Distinguishing and understanding thermogenic and biogenic sources of methane using multiply substituted isotopologues

D.A. Stolper \textsuperscript{a,d,*}, A.M. Martini \textsuperscript{b}, M. Clog \textsuperscript{a}, P.M. Douglas \textsuperscript{a}, S.S. Shusta \textsuperscript{c}, D.L. Valentine \textsuperscript{c}, A.L. Sessions \textsuperscript{a}, J.M. Eiler \textsuperscript{a}

\textsuperscript{a} Division of Geological and Planetary Sciences, 1200 E. California Blvd, Pasadena, CA, 91125, USA
\textsuperscript{b} Department of Geology, Amherst College, Amherst, MA, USA
\textsuperscript{c} Department of Earth Science and Marine Science Institute, University of California, Santa Barbara, CA, USA
\textsuperscript{d} Current Address: Department of Geosciences, Princeton University, Princeton, NJ, USA

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Abstract

Sources of methane to sedimentary environments are commonly identified and quantified using the stable isotopic compositions of methane. The methane "clumped-isotope geothermometer", based on the measurement of multiply substituted methane isotopologues ($^{13}$CH$_3$D and $^{12}$CH$_2$D$_2$), shows promise in adding new constraints to the sources and formational environments of both biogenic and thermogenic methane. However, questions remain about how this geothermometer behaves in systems with mixtures of biogenic and thermogenic gases and different biogenic environments. We have applied the methane clumped-isotope thermometer to a mixed biogenic–thermogenic system (Antrim Shale, USA) and to biogenic gas from gas seeps (Santa Barbara and Santa Monica Basin, USA), a pond on the Caltech campus, and methanogens grown in pure culture. We demonstrate that clumped-isotope based temperatures add new quantitative constraints to the relative amounts of biogenic vs. thermogenic gases in the Antrim Shale indicating a larger proportion (~50%) of thermogenic gas in the system than previously thought. Additionally, we find that the clumped-isotope temperature of biogenic methane appears related to the environmental settings in which the gas forms. In systems where methane generation rates appear to be slow (e.g., the Antrim Shale and gas seeps), microbial methane forms in or near both internal isotopic equilibrium and hydrogen-isotope equilibrium with environmental waters. In systems where methane forms rapidly, microbial methane is neither in internal isotopic equilibrium nor hydrogen-isotope equilibrium with environmental waters. A quantitative model of microbial methanogenesis that incorporates isotopes is proposed to explain these results.

1. INTRODUCTION

Understanding and quantifying the sources of methane in sedimentary environments is critical for studies of climate change, biogeochemical cycling, and energy exploration.

The two dominant processes that generate methane in nature are (Wuebbles and Hayhoe, 2002): (i) methane generated by methanogenic archaea, known as 'biogenic' methane; and (ii) methane generated during the thermally-activated breakdown of larger organic molecules, termed 'thermogenic' methane. Identifying the relative contributions of these sources to a given accumulation of methane can be challenging because gases tend to migrate and mix in the subsurface (Tissot and Welfle, 1978; England et al., 1987; Price and...
Schoell, 1995; Hunt, 1996). However, this identification is the necessary first step in understanding the (bio)geochemistry of methane formation, gas flow, and ecology of microorganisms in the subsurface.

In many environments, thermogenic and biogenic gases can be distinguished using average or ‘bulk’ carbon- and hydrogen-isotope compositions combined with the relative abundances of methane to other hydrocarbon gases (e.g., Bernard et al., 1976; Schoell, 1980, 1983; Whiticar et al., 1986; Chung et al., 1988; Whiticar, 1999). These molecular and isotopic ‘fingerprints’ are a powerful and important starting point for establishing the origin of methane in nature, but they are not foolproof—some gases can appear both thermogenic and biogenic in these frameworks (Martini et al., 1996, 1998). Additionally, the end-member compositions of thermogenic and biogenic gases are sufficiently variable that it can be difficult to quantify their contributions to mixtures solely using isotopic and compositional parameters. Furthermore, the fundamental controls of the isotopic composition—especially the hydrogen isotopic composition—of biogenic and thermogenic methane remains poorly understood (Burke, 1993; Sugimoto and Wada, 1995; Whiticar, 1999; Valentine et al., 2004; Schimmelmann et al., 2006; Yoshioka et al., 2008; Ni et al., 2011; Hattori et al., 2012; Kawagucci et al., 2014). Key questions to consider when interpreting the meaning of the isotopic composition of biogenic methane are the importance of kinetic vs. equilibrium isotope effects during methanogenesis and, by extension, how reversible are the enzymatic processes involved in methane generation (Blair, 1998; Whiticar, 1999; Valentine et al., 2004; Penning et al., 2005).

### 1.1. New techniques to identify the source of methane in the environment

A potentially powerful way to distinguish biogenic from thermogenic methane is through the direct determination of a gas’s formation temperature. For example, based on observations from environmental systems, biogenic gases in nature are generally thought to form below ~80 °C (Wilhelms et al., 2001; Valentine, 2011)—although laboratory methanogenic cultures can grow up to at least 122 °C (Takai et al., 2008). In contrast, based on both experiments and comparison to observations from sedimentary basins, thermogenic gases are thought to form above ~60 °C (Tissot and Welte, 1978; Quigley and Mackenzie, 1988; Hunt, 1996; Seewald et al., 1998; Seewald, 2003), and many models predict that most natural gases form at temperatures greater than ~150 °C (Quigley and Mackenzie, 1988; Seewald et al., 1998). Thus, knowledge of the formation temperature of a sample of methane could provide a fingerprint for its original formational environment and by extension, its source. Formation temperatures may be particularly distinctive and useful when combined with the established molecular and isotopic fingerprints discussed above as well as with existing geological and geochemical constraints.

New geothermometers based on the measurement of multiply substituted (‘clumped’) methane isotopologues (Ma et al., 2008; Tsuji et al., 2012; Stolper et al., 2014a,b; Ono et al., 2014a; Wang et al., in press) can constrain formation temperatures of methane. This is possible because the degree of clumped isotopic ordering, i.e., the clumped-isotope composition/distribution of a sample of methane (or any other molecule) that formed in isotopic equilibrium without further isotopic exchange after formation is a function of its formation temperature (e.g., Urey and Rittenberg, 1933; Wang et al., 2004; Eiler and Schauble, 2004; Schauble et al., 2006; Eiler, 2007, 2013; Ma et al., 2008; Yeung et al., 2012; Eiler et al., 2014a; Stolper et al., 2014a,b; Ono et al., 2014b; Wang et al., 2014; Webb and Miller, 2014). The potential to use clumped isotopes to measure methane formation temperatures has been demonstrated theoretically in several studies (Ma et al., 2008; Cao and Liu, 2012; Stolper et al., 2014a; Ono et al., 2014a; Webb and Miller, 2014).

The first demonstration that clumped-isotope distributions of methane could be measured at the sub-per-mil precision necessary for geothermometry along with an experimentally generated temperature calibration was by Stolper et al. (2014a) using mass spectrometry. Stolper et al. (2014a) also provided evidence that thermogenic methane can yield clumped-isotope temperatures related to its formation temperatures. Following this, Stolper et al. (2014b) showed that clumped-isotope temperatures of biogenic and thermogenic methane from sedimentary basins are consistent with expected gas formation temperatures. Additionally, Stolper et al. (2014b) discussed (and showed in the Supplementary Materials) that biogenic methane can form without achieving an equilibrium distribution of clumped-isotopes, i.e., out of clumped-isotope equilibrium. They hypothesized that biogenic methane that resulted in reasonable clumped-isotope temperatures (and thus, by inference, formation in clumped isotopic equilibrium) resulted from the partial reversibility of enzymes involved in methanogenesis. Using an independent infrared spectroscopic method for measuring methane clumped-isotope distributions, Ono et al. (2014a) confirmed the results of the mass spectrometric measurements of Stolper et al. (2014a,b) that thermogenic methane yields clumped-isotope temperatures consistent with their formational environments. This demonstration that two different analytical techniques yield similar quantitative determinations of clumped-isotope distributions and inferred temperatures is an important demonstration of the validity of the application of clumped-isotope thermometry to natural systems.

Although clumped-isotope distributions of methane appear to reflect equilibrium distributions imprinted at the time of formation in many settings, including for some microbially sourced gases, in addition to the results of Stolper et al. (2014b) discussed above, Stolper et al. (2014c), Gruen et al. (2014), and Ono et al. (2014b) all presented preliminary results indicating that biogenic methane forms out of clumped-isotope equilibrium such that the clumped-isotope temperatures yield apparent temperatures that are too high to be formation temperatures. Stolper et al. (2014c) suggested that this disequilibrium reflects the methane’s formation environment. Specifically, disequilibrium was suggested to occur in environments where methane generation rates are high, such as in organic-carbon-rich environments.
environments (like a pond). Conversely, in subsurface environments such as sedimentary basins, where methane generation rates are slower, methane was suggested to form in clumped isotopic equilibrium. Stolper et al. (2014c) also showed that the clumped isotopic composition of biogenic methane was linked to the difference in D/H ratios of methane vs. the water it formed in. Specifically, when methane was found to be in clumped isotopic equilibrium and yielded reasonable clumped-isotope temperatures, it was also in D/H equilibrium with its formational waters. However, when the methane appeared to be out of clumped isotopic equilibrium, it was out of D/H isotopic equilibrium with its source waters. Following this, Eiler et al. (2014b) presented a quantitative model of microbial methanogenesis based on the hypothesis of Stolper et al. (2014b) that the degree of enzymatic reversibility during methanogenesis controls the clumped isotopic composition of methane. This model successfully linked the clumped isotopic composition of methane and the isotopic difference between methane, water, and CO$_2$ to the overall reversibility of enzymes involved in methanogenesis. This preliminary work is developed and presented more fully here. Alternative hypotheses are that the generation of biogenic methane out of clumped isotopic equilibrium arises from kinetic isotope effects created via tunneling of hydrogen and deuterium atoms and/or other forms of kinetic isotope effect during hydrogenation reactions or reflect inheritance from substrates (Gruen et al., 2014; Ono et al., 2014b). In more recent work this same group (Wang et al., in press), confirmed that biogenic gases in some systems yield apparent methane clumped-isotope-based temperatures that are sensible, but that other biogenic systems yield apparent temperatures that represent methane formation of out of isotopic equilibrium. They additionally confirmed the correlation of D/H ratios of methane water vs. methane clumped-isotope compositions first presented by Stolper et al. (2014c) and developed a quantitative model similar to that shown by Eiler et al. (2014b) that also relates the generation of biogenic methane to enzymatic reversibility.

