsurface formation waters (Lundegard and Kharaka, 1993), was also not detected in these wells by Waldron et al. (2007).

The absence of major resonances in the substituted-aromatic region in the Antrim formation waters also rules out a major contribution of substituted succinates to the overall composition of the DOM. The protons bonded to the carbons alpha to the

carboxyl groups in a substituted succinate also produce resonances in the substituted-aromatic region (SDBSWeb : http://riodb01.ibase.aist.go.jp/sdbs/ (National Institute of Advanced Industrial Science and Technology, 2007)). The absence of major resonances in this region of the Antrim formation water DOM spectra indicate that these metabolites are not major components of the DOM. As the substituted succinate metabolism requires terminal electron acceptors (Wilkes et al., 2000; Elshahed et al., 2001; Widdel and Rabus, 2001; Aitken et al., 2004), and sulfate and other common terminal electron acceptors are absent in most Antrim formation waters (McIntosh and Walter, 2005), it it not surprising that these metabolites are not found in the Antrim.

The Antrim formation water DOM concentrations do not follow any clear trends. The high cell and DOM concentrations in well D2-26 may indicate that microbial activity is responsible for liberating more DOM from shale, or that the higher concentration of DOM at this site supports a denser microbial community. However, B4-18, which is also very biologically active, has a much lower DOM concentration than B1-36, which does not have detectable indicators of biological activity. Microbiological activity may also have the effect of more efficiently consuming and remineralizing DOM. It cannot be assumed that the TOC of the shale are homogenous at all three well locations, or that DOM produced from the shale would be homogenous at all the sites. The DOM concentration in each particular well is likely controlled by the interaction of multiple factors including the type and concentration of organic matter present in the local shale, the liberation of DOM from the kerogen by microbiological activity, and the consumption of DOM by the organisms present.

4.5 Conclusions

The intensity of resonances in the aliphatic and aromatic functional group regions increase with decreasing formation water salinity. Waldron et al. (2007) have shown that salinity controls microbiological substrate utilization in the same wells in the Antrim, and cell counts and extractable DNA follow a similar inverse relationship with salinity as aliphatic and aromatic functional group intensities. These data show that salinity, and microbial activity in turn, may control the microbial generation or the liberation of aliphatic and aromatic compounds from shale kerogen. Carbohydrate resonances may also reflect microbiological activity in the sampled wells. The well with the least evidence of biological activity, B1-36, had far greater diversity and intensity of carbohydrate resonances that the more biologically active wells, possibly as a result of more complete re-consumption of carbohydrates in the more active wells. Finally, the absence of significant resonances in the carbonyl and substituted aromatic regions of the all three DOM spectra indicate a lack of carboxylic acids in the sampled formation waters, which are typically common in organic rich subsurface formation waters. This may also reflect consumption of these compounds by the microbiological community present in these wells, and may reflect one mechanism by which shale organic matter enters the trophic chain leading to methanogenesis in the Antrim.

Table 4.1. Geochemical, dissolved organic carbon, cell count, and DNA data for each of the sampled wells.

Well	Depth (m)[a]	Alkalinity (meq liter^{-1})	[Cl-] (mM)	[Na+] (mM)	Dissolved Organic Carbon (mg/L)	Total Cell Counts (10^3 cells mL^{-1})	DNA (ng mL^{-1})
D2-26	302	32.1	8.4	60	12.8	6.2	0.004
B4-18	296	34.1	200	163	4.3	5.1	0.020
B1-36	520	6.3	3490	1363	7.3	BDL[b]	BDL

[a] Depth below land surface

[b] Below detectable limits

Table 4.2. Functional group distribution of Antrim well DOM as percentage of total integrated area.

Functional Group Region	ppm	D2-26	B4-18	B1-36
Aliphatic	0.1-1.8	70.9	69.4	48.8
Carbonyl	1.8-2.2	3.0	5.6	3.3
Substituted Aromatic	2.2-3.0	3.3	6.2	2.4
Carbohydrate	3.0-5.5	12.8	12.4	25.8
Aromatic	6.5-8.0	10.0	6.5	19.7

Table 4.3. Relative aliphatic peak areas as a percentage of total aliphatic area for D2-26, B4-18, and B1-36.

	Percent of Total Integrated Aliphatic Area		
Peak Number	D2-26	B4-18	B1-36
1	2.4	NP[a]	NP
2	30.5	29.6	30.1
5	56.2	57.3	33.7
6	6.5	7.8	31.9
7	4.3	5.3	4.4

[a] NP, no peak at this position.

Table 4.4. Relative aromatic peak areas as a percentage of total aromatic area for D2-26, B4-18, and B1-36.

	Percent of Total Integrated Aromatic Area		
Peak Number	D2-26	B4-18	B1-36
28	25.1	NP[a]	1.0
29	0.0	4.4	39.6
30	24.9	NP	2.8
31	NP	4.8	42.9
32	5.6	14.8	NP
33	7.2	NP	3.5
34	14.8	23.5	2.9
35	17.3	34.2	4.2

[a] NP, no peak at this position.

Table 4.5. Functional group distribution of Antrim formation water DOM as percentage of total integrated area (Recalculated to exclude possible contaminant peaks).