1.2. This study

Although initial results demonstrate promise in identifying the environment where methane forms, including distinguishing biogenic from thermogenic methane, clumped-isotope techniques have so far only been applied to methane from a small number of natural environments, most of which were dominantly either biogenic or thermogenic in origin (Stolper et al., 2014b; Wang et al., in press). Thus, it remains unclear whether these temperature constraints can yield quantitative insights into systems that contain a mixture of thermogenic and biogenic gases. Such mixtures could be difficult to untangle due to non-linear and unintuitive ‘mixing effects’ on the clumped isotope systematics of many gases [discussed below and more broadly in Eiler and Schauble (2004) and Eiler (2013)]. Additionally, distinguishing biogenic methane that forms out of isotopic equilibrium from thermogenic methane might be challenging (Gruen et al., 2014; Eiler et al., 2014b; Stolper et al., 2014b,c; Wang et al., in press).

To explore these issues, we have measured abundances of clumped isotope distributions of methane in gases from the northern and western margin of the Antrim Shale in the Michigan Basin, which is known to contain a mixture of biogenic and thermogenic gases (Martini et al., 1996, 1998, 2003). We demonstrate that the measurements provide quantitative, novel insights into the origin of gases in the system. Specifically, the results indicate a higher abundance of thermogenic gases than previously inferred and add geologically grounded constraints on the source of the thermogenic component.

To place our results from the Antrim Shale system into a broader context, we also measured biogenic gases from laboratory cultures of methanogens and two other natural environments (gas seeps from the Santa Barbara and Santa Monica Basins and a pond from the Caltech campus). We demonstrate that the measured difference in the D/H ratio of biogenic methane versus that of the water in which the methane forms is correlated to the clumped-isotope composition. Using this relationship, we demonstrate that when methane is in clumped isotopic equilibrium, it is in heterogeneous equilibrium with water as well. We argue that this relationship is indicative of the importance of enzymatic reversibility in setting the isotopic composition of biogenic methane. The combined results from the Antrim Shale and these other settings indicate that the clumped isotopic composition of biogenic methane is sensitive to the environment in which the organisms grow. Specifically, as hypothesized by Stolper et al. (2014c), in systems where rates of microbial methanogenesis appear to be low, methane is formed in isotopic equilibrium with itself and other phases such as water. In contrast, rapid methane generation rates promote the formation of methane out of isotopic equilibrium with both itself and with other phases.

Based on these findings, following the initial work of Eiler et al. (2014b), we develop a quantitative model linking the physiology of methanogens to the measured clumped-isotope abundances of biogenic gases through the assumption that the enzymes involved in methanogenesis can operate reversibly. This model considers both kinetic- and equilibrium-based controls on the generation and isotopic composition of methane. We show that for fully reversible enzymatic conditions methane is generated in both internal isotopic (homogenous phase) and heterogeneous phase equilibrium with water and CO$_2$. In contrast, for partially irreversible enzymatic conditions, methane is formed out of both internal isotopic equilibrium and out of equilibrium with water and CO$_2$. This work highlights the key role clumped-isotope measurements can play in elucidating the thermodynamic conditions under which biogenic methane is generated.

2. GEOLOGICAL AND GEOCHEMICAL CONTEXT OF THE ANTRIM SHALE

The Antrim Shale subcrops below glacial drift deposits in the Michigan Basin (Fig. 1). It is Devonian in age, having formed during the Acadian orogeny as a distal facies of the Catskills delta complex (Gutschick and Sandberg, 1991).
The northern margin of the shale contains economically significant accumulations of natural gas (Martini et al., 1996, 1998) with over 12,000 wells drilled (Martini et al., 2008). It is considered to be an ‘unconventional’ shale-gas system in which the gases were formed and are stored in situ. There is evidence based on both isotopic and chemical constraints for extensive fluid migration within the northern margin of the Antrim Shale: specifically water from basinal brines is thought to have mixed in the northern margin with meteoric waters (Wilson and Long, 1993; Martini et al., 1998; McIntosh and Walter, 2005). This mixing is facilitated by fluid migration along extensive vertical fracture networks (Apotria and Kaiser, 1994; Martini et al., 1998) that are potentially important conduits for gas migration.

A key distinguishing feature of the northern margin Antrim Shale gases is that they are thought to be dominantly (>80%) microbial in origin with the remaining fraction (<20%) thermogenic (Martini et al., 1996, 1998). In fact, the Antrim Shale has been used as a model for microbially generated, unconventional shale-gas systems (Curtis, 2002). In addition to their economic importance, these systems have been proposed to play a role in regulating climate between glacial/interglacial cycles via storage and release of methane to the atmosphere (Formolo et al., 2008; Kirk et al., 2012; Wuchter et al., 2013); (ii) δD values\(^1\) of methane and water extracted (co-produced) with the methane are linearly correlated. Following Schoell (1980), this correlation was interpreted to indicate a biological origin for most (>80%) of the gas (Martini et al., 1996, 1998); and (iii) δ\(^13\)C values of co-produced CO\(_2\) are high (>20‰; Martini et al., 1996, 1998, 2003), indicative of active hydrogenotrophic (CO\(_2\) + H\(_2\)) methanogenesis in a closed system (e.g., Claypool and Kaplan, 1974). It is important to note that only the biogenic methane is hypothesized to have formed within the shales. The original formation site of the thermogenic component of the gases is poorly constrained. Proposed sources of the thermogenic gases include the northern margin itself (i.e., that it is self-sourced) or migration of gases into the northern margin along the fracture networks discussed above from more deeply buried expressions of the Antrim Shale in the mid-basin or older source rocks in the Michigan Basin (Martini et al., 1996, 1998, 2003). Consequently, the Antrim Shale provides a type example for studying both the mixing of thermogenic and biogenic gases in a relatively...
simple system and the behavior of methanogenic archaea in the ‘deep biosphere’ (Edwards et al., 2012).

Measurements of clumped isotopes provide an independent method to quantify the ratio of thermogenic and biogenic gases in the northern margin and thus test the proposal that the gases are dominantly microbial in origin. Specifically, if northern and western margin gases are dominantly biogenic and formed in internal isotopic equilibrium, the clumped-isotope temperatures should reflect current ambient temperatures in the shale (~10–30 ºC; Martini et al., 1998).

3. METHODS

3.1. General methods and sample collection

We studied methane samples from a variety of economic wells from the northern margin of the Antrim Shale, and one sample from the western margin (Table 1). For each sample, we measured the δD and δ13C values of methane (δ13CCH4 and δDCH4), relative abundances of C1–C4 alkenes, δD and δ18O values of co-produced waters (δDH2O,liquid and δD18O,liquid), and δ13C values of CO2 (δ13CCO2) in the gas phase. Clumped-isotope compositions of methane were also measured, and are reported as the combined signal of the mass-18 isotopologues (13CH2D ±5% for C1–3 and δ18R DCH4 ±5% for δDCH4) for some samples.

Antrim Shale gases were sampled in duplicate directly from wellheads in stainless steel containers. One set was sent to Isotech Labs (Champaign, IL) and the other set sent to Caltech. At Isotech, δ13C values of CO2 were measured using dual-inlet mass spectrometry with an external reproducibility of 10%. Relative precision was typically ±5% for C1–3 hydrocarbons and ±10% for larger hydrocarbons. δD and δ18O of co-produced waters were measured at Smith College using a Picarro L1102-i cavity ring-down spectroscopy analyzer. Precision was 0.2‰ for δ18O and 0.5‰ for δD.

For Santa Barbara and Santa Monica Basin samples, gases were collected at the sea floor using an inverted funnel equipped with a stainless steel cylinder using the R/V Jason during cruise AT26-06 of the R/V Atlantis. Seeping gases were trapped using the inverted funnel and sealed in the cylinder for transport to the R/V Atlantis where they were subsampled for subsequent analyses. One subsample was used to measure gas composition at the University of California, Santa Barbara using the method described by Duly et al. (2007). Another subsample was shipped to Caltech for analysis of clumped isotopes.

One sample of methane was extracted from a pond on Caltech’s campus. A vacuum-evacuated 1 L Pyrex flask was connected to a vessel for collecting gas from sedimentary systems. Gases were released from the pond sediments manually through agitation and collected in the headspace of the initial vessel. Atmospheric gases were eliminated by first filling the vessel with pond water. The evacuated flask was then connected to the vessel via a rubber tube and opened, filling with pond-derived gas.

3.2. Description of clumped-isotope and ethane analyses and external reproducibility

Prior to isotopic analyses, methane and ethane were purified on a glass vacuum line as described in Stolper et al. (2014a), Stolper et al. (2014b), and Clog et al. (2014), δ13C, δD, and clumped-isotope compositions of methane and δ13C of ethane were measured via mass spectrometry on a prototype, high-resolution isotope-ratio mass spectrometer, the MAT 253 Ultra (Eiler et al., 2013), at Caltech as described in Stolper et al. (2014a) and Stolper et al. (2014b) for methane and Clog et al. (2014) for ethane. External precisions for δDH2O measurements were typically 0.13‰, 0.06‰ for δ13CCO2 measurements and 0.03‰ for δ13CC2H6 measurements.

Clumped-isotope compositions are expressed using Δ13H notation (Stolper et al., 2014a) where

\[ \Delta_{18} = \left( \frac{^{18}R}{^{18}R^*} - 1 \right) \times 1000, \]  
and

\[ ^{18}R = \left( \frac{13CH_3D + 13CH_2D_2}{13CH_4} \right)/13CH_4. \]

The brackets denote the molar concentration of the isotopologue as a fraction of all molecules of the compound of interest. R* is the ratio expected for a random distribution of isotopes amongst all isotopologues (Stolper et al., 2014a). Δ13H values are reported in a reference frame in which 0‰ represents a random isotopic distribution. All samples are compared to (i.e., referenced against) a laboratory standard with a Δ13H value in this reference frame of 2.981‰, which was previously calibrated in the study of Stolper et al. (2014a). Δ13H values are converted into temperature in Kelvin via the equation (Stolper et al., 2014a):

\[ \Delta_{18} = -0.0117 \left( \frac{10^6}{T^2} \right) + 0.708 \left( \frac{10^6}{T} \right) - 0.337. \]

Samples were measured over 8 analytical sessions spanning 10 months including some overlap with measurement sessions for data reported in Stolper et al. (2014b).
External precision of measurements was monitored three ways. First, a standard differing in δD (+56‰), δ13C (+33‰), and δ18 (+92‰) as compared to the laboratory internal standard was measured multiple times in each analytical session. External precision for the Δ18 value across all measurements is ±0.23‰ (1 standard deviation, σ; n = 19), and differs in Δ18 by <0.04‰ from the long-term average of the standard over 2.5 years of measurements (being slightly too positive). Second, gases equilibrated on nickel catalysts at 500 °C were measured in all sessions (Stolper et al., 2014a). External precision across the sessions is ±0.28‰ (1σ; n = 17) and differs by <0.06‰ in Δ18 from the long-term (2.5 year) average (again being slightly too positive). Additionally, no statistically significant dependence of Δ18 values on the bulk isotopic composition of the heated gases (monitored using a 300‰ range in δ18 values) was observed (Fig. A1). Third, 8 Antrim Shale gases and 1 Santa Monica Basin gas were measured multiple times in different sessions. Additionally, the Antrim Shale samples were purified by different users and contain significant quantities of gases other than methane. Thus, replicate analysis of these samples should provide constraints on the overall reproducibility of the Δ18 measurements that incorporates both sample preparation and mass-spectrometric measurements. The external precision for Δ18 for the samples is ±0.24‰ (1σ, n = 20). One Antrim sample (North Charlton A1-18), measured 3 times, yielded a single outlier Δ18 value compared to the others and this value is excluded from all further discussion. Exclusion of this outlier yields an external precision for Δ18 measurements of 0.17‰ (1σ, n = 19).

We thus conclude that Δ18 external precisions are ~0.25‰ consistent with internal, counting-statistics-derived errors for a single measurement of 0.24 to 0.27‰ (Stolper et al., 2014a). In two sessions, drift in the standard used to normalize data from a measurement session to a unified δD and Δ18 reference frame (as described in Stolper et al., 2014a) was observed. We corrected for this drift by measuring the standard used for the correction multiple times over the course of these sessions and assumed the values required for the correction for a sample measured on a different day changed linearly with time between observations of the standard. The quality of the correction was checked through multiple measurements of a secondary standard (the one described above which differs in δD, δ13C and δ18 as compared to the laboratory reference standard) in all sessions and re-analysis of at least some samples in other sessions. For both sessions, final average Δ18 values of the secondary standard were within 1 standard error (s.e.) of its long-term average (longer term average Δ18 = 1.71): 1.79 ± 0.07 (1 s.e., n = 2) and 1.56 ± 0.15 (1 s.e., n = 4). Additionally, the overall external precision of the Antrim Shale samples, including those observed in these sessions, does not differ from the external precision of other measured standards and 500 °C heated gases.

3.3. Methanogen pure-culture growth conditions and considerations

Pure cultures of Methanothermobacter marburgensis and Methanosarcina barkeri (species of methanogens) were grown at the University of California, Santa Barbara in an H2/CO2 atmosphere following Boone et al. (1989), at constant temperatures from 30 to 70 °C. Samples were shaken to ensure mixing of H2 into the solution. Before sampling, methane, culture media was removed from the culture bottles. Methane was removed from the culture bottles with a gas-tight syringe (Vici), injected onto a vacuum glass line, and then purified as described in Stolper et al. (2014a,b). These samples are more depleted in δ18 relative to our working gas standard by ~200 to 400‰, and are outside of the normal range of bulk isotopic composition of samples usually measured. To ensure no measureable bias exists for measurements of gases with significantly more depleted δ18 values than the standards, two biogenic gases (δ18 = −180 to −200‰) were heated in the presence of nickel catalysts to promote internal isotopic equilibrium at 500 °C following Stolper et al. (2014a). These gases yielded a Δ18 value of 1.03 ± 0.28 (1 s.e.), identical within error to the long term average for 500 °C heated gases, 1.06 ± 0.05 (n = 21, 1 s.e.). However we note that two samples measured were significantly more depleted in δ18 (by ~200‰) than the biogenic gases catalytically equilibrated at 500 °C (the 30 °C and 37 °C experiments). Thus there is the potential that an analytical artifact for these very low δ18 samples exists that was not tested for by our study of heated, low δ18 gases. Regardless, any inaccuracies up to ~±2‰ for these samples, i.e., an order of magnitude larger than the stated 1σ error bars, would not affect any interpretation of these measurements.

δD2H2O,liquid for one pure-culture experiment (65 °C M. marburgensis experiment) and the Caltech Pond samples were measured at Caltech using a spectroscopic Liquid-Water Isotope Analyzer (Los Gatos Research Inc.) as described in Feakins and Sessions (2010). Samples were standardized to the VSMOW/VSLAP scale by measuring standards of known isotopic composition that bracketed the δD values of the samples. Typical precision for δD2H2O,liquid measurements for this instrument is ~<1‰ (Feakins and Sessions, 2010). δD2H2O,liquid values were not measured for all culture media, which were prepared with the same water source but at different times. We assume that all media had the same δD value with a conservative 1σ error of 25‰ for the instances where the value was not measured.

3.4. Calculations of expected differences in δD2H2O vs. δDCH4

We calculated the expected equilibrium isotopic difference between δDCH4 and δD2H2O,liquid, i.e., αCH4-H2O,liquid. Where αA-B = (RA/RB). To do this, we first calculated αCH4-H2O,liquid (where ‘H2O, vapor’ refers to water in the vapor phase) as a function of temperature using the theoretically derived data from Richet et al. (1977). The derived equations are given in Table 2. In Richet et al. (1977), two theoretical datasets are available to calculate
α_{CH4-H2O,vapor}, one that incorporates anharmonicity in the reduced partition functions of methane and one that does not (i.e., is harmonic). These models disagree strongly at low temperatures, yielding, for example, a 70% difference in α_{CH4-H2O,vapor} at 20 °C. Perhaps surprisingly, there are no independent experimentally or environmentally derived constraints to indicate which is correct. In the face of these uncertainties, we simply used the average of the two model estimates at a given temperature to calculate α_{CH4-H2O,vapor}. This estimate was then combined with the experimentally derived fractionation factor between liquid water and vapor from Horita and Wesolowski (1994), resulting in an estimated value for α_{CH4-H2O,liquid}. We did not use the experimentally calibrated α_{CH4-H2O,liquid} calibration provided by Horibe and Craig (1995) (in combination with other fractionation factors to arrive at α_{CH4-H2O,liquid}) as it is only calibrated at temperatures greater than 200 °C. As our interest in using this calibration (see below) is for microbial environments, which range in temperature from ~0 to 120 °C (all samples examined here were generated below 70 °C), we consider it more accurate to use the theoretical forms given in Richet al. (1977). We note though, that the calibration of α_{CH4-H2O,liquid} from Horibe and Craig (1995) combined with the calibration for α_{H2O,liquid} from Richet et al. (1977) and α_{H2O,liquid-H2O,vapor} from Horita and Wesolowski (1994) results in a calibration that falls between those derived using the harmonic and anharmonic calculations for α_{CH4-H2O,vapor} from Richet et al. (1977) with α_{H2O,liquid-H2O,vapor} from Horita and Wesolowski (1994) discussed above.

### 4. MIXED-SOURCE GASES OF THE ANTRIM SHALE

#### 4.1. Stable-isotope systematics and chemical/physical measurements of the Antrim Shale

Measured isotopic and physical/chemical values for Antrim Shale gases are provided in Table 3 and Fig. 2. Samples measured in this study are similar in isotopic composition and C1/C2–4 ratios to those measured previously by others (Fig. 2a, c, and e; Martini et al., 1996, 1998, 2003). Gas-well temperatures range from 16 to 24 °C (Table 3).

The δ13C_C4H4 values of all measured gases vary by less than 3‰, ranging from −50‰ to −53‰ (Fig. 2c and d). Historically, δ13C_C4H4 values <−60‰ have often been considered indicative of biogenic gases, while those ~>−50‰ indicative of thermogenic gases (Schoell, 1980; Whiticar, 1999), though the actual ranges in nature may have a larger overlap (e.g., Tang et al., 2000; Valentine et al., 2004). Regardless, the δ13C values observed in the Antrim Shale gases fall between these historic ranges and, as discussed by others, do not yield strong constraints on the origin of the methane (Martini et al., 1996, 1998; Whiticar, 1999).

As observed in other northern margin gases, all δ13C_CO2 values are >20‰ (Table 3; Martini et al., 1996, 1998, 2003). Also, δD_CH4 and δD_H2O,liquid values are correlated, following a similar linear trend as observed previously (Fig. 2e). As discussed above, these two observations were previously used by Martini et al. (1996) to hypothesize that the northern margin gases are dominantly biogenic in origin. However, a proportion of thermogenic gases must be present in the samples because some contain significant quantities of C2–4 alkanes with C1/C2–4 ratios as low as 8 (i.e., 11% C2–4 alkanes relative to all C1,4 alkanes; Table 3). This is because C2–4 alkanes are thought to be of thermogenic origin in the Antrim Shale (Martini et al., 2003). Similarly low C1/C2–4 ratios (C1/C2–4 = 12; Fig. 2a) occur in other northern margin gases (Martini et al., 1998). Finally, as observed in previous studies (Martini et al., 1996, 1998), the C1/C2–4 ratio is related to δD_CH4 values, such that gases with more enriched δD_CH4 values have lower C1/C2–4 ratios (Fig. 2a). This relationship is consistent with gases with more enriched δD_CH4 values having a greater proportion of thermogenic gas.