Functional Group Region	ppm	D2-26	B4-18	B1-36
Aliphatic	0.1-1.8	70.9	69.4	48.8
Carbonyl	1.8-2.2	3.0	5.6	4.9
Substituted Aromatic	2.2-3.0	3.3	6.2	3.5
Carbohydrate	3.0-5.5	12.8	12.4	37.8
Aromatic	6.5-8.0	10.0	6.5	5.1

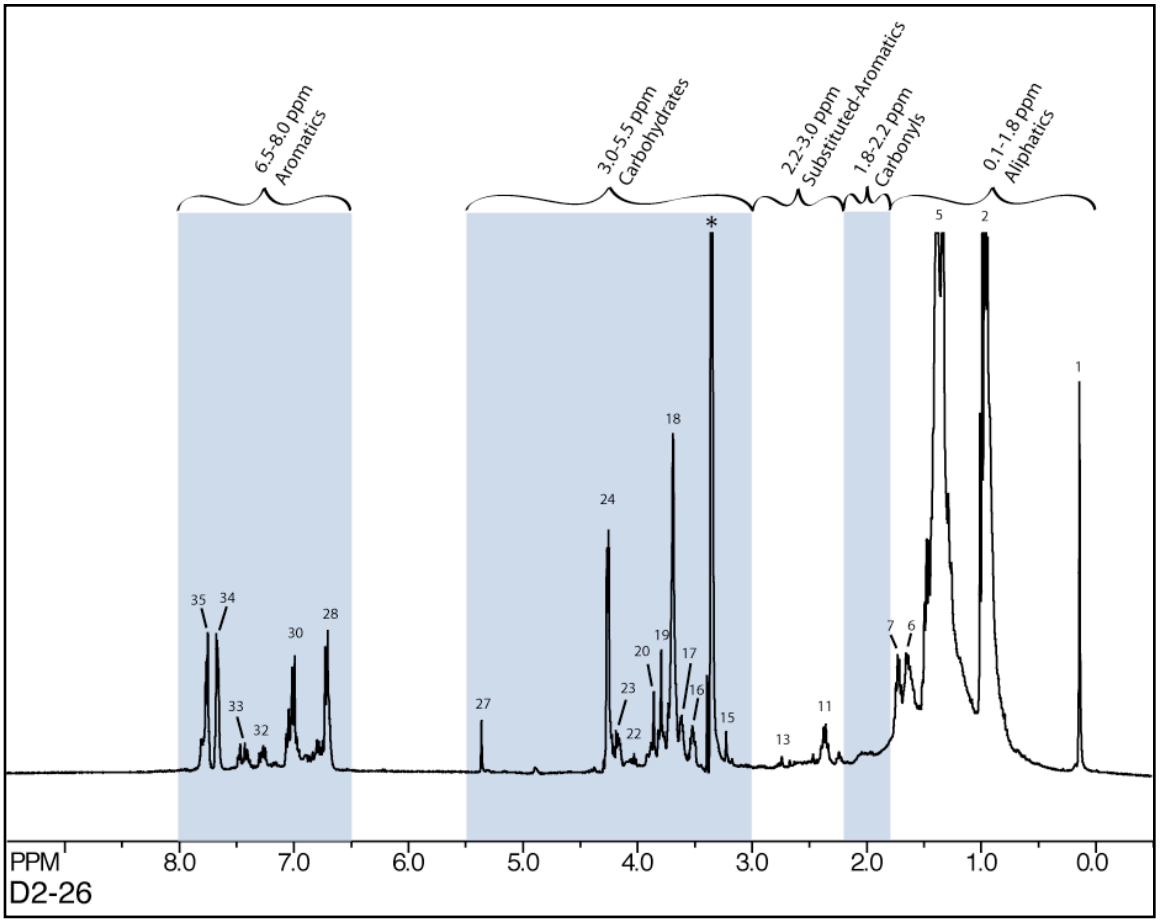

Figure 4.1. ^{1}H NMR spectrum of DOM from well D2-26. * denotes methanol solvent peak