#### 4.2. Δ18-based temperatures

Measured Δ18 temperatures average 67 °C, range from 40 to 115 °C (Table 3), and correlate with many of the other measured parameters. For example, a hyperbolic relationship exists between Δ18 temperatures and C1/C2–4 ratios, with hotter temperatures corresponding to lower C1/C2–4 values (Fig. 2b). Δ18 temperatures are also linearly correlated with δD_H2O,liquid (Fig. 2f) and δD_CH4 (Fig. 2g), with hotter temperatures corresponding to more enriched δD_H2O,liquid and δD_CH4 values. Despite the relatively small variation of δ13C_CH4 in Antrim shale gases (and a general lack of correlation with other geochemical properties), we note a weak linear relationship between δ13C_CH4 and Δ18 temperatures (r² = 0.31; Fig. 2d). This relationship or lack thereof is discussed in more detail below in the context of a quantitative mixing model (Section 4.2).

The relationships between Δ18 temperatures and various other isotopic and compositional parameters support the hypothesis that the northern margin gases are a mixture of lower temperature biogenic gases with higher

---

**Table 2**

<table>
<thead>
<tr>
<th>Type</th>
<th>Equation a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harmonic</td>
<td>α_{CH4-H2O,vapor} = 1000ln(α_{CH4H2O,vapor}) = 0.857(1000/T)² − 14.412(1000/T)¹ + 88.483(1000/T)⁰ − 209.56(1000/T)¹ + 60.553</td>
</tr>
<tr>
<td>Anharmonic</td>
<td>α_{CH4-H2O,vapor} = 1.101(1000/T)² − 11.537(1000/T)¹ + 38.890(1000/T)⁰ − 20.448(1000/T)¹</td>
</tr>
<tr>
<td>Harmonic</td>
<td>α_{CH4-H2O,vapor} = 1000ln(α_{CH4H2O,vapor}) = 0.619(1000/T)³ − 6.628(1000/T)² − 6.025(1000/T) + 3.071</td>
</tr>
</tbody>
</table>

a Equations derived from Richet et al. (1977).
b T is in Kelvin.
temperatures of the biogenic end members could be influenced by kinetic isotope effects that have been observed in some microbial systems (Stolper et al., 2014b,c; Gruen et al., 2014; Ono et al., 2014b; Eiler et al., 2014b; Wang et al., in press). Thus, it is not clear whether we should interpret the low measured $\Delta_{18}$ temperatures of biogenic gases as an indication of their actual formation temperatures, or instead as some non-equilibrium isotopic signature. As will be discussed and developed in the following sections, multiple lines of evidence indicate that the $\Delta_{18}$-based temperatures of the biogenic end-member gases are $\sim 20^\circ$C, the current temperature of the shales, and thus that the biogenic component of the gas was formed in clumped isotopic equilibrium.

### 4.3. Mixing model

The relationships between $\Delta_{18}$ temperature and $\delta^{13}$CH$_4$, $\delta^{18}$H$_2$O, and $C_1/C_2$ ratios suggests that the $\Delta_{18}$ temperatures relate to the amount of thermogenic and biogenic gas in the system. In order to explore the feasibility of this quantitatively, a mixing model was created. This model assumes that there are two end members of gases mixed together, one a biogenic gas formed at lower temperatures and the other a thermogenic gas formed at higher temperatures. In order to create this model, end-member values for the $\Delta_{18}$ temperatures, $\delta^{13}$CH$_4$ and $\delta^{18}$H$_2$O values, and $C_1/C_2$ ratios had to be assigned. In choosing these end-member values, we incorporated as many external constraints as possible. Thus the model can serve as a test of the plausibility of the measured $\Delta_{18}$ temperatures. I.e., we can ask whether the mixing model is both self-consistent with one or more of the trends defined by the samples studied here and, more broadly, with the other measured properties of these and related gases.

Assumed compositions for the thermogenic and biogenic end-member components of our mixing model are given in Table 4. Of these, two compositional parameters ($\delta^{13}$CH$_4$ and $\Delta_{18}$ of both end members) are discussed in detail here. $\delta^{13}$CH$_4$ end-member values were chosen assuming, as observed by the weak linear relationship between $\delta^{13}$CH$_4$ and $\Delta_{18}$ temperature (Fig. 2d), that the biogenic end member has a more enriched $\delta^{13}$CH$_4$ value than the thermogenic end member. Then, the highest measured $\delta^{13}$CH$_4$ value of a northern margin gas with $C_1/C_2 < 100$ for the thermogenic component (Table 4). However, the potential range of $\delta^{13}$CH$_4$ end-member values varies by $\sim 50$.

<table>
<thead>
<tr>
<th>Sample Location</th>
<th>$\Delta_{18}$ Temperature</th>
<th>$\delta^{13}$CH$_4$</th>
<th>$\delta^{18}$H$_2$O</th>
<th>$C_1/C_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bircher C3-24</td>
<td>20</td>
<td>-28.6</td>
<td>6.0</td>
<td>45</td>
</tr>
<tr>
<td>Lake Huron D4-15</td>
<td>16</td>
<td>-25.0</td>
<td>5.8</td>
<td>40</td>
</tr>
<tr>
<td>North Charlton A18</td>
<td>16</td>
<td>-29.0</td>
<td>6.0</td>
<td>40</td>
</tr>
<tr>
<td>State Charlton C4-31</td>
<td>16</td>
<td>-22.0</td>
<td>5.8</td>
<td>40</td>
</tr>
<tr>
<td>State Charlton A3-32</td>
<td>16</td>
<td>-21.0</td>
<td>5.8</td>
<td>40</td>
</tr>
<tr>
<td>Weber A4-10</td>
<td>16</td>
<td>-24.0</td>
<td>6.0</td>
<td>45</td>
</tr>
</tbody>
</table>

* Referenced to VSMOW.  
* $\Delta_{18}$-based temperature.  
* $\delta^{13}$CH$_4$-based temperature.  
* From Kerk et al. (2012).
Fig. 2. Measured data from Antrim Shale samples. Error bars are all 1σ and are not displayed if they are smaller than the symbol. Data from previous studies come from Martini et al. (1996, 1998, 2003). Panels on the left plot properties vs. δDCH4. Panels on the right plot properties vs. Δ13C-based temperatures. (A) C1/C2+4 vs. δDCH4. (B) C1/C2+4 vs. Δ13C-based temperatures. (C) δ13C vs. δDCH4. (D) δ13C vs. Δ13C-based temperatures. (E) δDH2O,liquid vs. δDCH4. (F) δDH2O,liquid vs. Δ13C-based temperatures. (G) δDCH4 vs. Δ13C-based temperatures.
significantly larger than the range of samples measured here (3‰; Fig. A2). Thus, our choice of end-member $\delta^{13}$C$_{CH_4}$ values can be thought of as plausible but non-unique. Importantly, the $\delta^{13}$C$_{CH_4}$ variations among Antrim methane samples are sufficiently small and poorly correlated with other isotopic and molecular indices that this uncertainty has little bearing on other elements of our model: Any choice of $\delta^{13}$C$_{CH_4}$ values from the possible range has no significant quantitative impact on the resultant model (Fig. A2). For these reasons we include $\delta^{13}$C values in Table 4 in the interest of completeness but do not discuss them further.

Fig. 3. Comparison of the end-member mixing models with or without oxidation of C$_{2-4}$ hydrocarbons. Error bars are all 1σ and are not displayed if they are smaller than the symbol. For C, D, and E, both mixing models yields identical results as the oxidation of C$_{2-4}$ hydrocarbons has no effect on the methane’s or the formation water’s isotopic composition. Panels on the left plot properties vs. $\delta$D$_{CH_4}$. Panels on the right plot properties vs. $\Delta^{18}$-based temperatures. (A) C$_{1}$/C$_{2-4}$ vs. $\delta$D$_{CH_4}$. (B) C$_{1}$/C$_{2-4}$ vs. $\Delta^{18}$-based temperatures. (C) $\delta$D$_{H_2O,liquid}$ vs. $\delta$D$_{CH_4}$. (D) $\delta$D$_{H_2O,liquid}$ vs. $\Delta^{18}$-based temperatures. For this mixing space, it is presumed that the mixing ratios of the gases are equivalent to the mixing ratios of the waters. (E) $\delta$D$_{CH_4}$ vs. $\Delta^{18}$-based temperatures.
For this model, we have assumed that the biogenic Δ18 temperature reflects current temperatures in the Antrim Shale (~20 °C). We will validate this in a variety of ways in the following sections, but in first constructing this mixing model, we simply adopt it as an assumption. We do not know of any independent constraints on the maximum formation temperature of the thermogenic end member, because its source is not known (Martini et al., 1996, 1998). We estimated this temperature (T_A18) using the observed linear relationship between ΔDCH4 and Δ18 temperature (Fig. 2g; T_A18 (°C) = 2.33 × ΔD + 626) with independent constraints on the maximum ΔDCH4 of the thermogenic end member, −207/°C (from the northern margin; Martini et al., 1998). For this analysis, we excluded the one significant outlier point in Fig. 2g (State Charlestown A3-32, ΔD = −211/°C, T_A18 = 67 °C). Application of our regression then implies a maximum Δ18 temperature of 144 °C (±11 °C, 1σ). Although this temperature is higher than those estimated for maximum burial temperatures of the northern margin of the Antrim Shale (see below), it is consistent with previously measured peak temperatures (based on fluid inclusions) of ~150 °C (and perhaps as high as 175 °C) in the deeper-buried Devonian-aged, oil-producing Dundee Formation in the Michigan Basin (Luczaj et al., 2006) and previous suggestions that the thermogenic gases could have formed outside of the northern margin (Martini et al., 1996, 1998).