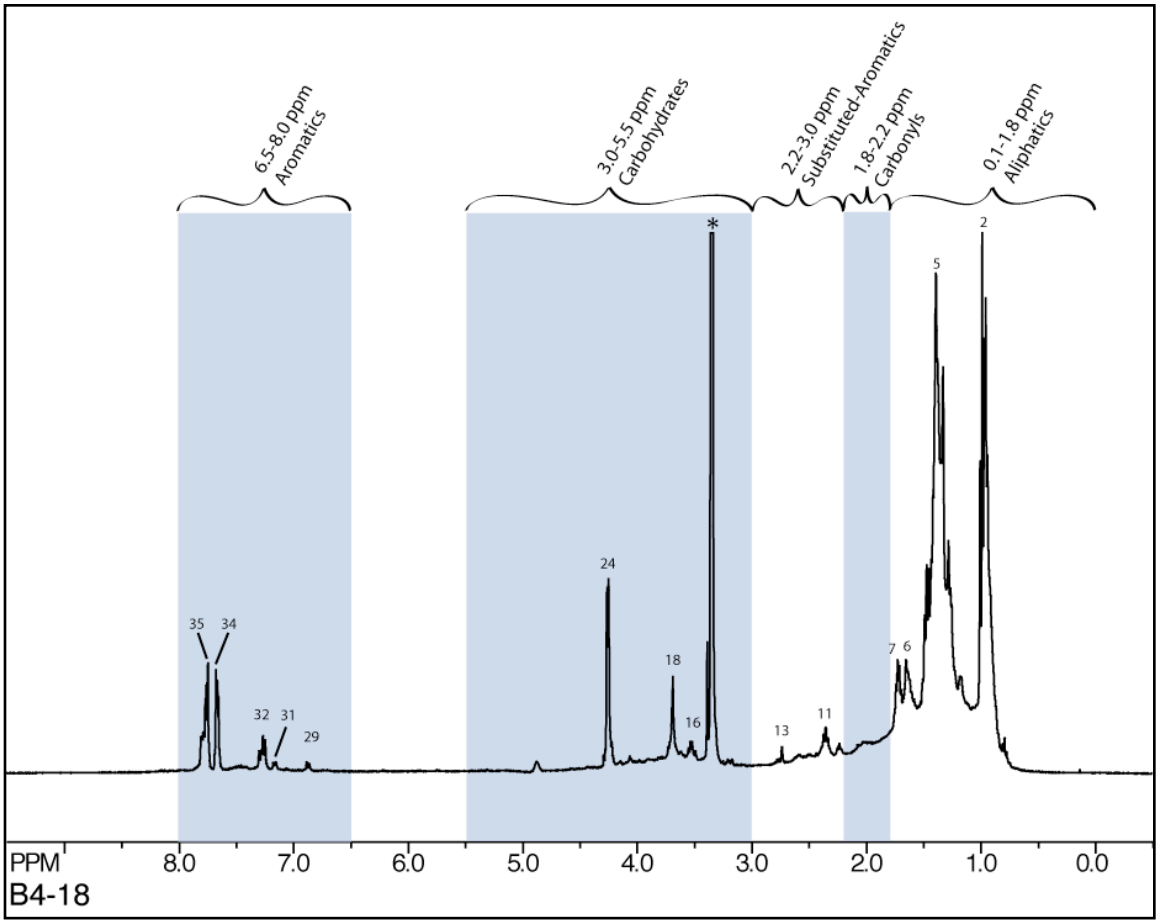

Figure 4.2. ^{1}H NMR spectrum of DOM from well B4-18. * denotes methanol solvent peak

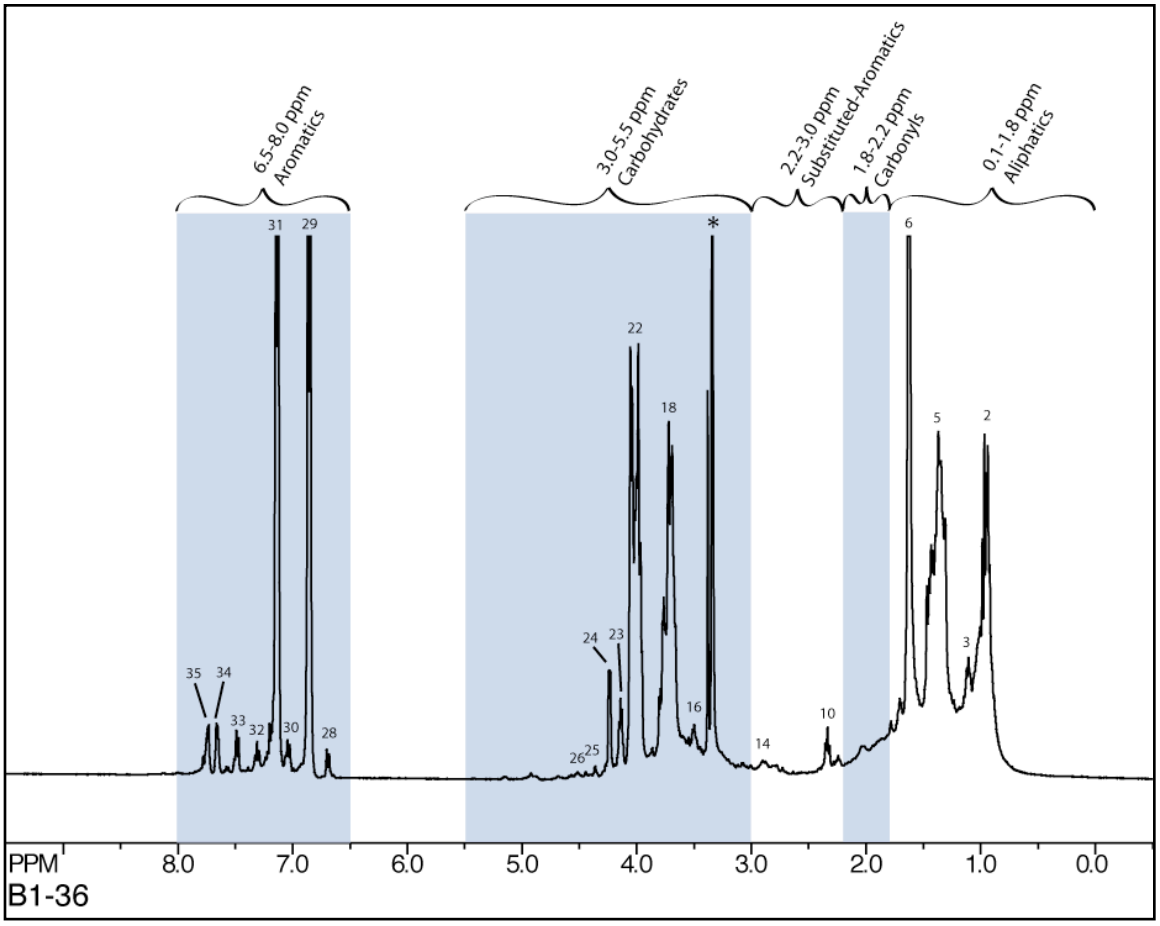

Figure 4.3. ^{1}H NMR spectrum of DOM from well B1-36. * denotes methanol solvent peak