A key aspect of mixing of clumped isotopic compositions to consider is that Δ18-based temperatures of a mixture are not linear functions of the Δ18-based temperatures of the end members. This non-linearity occurs because: (i) Δ18 values have a non-linear dependence on temperature (Eq. (3) above)—thus, although Δ18 values can be linear mixtures of end members, Δ18-based temperatures need not; and (ii), Δ18 values of mixtures depend not only on the Δ18 value of the end members, but the ΔDCH4 and δ34CH4 values as well (Stolper et al., 2014a,b). In the Antrim Shale, the δ34CH4 and δDCH4 values of the biogenic and thermogenic end members do not differ enough for this second non-linearity to matter: for example, the differences in Δ18 values of the mixtures using the approximation that Δ18 values mix linearly vs. doing the calculation precisely are less than 0.1%/°C. This is less than half the analytical precision of a single measurement and thus unimportant for all conclusions made here—for the calculations here, all mixing is done exactly and all non-linearities included.

Modeled isotopic compositions for mixtures of these two end-member gases are shown in Fig. 3. The model agrees well with measured data for δDCH4 vs. δDH2O (Fig. 3c). Δ18 temperatures vs. δDH2O (Fig. 3d), and δDCH4 vs. Δ18 temperature (Fig. 3e). Poorer matches between the mixing model and the measured data occur for C1/C2-4 vs. δDCH4 (Fig. 3a) and vs. Δ18 temperature (Fig. 3b). The poor match of C1/C2-4 ratios vs. the isotopic values was expected because the abundances of ethane, propane, and butane are not thought to be controlled solely by mixing of end members in the Antrim Shale, but instead by a combination of mixing and microbial oxidation (Martini et al., 2003). The oxidation of ethane and propane is supported by the correlation of δ13C2H6 with the concentration of ethane such that as ethane concentrations decrease (C1/C2-4 rises), δ13C2H6 values increase (Fig. 4; Martini et al., 2003). A significant change in the δ13C value

![Fig. 4. Mixing model’s prediction of the δ13C of ethane as a function of the C1/C2-4 ratio. δ13C values of ethane are from this study and from previous measurements (from Martini et al. (2003) and Kirk et al. (2012)). Fractionation factors (α) for ethane oxidation are equal to (1000 + δ13C2H6,respired)/(1000 + δ13C2H6, residual), where ‘respired’ refers to the ethane that is respired by microbes and ‘residual’ refers to the remaining ethane. α values for ethane oxidation are all from Kinnaman et al. (2007). The thermogenic end member is assumed to have a δ13C value of −52.9 %/°C and C1/C2-4 of 5.8. See text for details.](image-url)
of ethane cannot occur solely due to mixing when one end member contains no or very little ethane. Oxidative consumption of ethane, on the other hand, will cause $\delta^{13}C_{C_2H_6}$ values to increase (Kinnaman et al., 2007; Kniemeyer et al., 2007; Valentine et al., 2010).

We incorporated this microbial hydrocarbon oxidation into the mixing model by assuming that its extent is correlated with the amount of biogenic gas in the mixture. We have modeled this by assuming that the amount of microbial oxidation is equal to the mixing ratio of biogenic gas raised to a power (with maximum and minimum mixing ratios of 0 and 1 respectively). We found the power, 2.1, that best fit the data by minimizing the combined residuals of the modeled and measured $\log(C_i/C_{2-4})$ values as a function of $\delta D_{CH_4}$ and $\Delta_{18}$ temperature (Fig. 3a and b). Importantly, both the $C_i/C_{2-4}$ vs. $\delta D_{CH_4}$ and vs. $\Delta_{18}$ temperature can be fit with the same amount of oxidation as a function of the biogenic mixing ratio. Although this is an arbitrary parameterization of microbial oxidation, the essential point is that the data are consistent with increasing $C_{2-4}$ oxidation at the same time more microbial gas is added. Importantly, both the $C_i/C_{2-4}$ vs. $\delta D_{CH_4}$ and vs. $\Delta_{18}$ temperature relationships can be fit with the same parameterization.

In contrast to ethane and propane, we have assumed that methane oxidation is not significant in the northern margin following Martini et al. (2003). This is based on the relatively small range of $\delta^{13}C_{C_2H_6}$ values (<3‰) and on the absence of covariation between $\delta D_{CH_4}$ and $\delta^{13}C_{C_2H_4}$ (Fig. 2c). Significant amounts of methane oxidation generally result in measurable increases in both the $\delta^{13}C$ and $\delta D$ values of the residual methane (Whiticar, 1999). Additionally, $C_{2-4}$ alkanes are often oxidized orders of magnitude more rapidly than methane in natural systems (Valentine et al., 2010). However, we note that recent laboratory manipulations suggest that anaerobic microbial oxidation of methane may be occurring within the Antrim Shale (Wuchter et al., 2013). Regardless, the effect of microbial oxidation on $\Delta_{18}$ values of residual methane is unknown and thus currently difficult to incorporate into our model.

4.4. A test of the mixing model

Importantly, the model can be tested using an entirely independent constraint, the $\delta^{13}C$ of ethane. Specifically, the model calculates the amount of ethane oxidation occurring as a function of the mixing ratio of biogenic relative to thermogenic gases (or $C_i/C_{2-4}$ ratios). Therefore, if the carbon isotopic fractionation associated with microbial ethane oxidation is known, the model explicitly predicts the $\delta^{13}C_{C_2H_6}$ of the residual ethane. We performed this test using a Rayleigh distillation model (Criss, 1999) with a range of independently measured carbon-isotope fractionation factors for microbial ethane oxidation from Kinnaman et al. (2007), an initial $C_i/C_{2-4}$ ratio of 5.8 for the thermogenic end member (as in the model above), and initial $\delta^{13}C_{C_2H_4}$ value of $-52.9$, the lowest observed $\delta^{13}C_{C_2H_4}$ from the northern margin gases. We note that the fractionation factors used here are for aerobic ethane oxidation as these are the only known isotopic fractionation factors for microbial ethane oxidation.

The model’s predicted $\delta^{13}C_{C_2H_6}$ vs. $C_i/C_{2-4}$ values are consistent with the measured values from wells sampled in this study and other measured wells (Fig. 4). Although some measurements fall outside of the predicted envelope of values (Fig. 4), these divergences are not surprising as it is unlikely that all samples start with the exact same $C_i/C_{2-4}$ ratio or ethane $\delta^{18}C$ value. Regardless, the agreement between the modeled vs. measured $\delta^{13}C_{C_2H_6}$ values further demonstrates the plausibility of the mixing/oxidation model we present and provides an independent check on the calculated amount of oxidation occurring in the samples. The model also helps to validate our assumption that methanogens in the Antrim Shale generate methane with a $\Delta_{18}$-based temperature near $-20$°C. If that temperature was 40 to 45°C, with no microbial oxidation of $C_{2-4}$ gases, then the measured $\Delta_{18}$ vs. $C_i/C_{2-4}$ data could be reproduced by the model, but the $\delta D_{CH_4}$ vs. $C_i/C_{2-4}$ would still result in a misfit. Adding microbial oxidation to the model would correct the fit for $C_i/C_{2-4}$ vs. $\delta D_{CH_4}$, but would then result in a misfit for $\Delta_{18}$ vs. $C_i/C_{2-4}$. In other words, only model parameters with biogenic methane at or near internal isotopic equilibrium at $-20$°C can satisfy all of the measured isotopic and compositional relationships.

4.5. A discrepancy between $\Delta_{18}$ temperatures and previous estimates of the amount of thermogenic gas in the Antrim Shale

Although the model describes the observed trends in both the isotopic and compositional parameters, it contrasts with previous studies in that it requires a larger thermogenic contribution (20 to 80% thermogenic) to gases from the northern margin of the Antrim Shale (Table 5) than previously proposed (<20%; Martini et al., 1996, 1998, 2003). It is important to consider that the values in Table 5 are modeled results and depend on the assumed $\Delta_{18}$ and $\delta D_{CH_4}$ value of the thermogenic end member, with higher $\Delta_{18}$-based temperatures and/or more depleted $\delta D_{CH_4}$ values resulting in lower required amounts of the thermogenic component or vice-versa. For example, choosing the formation temperature for the thermogenic end member to be the maximum $\Delta_{18}$ temperature observed, 115°C, changes the average thermogenic content of the gases to 50% (±28%, 1σ). Regardless, the higher-than-expected proportions of thermogenic gas (>20%) for some samples are an inescapable conclusion of the $\Delta_{18}$ temperatures: Even if the thermogenic component gases formed at infinite temperatures ($\Delta_{18} = 0$), mixing with a low temperature (~20°C) gas would result in the average $\Delta_{18}$ temperature for the northern margin gases measured here, 67°C, to be 25% thermogenic.

Furthermore, the maximum amount of $C_{2-4}$ relative to $C_{1-4}$ alkanes measured in the northern margin gases supports a higher-than-expected proportion of thermogenic gases. Specifically, in the northern margin, $C_{2-4}$ alkanes can represent up to ~10% of all $C_{1-4}$ alkanes (Table 3). If all gases were >80% biogenic, then the original thermogenic end member would need to be at least 50% $C_{2-4}$ (assuming...
Table 4
Thermogenic and biogenic end member values for mixing calculation

<table>
<thead>
<tr>
<th>Thermogenic end member</th>
<th>Biogenic end member</th>
</tr>
</thead>
<tbody>
<tr>
<td>δDCH4 (‰)</td>
<td>–207d</td>
</tr>
<tr>
<td>δ13CCH4 (‰)</td>
<td>–55c</td>
</tr>
<tr>
<td>δD2H2O,liquid (‰)</td>
<td>–11f</td>
</tr>
<tr>
<td>Δ18-alkanes T (°C)</td>
<td>144g</td>
</tr>
<tr>
<td>C1/C2-4</td>
<td>5.8h</td>
</tr>
<tr>
<td></td>
<td>∞i</td>
</tr>
</tbody>
</table>

* a Source: Maximum value of northern margin gases from the Antrim Shale (Martini et al., 2003).
* b Source: Minimum value of northern margin gases from the Antrim Shale (Martini et al., 1998).
* c Source: As discussed in the text.
* d Maximum and minimum δD2H2O,liquid were taken from units within the Antrim Shale, but outside of the northern margin. We have used samples from outside of the northern margin due to the large degree of fluid migration and mixing in the Michigan Basin (Wilson and Long, 1993; Martini et al., 1996, 1998; McIntosh and Walter, 2005), which lessens than chance of observing end-member compositions in the northern margin.
* e Source: Maximum value of formation waters within the Antrim Shale (Wilson and Long, 1993). Specifically from the Berea Sandstone.
* f Source: Minimum value of formation waters in the Antrim Shale (Martini et al., 2003). Specifically from the western margin.
* g Source: Estimated as discussed in the text.
* h Source: Average temperature of wells measured in this study (Table 3).
* i Source: Average value for thermogenic gases from the central basin of the Antrim Shale and from Silurian formation gases (Martini et al., 1998, 2003).
* j Biogenic gases are assumed to generate pure methane.