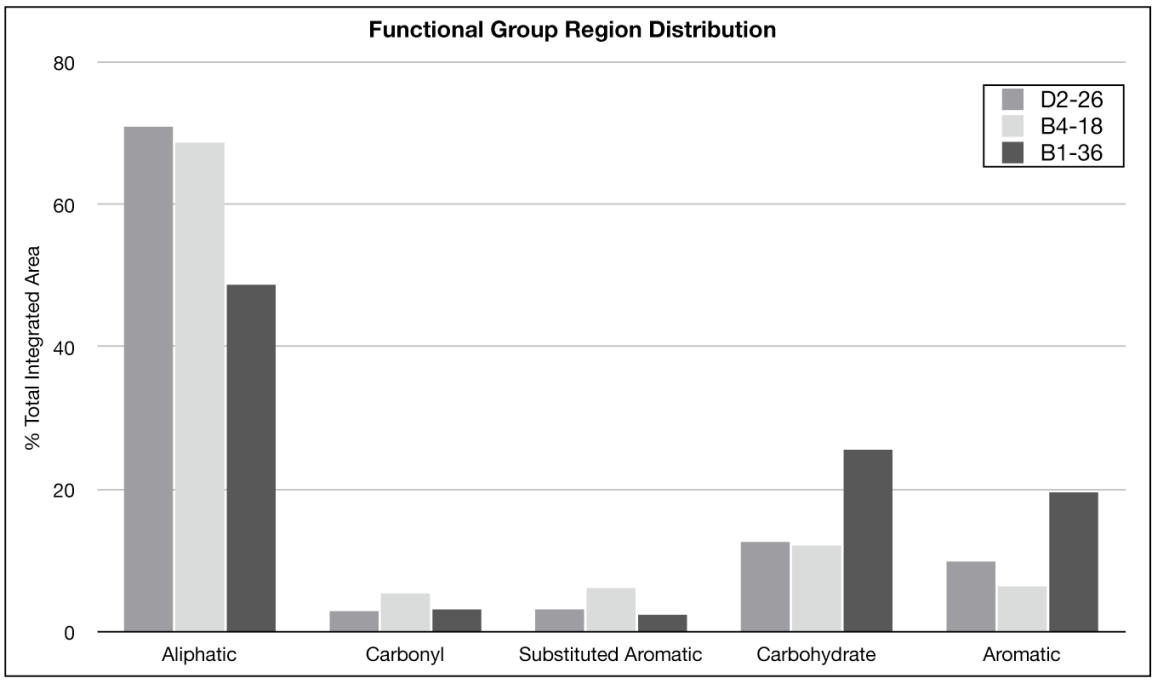

Figure 4.4. Bar graph displaying functional group distribution of each Antrim well as percentage of total integrated area.