Thus, a significant question is why the original estimate for the amount of thermogenic relative to biogenic gas differs from those based on the constraints provided by the Δ18 temperatures and δDCH4 values in the mixing/oxidation model. The previous estimation was based on the linear relationship observed between δDCH4 and δD2H2O. Because this slope was similar to those observed in some natural settings dominated by hydrogenotrophic methanogens (e.g., Schoell, 1980), it was assumed that most of the gases were biogenic in origin, with a minor thermogenic component included to accommodate the presence of C2+-alkanes. This is a straightforward interpretation of the cause of the relationship between δDCH4 and δD2H2O. However, this relationship is not unique and can also be interpreted as a mixing line as above. When the additional constraints provided by the C1-4 concentrations and Δ18 temperatures are also included in the analysis, significant proportions of thermogenic gases become required in many of the samples. We stress that despite this greater-than-expected proportion of thermogenic gas in the samples, the Δ18 values indicate that biogenic gases also represent a significant, and in many cases dominant proportion of gases in the Antrim Shale (Table 5; Martini et al., 1996, 1998, 2003).

4.6. Potential implications of measured Δ18 values and the mixing/oxidation model for the generation and source of hydrocarbon gases in the Antrim Shale

If this mixing/oxidation model is correct, it has significant implications for the history of gas generation and migration in the Antrim Shale. Specifically, the formation temperature of the thermogenic component is, at a minimum, 115 °C, and, as modeled here, ~140 °C. The northern margin of the Antrim Shale, based on vitrinite reflectance data (R0 between 0.4 and 0.6% (Cercone and Pollack, 1991) has remained below ~60 °C (Hunt, 1996). The Δ18 temperatures, therefore, demonstrate that the thermogenic components are unlikely to be locally sourced. Formations that could have reached these elevated temperatures include (i) the Antrim Shale in the central basin, which has vitrinite reflectance values as high as 1.0% (Martini et al., 2003), consistent with maximum temperatures of ~160 °C (Hunt, 1996); and (ii) more deeply buried Silurian and older strata underlying the Antrim Shale, that likely reached maximum burial temperatures above 100 °C (Cercone and Pollack, 1991) to 150 °C (Luczaj et al., 2006).

An additional constraint on the origin of the thermogenic gases in the northern margin is the predicted minimum ΔDCH4 value of ~207/1000 for the thermogenic component. This value may eliminate the central basin of the Antrim Shale as a potential source because gases there exhibit δDCH4 values between ~270/1000 to ~280/1000, though only two gases have been measured (Martini et al., 2003). Silurian-aged (or older) thermogenic gases derived from below the Antrim Shale are also plausible sources: stratigraphically equivalent formations from the Appalachian Basin in other Devonian, Silurian, and Ordovician aged formations commonly contain methane with δD values greater than ~200/1000 (Osborn and McIntosh, 2010).

Table 5
Modeled relative concentrations of biogenic gas in Antrim Shale samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Proportion of biogenic gas (%) ± a (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butcher C3-24</td>
<td>75 ± 6.1</td>
</tr>
<tr>
<td>Lake Horicon D4-15</td>
<td>49 ± 1.5</td>
</tr>
<tr>
<td>Marlatt 1-7</td>
<td>66 ± 8.9</td>
</tr>
<tr>
<td>North Charlton A1-18</td>
<td>77 ± 4.1</td>
</tr>
<tr>
<td>State Charlton C4-18</td>
<td>44 ± 0.1</td>
</tr>
<tr>
<td>State Charlton C4-31</td>
<td>65 ± 3.4</td>
</tr>
<tr>
<td>State Loud B4-28</td>
<td>26 ± 6.4</td>
</tr>
<tr>
<td>State Loud C2-31</td>
<td>18 ± 0.2</td>
</tr>
<tr>
<td>State Charlton A3-32</td>
<td>54 ± 7.8</td>
</tr>
<tr>
<td>Weber A4-10</td>
<td>75 ± 8.4</td>
</tr>
</tbody>
</table>

* a Errors are derived by propagating the 1σ errors of the Δ18 values of the samples through the calculation. They do not include the potential errors associated with our estimates of the end-member compositions.
Large scale mixing of gases from deeper portions of the basin, whether from the Antrim Shale or other units, could be reflected not only in the hydrocarbon composition of the gases, but in the chemical and isotopic compositions of the co-produced waters. This is because mixing of biogenic and thermogenic gases may and perhaps should co-occur with mixing of deeper basinal brines, which carry thermogenic gas, and fresher surficial waters, which contain biogenic gas. This prediction is borne out: the $\Delta_{18}$-based temperatures correlate strongly with the $\delta^D$ values of the co-produced waters (Fig. 2f), with hotter temperatures correlating to enriched $\delta^D_{H_2O}$ values. As demonstrated by Wilson and Long (1993) and Martini et al. (1998), $\delta^D_{H_2O}$ values of waters in the Michigan Basin and northern margin of the Antrim Shale correlate with the salt composition of the waters. Specifically, more depleted $\delta^D_{H_2O}$ values correlate with a low salt, meteoric water end member while more enriched $\delta^D_{H_2O}$ values correspond to waters with a higher salt content. Wilson and Long (1993) and Martini et al. (1998) interpreted this relationship to indicate the mixing of basinal brines with meteoric water in the Michigan Basin, which is consistent with hydrological models of the Michigan Basin (e.g., McIntosh, 2011) and tracer studies using radiogenic isotopes (e.g., Ma et al., 2005).

Thus, the relationship between $\Delta_{18}$ temperatures and $\delta^D_{H_2O}$ values is consistent with previous geological, geochemical, and hydrological studies of Michigan Basin fluids and demonstrates the importance of interpreting clumped-isotope compositions of gases in both an environmental and geological context.

In summary, our finding of relatively high apparent temperatures for methane in Antrim Shale gases leads to two key conclusions: (i) there is more thermogenic gas in the system than previously proposed and (ii) that thermogenic component of the gas is probably derived from depth (from the basin center or below the northern margin of the Antrim Shale) and migrated to the shallow depths the Antrim Shale currently occupies on the basin rim. This is significant because the Antrim Shale is considered an end member of unconventional gas reservoirs in which the vast majority of gas formed in situ via microbial methanogenesis (Curtis, 2002) with the thermogenic component largely sidelined in subsequent interpretations. We interpret the $\Delta_{18}$ temperatures to indicate a different story: the biogenic gases are indeed derived in situ and represent ~50% of the gas. However the thermogenic component is an important contributor to the system (also ~50%) and formed elsewhere, mixing with the biogenic gas via migration. Thus the northern margin of the Antrim Shale can be considered a pseudo unconventional system—about half of the gas formed in situ while the other half formed elsewhere and migrated to the shale. Thus care should be taken in the use of the Antrim Shale as the type model for biogenic natural gas deposits (Curtis, 2002). Additionally, the importance of the relative amounts of biogenic and thermogenic gases in the Antrim Shale should factor into future calculations that suggest such deposits are major contributors of methane to the inventory of greenhouse gases in the atmosphere (Formolo et al., 2008). This alternative conclusion demonstrates the potential of $\Delta_{18}$ temperatures in untangling and quantifying the sources of gas to economically important gas reservoirs.

### 5. The Meaning of $\Delta_{18}$ Temperatures for Naturally Occurring Biogenic Samples

In the Antrim Shale, samples that yielded the lowest $\Delta_{18}$ temperatures have properties consistent with a dominantly biogenic origin. Similarly low $\Delta_{18}$ temperatures for biological samples were previously observed in biogenic methane derived from biodegraded oils in the Gulf of Mexico (Stolper et al., 2014b) and other sedimentary systems (Wang et al., in press). A simple interpretation of these measurements is that, in these environments, methanogens generate methane in or close to internal isotopic equilibrium. Two key questions brought up by these results are: (i) what are the driving biochemical reasons that biogenic methane in many natural settings are near internal isotopic equilibrium; and (ii) how common is this result and is it dependent on the environmental setting. To probe these questions, and thus help inform our interpretation of the measured composition of the Antrim biogenic component, we additionally measured methane created by methanogens from low-temperature seep environments (Santa Barbara and Santa Monica Basins), a pond from the Caltech campus, and methane generated by methanogens in culture incubations (Tables 6 and 7, Fig. 5).

Methane samples from Santa Barbara and Santa Monica Basins yield $\Delta_{18}$-based temperatures from 6 to 16°C (Table 6) and are within 2σ error in all cases of their present bottom-water temperatures (5 to 9°C; Table 6). We consider these samples to be nearly pure biogenic gas because they have low $\delta^{13}C$ values (~66‰ to ~70‰), generally indicative of microbially derived methane (Whiticar, 1999), and are nearly pure methane (>99.5%).

In contrast, the pond sample yielded a negative $\Delta_{18}$ value (Fig. 5; Table 6), which is not thermodynamically possible for any sample in internal isotopic equilibrium at any temperature. This sample demonstrates that natural biogenic methane can yield $\Delta_{18}$ values strongly out of equilibrium. Data from laboratory cultures confirm this observation: all methane from cultured methanogens yield $\Delta_{18}$ values that differ from those predicted by their known growth temperatures, and also yield, in some cases, samples with negative $\Delta_{18}$ values (Table 7, Fig. 5). As discussed above, that such disequilibrium can occur was previously discussed and demonstrated in Stolper et al. (2014b) and presented in preliminary reports by this group (Stolper et al., 2014c; Eiler et al., 2014b) and preliminary reports by others (Gruen et al., 2014). Such observations have also recently been confirmed by Wang et al. (in press).