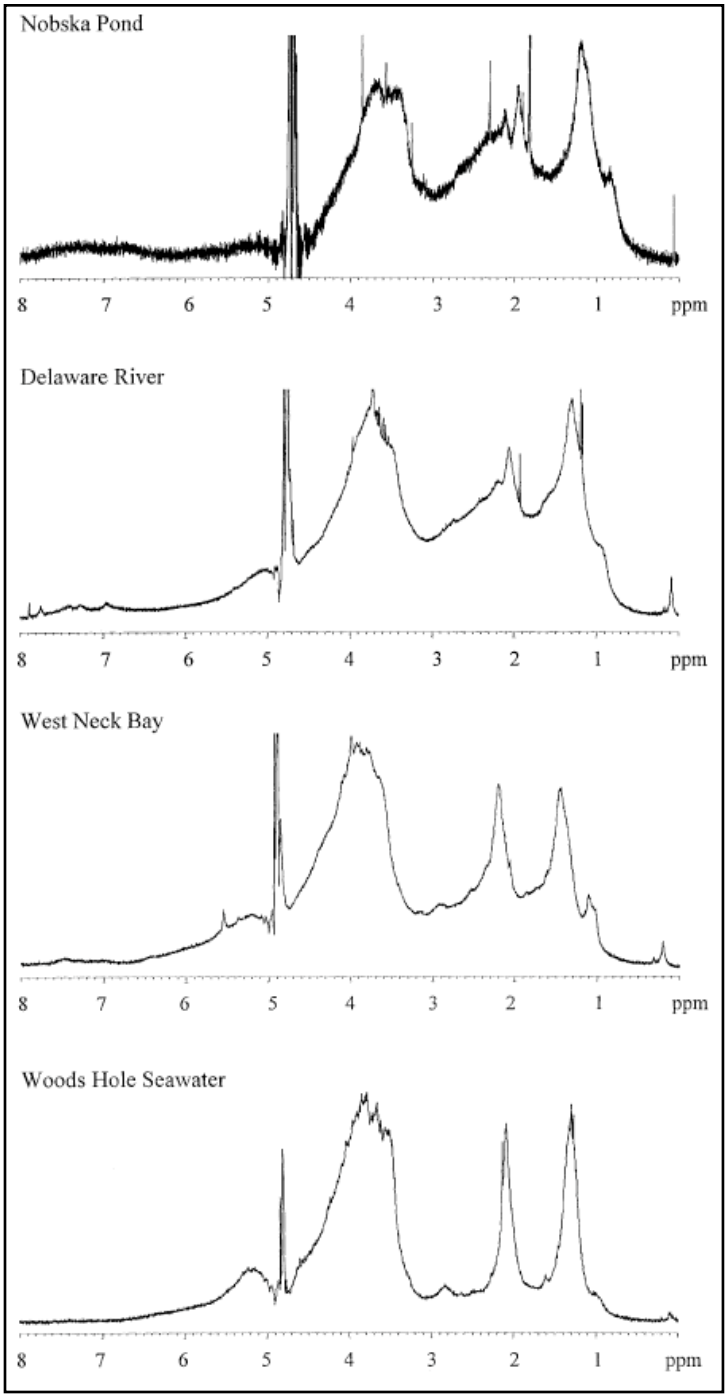

Figure 4.5. ^{1}H NMR spectra for DOM extracted by ultrafiltration from Nobska Pond, Delaware River, West Neck Bay, and Woods Hole Seawater. From Repeta et al., 2002.

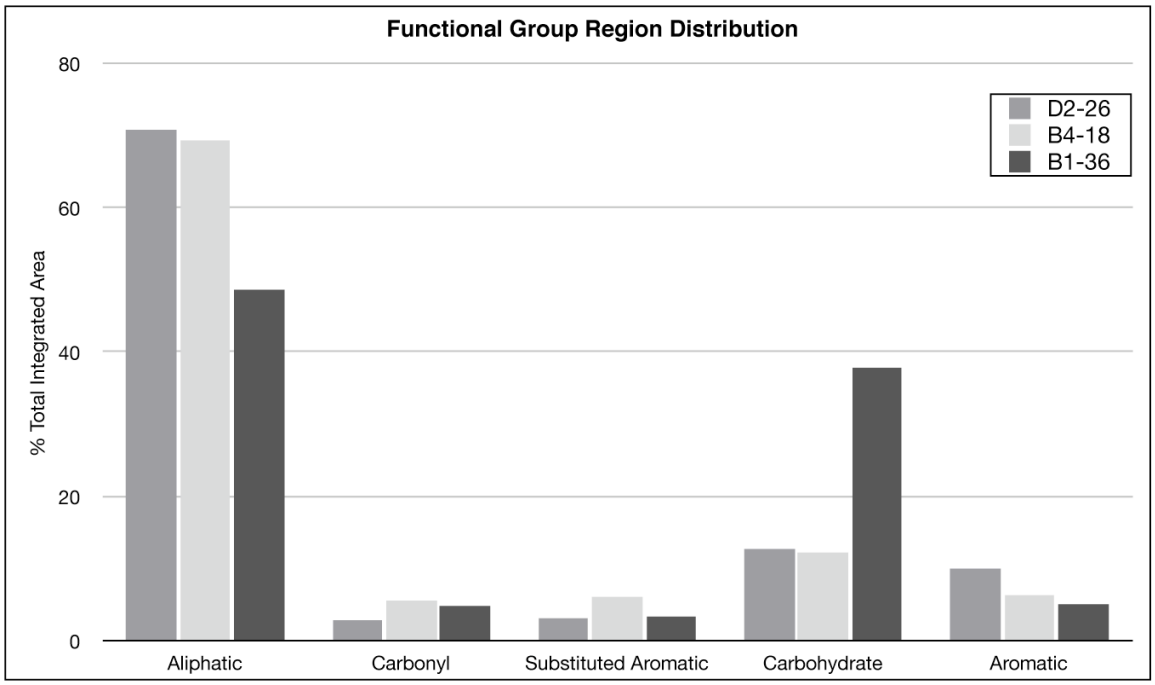

Figure 4.6. Bar graph of recalculated functional group distribution of Antrim well DOM as a percentage of total integrated area excluding the possible contaminant peaks 6, 29, and 31 in sample B1-36.

CHAPTER 5

SYNTHESIS OF RESEARCH, ADDITIONAL RESEARCH, AND CONCLUSIONS

5.1 Synthesis of Research

This research aimed to test three hypotheses about the Antrim microbial community, the sources of energy and organic carbon that feed it, and the metabolic roles that are involved in a trophic chain leading to methanogenesis in the Antrim. The first hypothesis states the Antrim microbial community consumes shale-derived dissolved organic matter as its primary source of energy and organic carbon. The second hypothesis states the consumers of shale-derived DOM utilized either a fermentative or sulfate-reducing metabolism. Finally, the third hypothesis states that either sulfate-reducing bacteria or fermentative bacteria initiate a trophic chain in the Antrim by consuming this shale-derived DOM and thus leave a distinctive signature in the Antrim DOM that can be identified and used to determine which of the two metabolisms is occurring in the Antrim. The previous chapters have described the experiments that were conducted to test these hypotheses. The first experiment was a series of enrichment cultures that utilized a laboratory generated shale-derived DOM as a medium to encourage growth of fermentative and sulfate-reducing bacteria using Antrim formation water as an inoculum. The second experiment utilized these sulfate-reducing and fermentative enrichments in larger scale microcosms so that the DOM could be characterized before and after growth to determine which components of DOM were consumed and produced in each of the microcosms. The final experiment isolated and characterized the DOM from three wells producing water from the Antrim Formation to

determine the composition of Antrim formation water DOM and the geochemical and microbiological controls on its composition.

The previous chapters have discussed the results of the individual components of this research. Although useful analyses can be made from each experiment individually, a true testing of the research hypotheses requires that the results of the three experiments be synthesized and analyzed together. The enrichment experiments provide evidence that support all three hypotheses, but comparisons between the microcosm and Antrim formation water DOM experiments were necessary to fully test the hypotheses. Ultimately, the differences in the NMR spectra of the sulfate-reducing and fermentative microcosms were too subtle to allow for the second and third hypotheses to be fully tested. However, comparisons between the laboratory experiments and the DOM collected from the Antrim provided several significant insights into the controls on the character of DOM in the Antrim formation waters.

5.2 Enrichment Experiment

The enrichment and successful growth from the Antrim well waters of fermentative and sulfate-reducing bacteria demonstrates that Antrim microorganisms are capable of growth using shale-derived dissolved organic matter as the only source of energy and organic carbon. Further, the production of methane in enrichment FERM 4 demonstrates that a complete methanogenic community can be supported using the water soluble components of shale as the only source of organic carbon. This experiment provides evidence that members of the Antrim microbial community are capable of growth on shale-derived DOM without requiring direct access the shale itself. However, further

experiments were needed to characterize the laboratory-generated shale-derived DOM and the components of the DOM that are consumed and produced by the enriched microorganisms, and to put the laboratory experiments in the context of the actual conditions in the Antrim.

5.3 Characterization of Laboratory and Antrim Formation Water DOM

The microcosm experiments allowed the laboratory generated shale-derived DOM to be studied in more detail and allowed for the examination of the production and consumption of DOM components by the microorganisms enriched from the Antrim. Comparisons of the SDDS and microcosm DOM with Antrim formation water DOM allowed the results of the laboratory experiments to be put in the context of actual conditions in the Antrim. Conversely, the microcosm experiments provided a simplified model of conditions in the Antrim, providing a context for further examination of the data collected from the Antrim DOM.

5.3.1 DOM Concentrations in the SDDS and Antrim Formation Waters

Because there were no detectable indications of biological activity in well B1-36 (Waldron et al. 2007), the mid-range DOM concentration of water from well B1-36 relative to the other two wells may be representative of a DOM concentration that reflects a dissolution of organic shale components with minimal influence from microbiological activity. If we assume that the measured SDDS DOM concentration of 4.08 mg/L is low due to loss of organic matter in the sample used for TOC measurement, and the true DOM concentration of the SDDS is greater than the 6.42 mg/L average DOM concentration of the fermentative microcosm, then it is similar to the 7.3 mg/L DOM

concentration of B1-36, suggesting that this DOM concentration of approximately 7 mg/L may represent an abiotic DOM equilibrium value for Antrim shale and water. If we assume that the DOM concentration of well B1-36 represents an abiotic baseline value, then the wells with high levels of biological activity, D2-26 and B4-18, represent DOM concentrations that reflect either significant consumption of DOM, and thus lower DOM concentrations, or additional liberation of DOM from the source shale by microbiological activity. Of course, it cannot be assumed that either the shale used for the SDDS or the shale present at each well site are homogenous, so these interpretations must be treated with caution.

5.3.2 Comparison of Laboratory and Field DOM NMR Spectra

This research originally envisioned that the NMR spectra of the sulfate-reducing and fermentative microcosms could be compared with the NMR spectra of Antrim formation water DOM to determine which metabolic pathway, sulfate-reduction or fermentation, was dominant in the Antrim. NMR analyses on shale-derived DOM microcosms demonstrated that SRB and fermentative bacteria have subtle but detectable differences in the consumption and production of DOM relative to the SDDS. However, the subtlety of the differences in the NMR spectra of the sulfate-reducing and fermentative microcosms make a concrete determination of metabolic characteristics from DOM composition unfeasible. Further, differences between the SDDS and microcosm DOM and the DOM collected from the Antrim formation waters indicate that the laboratory experiments do not fully simulate the conditions occurring in the Antrim. Still, useful analyses can be made from the data obtained.

The NMR spectra of the SDDS, the microcosms, and the Antrim well waters have similar overall shapes and features. The dominant features in all of these spectra are mostly in the aliphatic and carbohydrate regions. In this way these spectra are all similar to the NMR spectra obtained by other workers from differing environmental waters. The NMR spectrum that Kaiser et al. (2003) obtained from the River Tagliamento in Italy shares these broad features. Kaiser et al.(2003) suggested that the DOM from the River Tagliamento was dominated by microbially derived aliphatic esters as well as fatty acids and sugars derived from terrestrial plants. The DOM from the SDDS, microcosms, and Antrim formation waters may be similarly composed of microbial components, fatty acids, and sugars, but these must be produced by either the anaerobic subsurface community, or from ancient sources bound to the shale organic matter and preserved through diagenesis.

Neither the sulfate-reducing or fermentative microcosms exactly matches any of the Antrim DOM, but there are broad similarities. The peak 2 is consistently large in the Antrim well waters, but the peaks at the same position in the SDDS and microcosm spectra is relatively small. Peak 4 is not present in any of the Antrim well waters, but a large peak at this position is present in the SDDS and microcosm spectra. Peaks 6 and 7 are present in all of the spectra, appearing as relatively equally sized peaks in all but the sulfate-reducer microcosm, where peak 7 is significantly larger than peak 6.