The interpretation of $\Delta_{18}$ values for methane from pure culture experiments is ambiguous because the observed disequilibrium could result from several different processes. For example, production of methane with continuously changing $\delta^D$ and $\delta^{13}C$ values due to Rayleigh distillation or changes in isotopic fractionation factors (Valentine et al., 2004), even if $\Delta_{18}$ values represented internal isotopic equilibrium, could result in disequilibrium $\Delta_{18}$ values due to the potential non-linear mixing effects on $\Delta_{18}$ values (Eiler et al., 2014d).
5.1. Comparison of $\Delta_{18}$ temperatures to the $\delta D_{\text{CH4}} - \delta D_{\text{H2O, liquid}}$ heterogeneous phase geothermometer

Other geothermometers can be used to calculate 'apparent equilibrium temperatures' (Zhang, 1994) of methane formation. For example, equilibrium D/H fractionations between methane and water (i.e., $\alpha_{\text{CH4-H2O, liquid}}$) are a function of temperature (Bottinga, 1969; Richet et al., 1977; Horibe and Craig, 1995). We calculated the departure of $\alpha_{\text{CH4-H2O, liquid}}$ from the expected value for equilibrium at the given environmental or experimental temperature as discussed in the methods section above (Section 3.4). This departure is given by the quantity, $\left(\frac{\text{[measured } \alpha_{\text{CH4-H2O, liquid}}\text{]} - \text{[predicted equilibrium } \alpha_{\text{CH4-H2O, liquid}}\text{]}}{\text{[predicted equilibrium } \alpha_{\text{CH4-H2O, liquid}}\text{]}}\right) \times 1000$. We also calculated departure from equilibrium for $\Delta_{18}$ values as $\left(\frac{\text{[measured } ^{18}\text{R}/^{18}\text{R}] - \text{[predicted equilibrium } ^{18}\text{R}/^{18}\text{R}]}}{\text{[predicted equilibrium } ^{18}\text{R}/^{18}\text{R}]\right)} \times 1000$. As given in equation 1, $^{18}\text{R}/^{18}\text{R}$ is $(\Delta_{18}/1000 + 1)$ and is analogous to $\alpha_{\text{CH4-H2O, liquid}}$ except it is for a single phase.

The departure from equilibrium in $\Delta_{18}$ and $\alpha_{\text{CH4-H2O, liquid}}$ are compared in Fig. 6. In this figure, a value of 0% indicates that the measured quantity is identical to that expected for isotopic equilibrium. The origin, therefore, represents methane that is both in internal isotopic equilibrium and in hydrogen-isotopic equilibrium with water. Two key observations can be made about the data plotted in Fig. 6: (i) there are multiple points that plot in and around the origin, representing samples in equilibrium. And (ii) the degree of disequilibrium in $\Delta_{18}$ is correlated with that in $\alpha_{\text{CH4-H2O, liquid}}$. Put another way, disequilibrium in isotopic clumping appears to be related to disequilibrium in bulk D/H compositions. As discussed, above this relationship was first identified by Stolper et al. (2014c) and has recently been confirmed by Wang et al. (in press).

A simple interpretation of this data array is that when biogenic methane is in internal isotopic equilibrium (and $\Delta_{18}$ values yield sensible temperatures), it is in isotopic equilibrium with its source waters and plots at the origin of Fig. 6. This was not an unforeseen result—we expect that it is unlikely at relatively low ($<100$ °C) temperatures that methane is capable of exchanging hydrogen solely with itself in order to attain internal isotopic equilibrium. Instead methane presumably exchanges hydrogen with some other phase in order to be in internal isotopic equilibrium. Although there are multiple sources of reduced H that are used by methanogens to reduce CO$_2$ to CH$_4$ (Thauer, 1998), it is generally assumed, based on many experimental results, that these hydrogen species are in rapid isotopic equilibrium with hydrogen in water and thus that the D/H ratio of water ultimately controls the D/H ratio of methane (Daniels et al., 1980; Schoell, 1980; Balabane et al., 1987; Sugimoto and Wada, 1995; Whiticar, 1999; Valentine et al., 2004; Yoshioka et al., 2004; Eiler, 2007, 2013; Stolper et al., 2014a,b). Alternatively, kinetic isotope effects during methane generation could create disequilibrium $\Delta_{18}$ values. It is thus significant that methane from a natural setting also exhibits a disequilibrium $\Delta_{18}$ value, indicating that such effects cannot be due solely to culturing artifacts.

### Table 6: Environmental biogenic samples

<table>
<thead>
<tr>
<th>Environmental Name</th>
<th>Sample Name</th>
<th>Lat</th>
<th>Long</th>
<th>Environmental T (°C)</th>
<th>Depth (m)</th>
<th>$\delta^{13}C($)</th>
<th>$\delta^{18}C(H_2O)($)</th>
<th>$\delta^{18}C(CH_4)$</th>
<th>$\Delta_{18}$</th>
<th>Expected $\delta^{18}C(CH_4)$</th>
<th>$\Delta_{18}$ based temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>J2-737 Santa Monica Basin</td>
<td>33.6397</td>
<td>118.6470</td>
<td>5.3</td>
<td>897</td>
<td>181.8</td>
<td>0.12</td>
<td>6.52</td>
<td>0.28</td>
<td>6.85</td>
<td>0.12</td>
<td>8</td>
</tr>
<tr>
<td>J2-744 Santa Barbara Basin</td>
<td>34.3962</td>
<td>120.1483</td>
<td>9.2</td>
<td>230</td>
<td>179.2</td>
<td>0.12</td>
<td>6.85</td>
<td>0.27</td>
<td>6.85</td>
<td>0.12</td>
<td>8</td>
</tr>
<tr>
<td>J2-749 Santa Monica Basin</td>
<td>33.6966</td>
<td>119.0536</td>
<td>6.0</td>
<td>566</td>
<td>169.5</td>
<td>0.12</td>
<td>6.67</td>
<td>0.01</td>
<td>6.52</td>
<td>0.27</td>
<td>6.85</td>
</tr>
<tr>
<td>Caltech Pond</td>
<td>34.1386</td>
<td>118.1255</td>
<td>20</td>
<td>&lt;2</td>
<td>319.5</td>
<td>0.11</td>
<td>61.2</td>
<td>0.01</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

- Referenced to VSMOW.
- Referenced to the VPDB scale.
- $\delta^{13}C($) and $\delta^{18}C(H_2O)($) are compared in Fig. 6. In this figure, a value of 0% indicates that the measured quantity is identical to that expected for isotopic equilibrium. The origin, therefore, represents methane that is both in internal isotopic equilibrium and in hydrogen-isotopic equilibrium with water. Two key observations can be made about the data plotted in Fig. 6: (i) there are multiple points that plot in and around the origin, representing samples in equilibrium. And (ii) the degree of disequilibrium in $\Delta_{18}$ is correlated with that in $\alpha_{\text{CH4-H2O, liquid}}$. Put another way, disequilibrium in isotopic clumping appears to be related to disequilibrium in bulk D/H compositions. As discussed, above this relationship was first identified by Stolper et al. (2014c) and has recently been confirmed by Wang et al. (in press).

A simple interpretation of this data array is that when biogenic methane is in internal isotopic equilibrium (and $\Delta_{18}$ values yield sensible temperatures), it is in isotopic equilibrium with its source waters and plots at the origin of Fig. 6. This was not an unforeseen result—we expect that it is unlikely at relatively low ($<100$ °C) temperatures that methane is capable of exchanging hydrogen solely with itself in order to attain internal isotopic equilibrium. Instead methane presumably exchanges hydrogen with some other phase in order to be in internal isotopic equilibrium. Although there are multiple sources of reduced H that are used by methanogens to reduce CO$_2$ to CH$_4$ (Thauer, 1998), it is generally assumed, based on many experimental results, that these hydrogen species are in rapid isotopic equilibrium with hydrogen in water and thus that the D/H ratio of water ultimately controls the D/H ratio of methane (Daniels et al., 1980; Schoell, 1980; Balabane et al., 1987; Sugimoto and Wada, 1995; Whiticar, 1999; Valentine et al., 2004; Yoshioka et al., 2004; Eiler, 2007, 2013; Stolper et al., 2014a,b). Alternatively, kinetic isotope effects during methane generation could create disequilibrium $\Delta_{18}$ values. It is thus significant that methane from a natural setting also exhibits a disequilibrium $\Delta_{18}$ value, indicating that such effects cannot be due solely to culturing artifacts.
Therefore, it may not be surprising that the $D_{18}$ values and $\alpha_{\text{CH}_4-\text{H}_2\text{O,liquid}}$ are related. This is discussed in more detail in the Appendix (specifically A2).

We note that there is scatter around the origin in Fig. 6, the point of mutual isotopic equilibrium between CH$_4$ and H$_2$O and internal isotopic equilibrium in CH$_4$. This could result from the fact that the hydrogen isotopic composition of cellular waters, which actually controls the value of $D_{18}$, are not necessarily equivalent to the isotopic composition of the environmental waters (e.g., Kreuzer-Martin et al., 2005) or that our estimates for the $D_{18}$ for the environmental pore waters (e.g., we assume the $D_{18}$ value is 0$\%$ for all marine systems) is incorrect. A similar comparison for $\delta^{13}$C values is not possible because $\delta^{13}$C values of dissolved inorganic carbon or CO$_2$ were not measured for any samples and are difficult to estimate for the environmental samples as they can vary strongly (up to $\sim$40$\%$) in sedimentary systems (Claypool and Kaplan, 1974).