Overall, the spectra from the laboratory generated DOM experiments are quite similar to each other, and differences are mostly in the relative intensities of the peaks present in all three samples. In contrast, the Antrim well water spectra are more complex,

both in the diversity of peaks present in each sample, as well as in the relative intensities of the peaks present.

The presence of low molecular weight compounds in both the laboratory and Antrim field samples can be inferred from the sharp well-defined peaks that appear in NMR spectra. The metal species that are commonly associated with DOM may cause rapid relaxation of ^{1}H resonances during NMR analysis, resulting in smoothing of peaks in NMR spectra (Simpson et al., 2001; Kim et al., 2003). An abundance of non-metal-bearing low molecular weight compounds, such as fatty acids and simple sugars, would have the effect of adding sharp and well defined peaks to the NMR spectrum. The fermentative and sulfate-reducing microcosm DOM NMR spectra had more of these sharp peaks than the SDDS, suggesting that such low molecular weight compounds were produced by microbiological activity. The NMR spectra from the Antrim wells have more jagged peaks and sharp peaks than the generally smooth NMR spectra of the SDDS and microcosms. These features in the NMR spectra of the Antrim formation water DOM may indicate that the DOM is composed of fewer large metal-bearing molecules and is more dominated by low molecular weight compounds compared to the laboratory samples. This difference in DOM composition may be attributable to production of low molecular weight acids and sugars by the microorganisms present in the Antrim, or by the liberation of such compounds from the shale by the activity of the microorganisms.

The most notable differences between NMR spectra of the laboratory-generated and Antrim well water DOM is the absence of major resonances in the carbonyl and substituted-aromatic regions in the Antrim formation waters, and the lack of major

aromatic resonances in the SDDS and microcosm spectra. The absence of major resonances in the carbonyl and substituted-aromatic regions in the Antrim well waters rules out a substantial presence of carboxylic acids in the Antrim well waters, as all carboxylic acids produce a resonance in this region (SDBSWeb : http://riodb01.ibase.aist.go.jp/sdbs/ (National Institute of Advanced Industrial Science and Technology, 2007)). Organic acids are often an important component of the DOM in subsurface waters (Lundegard and Kharaka, 1994). Laboratory experiments by Lewan and Fisher (1994) suggest that these acids may be bound to kerogen macromolecules by weak hydrogen bonds during the early diagenesis of the sediment and are subsequently easily released from the kerogen in aqueous solutions. The broad peak at position 12 in the substituted-aromatic region in the SDDS and both microcosm spectra may indicate the presence of these carboxylic acids. If these resonances are indeed produced by carboxylic acids, then it is likely that these acids were derived from the shale kerogen and were released into solution by the mechanism proposed by Lewan and Fisher (1994). The presence of resonances in the substitued-aromatic region of the SDDS spectra is notable as this indicates that an aqueous solution containing ground Antrim shale will produce such resonances. If the DOM in the formation waters of the Antrim is derived from the shale, then the spectra of the DOM obtained from the formation waters should also contain these resonances. As there are minor resonances in the substituted-aromatic regions of the Antrim well water spectra (peaks 10, 11, and 12) there may be a minor presence of carboxylic acids in the Antrim, but significantly less than the laboratory generated DOM would indicate would be initially available from the shale. The absence

in the Antrim formation water NMR spectra major of resonances in the substituted-aromatic region suggest that some compounds were consumed by the microbial community and are thus absent in the DOM.

The absence of major aromatic resonances in the NMR spectra of the laboratory generated DOM is a second major difference between the laboratory and field collected DOM. Since the microbial community in the microcosms did not have access to the shale itself, the presence of large aromatic resonances in the Antrim well waters but not in the laboratory generated ASE or microcosms could indicate that microbiological activity is required to liberate aromatics from the shale kerogen. However, there are very minor aromatic resonances in the sulfate-reducer and fermentative microcosms (peaks 34 and 35). These resonances are not present in the NMR spectra for the SDDS, suggesting that they were produced in the microcosms by the microbial community. There is some laboratory evidence to suggest that aromatic hydrocarbons can be produced as anaerobic hydrocarbon degradation products, including under methanogenic conditions (Widdel and Rabus, 2001; Harder and Foss, 1999). The large aromatic peaks in the NMR spectra of the Antrim well waters could therefore also be produced by microorganisms degrading shale-derived hydrocarbons.

5.4 Additional Research

The results of this research raise additional questions that can be addressed with further research. The experiments conducted as part of this research could not sufficiently test the proposed hypotheses. Expansion and modification of these experiments may provide the necessary data to fully test the hypotheses that guided this

research.

5.4.1 High Resolution Characterization of DOM

The lack of distinctive differences between the NMR spectra of the fermentative and sulfate-reducing microcosms was a principal obstacle in identifying characteristics of DOM composition that are diagnostic of either of the two metabolisms, and thus prevented a full testing the second and third hypotheses. These diagnostic characteristics of DOM were to be used as a tool for determining if either of the metabolic pathways occurs in the Antrim Shale. The differences in the NMR spectra of the microcosms that were detected indicate that some compositional differences exist; however, these differences are difficult to interpret with using ^{1}H NMR as the only analytical tool. ^{1}H NMR analyses are limited to functional group level characterization, and the compositional differences between the sulfate-reducing and fermentative microcosm DOM cannot be adequately resolved using this analytical technique. It is likely that the microbial community in the microcosms are consuming the DOM more selectively than at the functional group level. Another analytical technique, Electrospray Ionization Mass Spectroscopy (ESI-MS) can provide molecular compound level characterization of the DOM.

Electrospray Ionization Mass Spectroscopy (ESI-MS) is a relatively new analytical method for the characterization of DOM. Electrospray Ionization is an ionization technique that preserves the molecular structure of large molecules as they are introduced into a detector such as a mass spectrometer. Compound or molecular level characterization of DOM is possible when electrospray ionization is paired with a mass

spectrometer with sufficiently high resolution (Kim et al. 2003; Seitzinger et al. 2005; Kujawinski et al. 2004). Molecular level characterization of DOM requires mass resolving power of 1-20 ppm, or 10^{-4} to 10^{-5} atomic mass units (AMU) for a molecule with a mass of several hundred AMU. It is possible at this resolution to calculate a unique chemical formula for a given mass detected by the mass spectrometer.

The molecular level characterization afforded by high resolution ESI-MS may provide the necessary resolution to determine specific compositional differences between the components of DOM consumed and the metabolites produced by the communities in either the sulfate-reducing or fermentative microcosms. These data may be compared with ESI-MS data of Antrim formation waters to determine if any diagnostic compounds are present in the Antrim.

5.4.2 Shale Added Microcosms

One of the most notable differences between the conditions in the laboratory microcosm experiment and those occurring in the Antrim is direct access to shale as a source of additional organic matter. The microbial community present in the Antrim can potentially access and liberate additional organic matter from the shale, while the microcosm organisms were limited to the DOM present in solution at the start of the experiment. Adding ground shale to the microcosms would give the microbial community access to additional shale organic matter, and more accurately simulate actual conditions in the Antrim. The DOM from these shale-added microcosm experiments could then be compared with Antrim formation water DOM and sterile controls, and

could provide evidence that microbial activity plays a role in liberating additional DOM from shale.

5.4.3 Enhanced Microbiological Analyses

Additional enrichment experiments can be conducted to determine if other metabolic roles or terminal electron donors are present. Further, full clone libraries and 16S rRNA sequencing of the enrichment communities should be preformed so that a more complete picture of the microbial community present can be formed and more meaningful comparisons with other microbial communities can be made.

5.4.4 Expanded Sampling of Antrim Formation Water

The limited number of sample sites for collecting Antrim formation water DOM made it difficult to discern trends in DOM concentration and composition and their relationship to microbiological and geochemical conditions. The DOM of additional wells along the salinity and microbiological gradient of the Norther Producing Trend of the Michigan Basin should be examined to develop a larger picture of the controls on DOM composition in the Antrim.

5.5 Conclusions

The enrichment experiments showed that fermentative and sulfate-reducing members of the Antrim microbial community are capable of consuming shale-derived DOM, and that a complete methanogenic community can be sustained on shale-derived DOM as the only source of organic carbon and energy. These data confirm the hypotheses that microbial communities in the Antrim can consume the water soluble components of a shale, and that they can utilize fermentative and sulfate-reducing

metabolisms to consume these water soluble components of a shale. The successful enrichment of sulfate-reducing bacteria indicates that bacteria capable of utilizing sulfate as an electron acceptor are present in the Antrim despite the current absence of sulfate in the Antrim formation waters.

The results of the microcosm experiments show that the growth of organisms enriched from the Antrim on shale-derived DOM will produce detectable differences in DOM composition when examined using ^{1}H NMR analyses. Although the exact nature of the consumed and produced compounds could not be determined, the evidence gathered suggests that the communities in the fermentative and sulfate-reducing microcosms produce a different suite of metabolic products and may be selectively consuming different components of the shale-derived DOM. The presence of minor aromatic resonances in the NMR spectra of the FM and SRM microcosms and the strong aromatic resonances in the spectra of the Antrim formation waters may indicate that some aromatic compounds are produced by microbiological consumption of shale-derived DOM.

Resonances in the substituted-aromatic region of the NMR spectra of the SDDS may support findings by Lewan and Fisher (1994) that carboxylic acids can be produced from by the interaction of water and shale; however, the absence of significant resonances in the carbonyl and substituted aromatic regions of the Antrim formation water spectra indicate a lack of carboxylic acids in the sampled formation waters. This may reflect consumption of these compounds by the microbiological community present

in these wells, and may reflect one mechanism by which shale organic matter enters the trophic chain leading to methanogenesis in the Antrim.

The NMR analyses of the Antrim formation waters revealed that DOM composition in Antrim formation waters are not uniform through the geochemical and microbiological gradients. These data show that salinity, and microbial activity in turn, may control the generation or liberation of DOM components from the shale as wells as the consumption of components of the shale DOM.

5.6 Applications

This research will improve our understanding of the structure of methanogenic communities in black shales. Knowledge of which metabolic pathways and types of microorganisms are involved in the decomposition of shale organic matter into methane will improve our understanding of the environmental conditions that are necessary to support such communities. Knowledge of which components of shale organic matter are utilized by methanogenic communities will also have positive implications for gas exploration. Further, detailed knowledge of the preferred substrates, metabolic pathways, and types of organisms in a methanogenic community may lead to strategies for enhancing gas production in currently active wells, or even stimulating production in non-producing or underproducing areas.

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