### 5.2. An isotopic model of methanogenesis

To explore the relationship indicated by Fig. 6, we created a model for kinetic and equilibrium isotope effects associated with microbial methanogenesis. This model follows the mathematical framework introduced by Rees (1973) to describe the bulk isotopic composition of sulfide generated by sulfate-reducing bacteria and is influenced by the ideas presented in Blair (1998), Valentine et al. (2004), and Penning et al. (2005) that hypothesized and provided evidence that the bulk isotopic composition of methane is controlled in part by the overall reversibility of the enzymes involved in methanogenesis. The model also follows the initial ideas raised in Stolper et al. (2014b) that the reversibility of these enzymes also controls the clumped isotopic composition of methane. A full derivation of the model and assumptions implicit to it are given in the Appendix and follows the initial work presented by Eiler et al. (2014b). The model is similar in structure to that

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth T (°C)</th>
<th>$\delta D (%)$</th>
<th>$\pm^b$</th>
<th>$\delta^{13}$C (‰)</th>
<th>$\pm^b$</th>
<th>$\Lambda (%)$</th>
<th>$\pm^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. marburgensis</em></td>
<td>70</td>
<td>317.2</td>
<td>0.12</td>
<td>-54.7</td>
<td>0.01</td>
<td>1.16</td>
<td>0.25</td>
</tr>
<tr>
<td><em>M. marburgensis</em></td>
<td>65</td>
<td>367.3</td>
<td>0.11</td>
<td>-44.7</td>
<td>0.01</td>
<td>0.89</td>
<td>0.27</td>
</tr>
<tr>
<td><em>M. marburgensis</em></td>
<td>50</td>
<td>356.7</td>
<td>0.12</td>
<td>-40.1</td>
<td>0.01</td>
<td>0.78</td>
<td>0.26</td>
</tr>
<tr>
<td><em>M. marburgensis</em></td>
<td>45</td>
<td>317.9</td>
<td>0.1</td>
<td>-48.1</td>
<td>0.01</td>
<td>1.85</td>
<td>0.29</td>
</tr>
<tr>
<td><em>M. marburgensis</em></td>
<td>30</td>
<td>476.0</td>
<td>0.12</td>
<td>-65.1</td>
<td>0.01</td>
<td>-1.59</td>
<td>0.25</td>
</tr>
<tr>
<td><em>M. barkeri</em></td>
<td>37</td>
<td>-491.6</td>
<td>0.12</td>
<td>-56.7</td>
<td>0.01</td>
<td>-4.66</td>
<td>0.26</td>
</tr>
</tbody>
</table>

*a* Referenced to VSMOW.

*b* Errors are the standard deviation of a single measurement as all samples were only measured once.

*c* Referenced to the VPDB scale.

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Fig. 5. Measured or modeled $D_{18}$ values of biogenic gases from natural or experimental systems. The theoretically derived curve describes the expected $D_{18}$ value for methane isotopically equilibrated internally as a function of temperature and is from Stolper et al. (2014a). The Gulf of Mexico samples were reported in Stolper et al. (2014b). The Antrim biogenic model end-member point is the value used in the mixing model of the Antrim Shale samples to describe the biogenic end member and is thus required to be on the equilibrium curve.
recently proposed by Yoshinaga et al. (2014) for anaerobic methane oxidation and, as discussed above, Wang et al. (in press) for methanogenesis.

Three key assumptions are made in the model: (i) following the 'differential reversibility' hypothesis of Valentine et al. (2004), we assume that all metabolic intermediates between CO$_2$ and H$_2$ and a methyl group bound to a co-factor are in isotopic equilibrium (Fig. A3). (ii) We assume that H$_2$ and thus all methane precursors are in isotopic equilibrium with cellular H$_2$O (Valentine et al., 2004). (iii) We assign the rate-limiting step in methane production to be the final hydrogenation step catalyzed by methyl-coenzyme M reductase (MCR; Fig. A3). We note that the validity of the assumption (i), that all metabolic intermediates between the methyl group and CO$_2$ and H$_2$ are in isotopic equilibrium, is most valid for natural environments in which H$_2$ partial pressures are low and where there is a relatively small chemical potential contrast between the reactants and products of methanogenesis; however, as discussed in Valentine et al. (2004), in pure culture experiments where these chemical potential gradients are large, the metabolic intermediates may not be in isotopic equilibrium with CO$_2$ and H$_2$. In such a case, prior steps before MCR may be rate limiting. We discuss this in more detail below in Section 5.4 after the description of the model.

In the model, we vary the degree of reversibility of MCR to explore whether the trend seen in Fig. 6 can be reproduced (Fig. A3). The capacity for MCR to catalyze both the addition and removal of a hydrogen from methane, i.e. that it is reversible, has been demonstrated using isotopic labeling techniques on MCR from the methanogen *M. marburgensis* (Scheller et al., 2010). Moreover, the capacity of anaerobic methanotrophs to synthesize MCR has led to the hypothesis that these organisms use the enzymes of methanogenesis (starting with MCR) in reverse to consume methane (e.g., Hallam et al., 2004; Thauer, 2011).

The required inputs for the model are given in Table 8. Rate constants are given in this table for individual isotopologue ratios. The model describes the isotopic composition of methane formed by methanogens as a function of the reversibility of methyl-coenzyme M reductase. It is described in the text and Appendix.

![Fig. 6. Comparison of measured $\Delta_{18}$ values to those expected if the methane formed in internal isotopic equilibrium vs. the difference in measured $\delta^{18}$CH$_4$ and $\delta^{18}$H$_2$O,liquid values (expressed as a difference in ratios, $\alpha$CH$_4$-H$_2$O,liquid) compared to that expected for heterogeneous phase isotopic equilibrium. Deviations in measured $\Delta_{18}$ values vs. those expected for equilibrium are represented in this figure through the comparison of $^{18}R/^{18}R^*$ values. As given in Eq. (1), $^{18}R/^{18}R^* = (\Delta_{18}/1000 + 1)$. All samples near the origin, which is the point at which methane is both internally isotopically equilibrated amongst all isotopologues and isotopically equilibrated for hydrogen isotopes with H$_2$O are from marine samples and assumed to have a $\delta^{18}$H$_2$O,liquid = 0. The Caltech pond sample, the only environmental sample not located near the origin, was measured to have a $\delta^{18}$H$_2$O,liquid of -16. One pure-culture sample (the 65°C experiment) was measured to have a $\delta^{18}$H$_2$O,liquid of -37.8 for the culture media. We assume that this value is the same for all pure-culture experiments. We assume a conservative error for assignment of $\delta^{18}$H$_2$O,liquid of 25‰ to account for our estimations and potential for differences in the $\delta^{18}$H$_2$O,liquid of the environmental waters vs. the intracellular waters of the methanogens. The model describes the isotopic composition of methane formed by methanogens as a function of the reversibility of methyl-coenzyme M reductase. It is described in the text and Appendix.](image-url)
isotopologues. When a rate constant directly constrains a specific bulk fractionation factor between two phases (\(\alpha\)), \(\alpha\) is given as well. In the model, when MCR is assumed to be completely reversible, CH\(_4\) is required to be in isotopic equilibrium with CO\(_2\) and H\(_2\)O. The equilibrium isotopic fractionations between CH\(_4\) and H\(_2\)O and CO\(_2\) were calculated using \(\alpha_{CH4-H2O,liquid}\) as described above and \(\alpha_{CH4-CO2}\) values derived from Richet et al. (1977; Table 8). For the model, we assume an average temperature of 20 °C. Because the data in Fig. 6 come from a variety of different environments and environmental temperatures, this is clearly an approximation. However, the purpose of the model is to gain first-order insight into the meaning of \(\Delta_{18}\) values observed in biogenic samples, and thus we consider this approximation to be acceptable for the time being. We calculated the equilibrium constants for homogeneous phase internal isotopic equilibrium between \(^{13}\)CH\(_3\) and \(^{12}\)CH\(_2\)D with all other methane isotopologues (see Appendix) using the statistical mechanical model described in Stolper et al. (2014a).

In the model, when the rate of methane production exceeds that of consumption (through the reversal of MCR), a kinetic isotope effect can be expressed. We assume that the maximum kinetic hydrogen isotope effect (\(\alpha_{CH4-H2O,liquid}\)) is 0.528. This value is based on data from our study and is similar in magnitude to that found by pure-culture experiments on hydrogenotrophic methanogens from Balabane et al. (1987), 0.567. For the maximum kinetic carbon isotope \(\alpha_{CH4-CO2}\), we assume a value of 0.92. This is intermediate to the maximum value observed in pure-culture experiments (0.93; Botz et al., 1996; Conrad, 2005), and from natural settings (up to 0.91; Whiticar, 1999). These values are fixed in the model. We then varied the rate constants for the kinetic isotope effect for \(^{13}\)CH\(_3\)D and \(^{12}\)CH\(_2\)D formation relative to \(^{12}\)CH\(_4\) (Table 8) to achieve a best fit to the empirical data (Fig. 6). To first order, the relationship observed between \(\alpha_{CH4-H2O,liquid}\) and \(\Delta_{18}\) can be reproduced by our model.

5.3. Testing the model

The structure of our model and simple trend of the data in Fig. 6 make it clear that some combination of rate constants for the formation of \(^{13}\)CH\(_3\)D and \(^{12}\)CH\(_2\)D relative to \(^{12}\)CH\(_4\) will provide an adequate fit to the data. Nevertheless, the basic validity of the model can be tested by examining whether the required rate constants for \(^{13}\)CH\(_3\)D and \(^{12}\)CH\(_2\)D relative to \(^{12}\)CH\(_4\) formation are chemically reasonable, and quite similar relative to the isotope effects required for a single \(^{13}\)C or D substitution. Such a coincidence was not a forgone conclusion, and helps to demonstrate the self-consistency of our model.

Furthermore, the model is testable through the independent measurement of the abundances of \(^{13}\)CH\(_3\)D and \(^{12}\)CH\(_2\)D because only specific trends, based on this model, are allowed (Fig. 7). I.e., if their abundances can be measured independently this model can be tested explicitly (preliminary results for such measurements were presented by Eiler et al., 2014b).

5.4. Model considerations

The analysis above indicates that the model is plausible, makes testable predictions, and is consistent with the observed data (Fig. 6). However, the model makes several simplifications that we consider more closely here:

1. The model assumes that the final step of methanogenesis, the hydrogenation of a methyl group catalyzed by MCR, is the rate-limiting step, and that all previous steps are in isotopic equilibrium. However, in some pure-culture experiments it is thought that under high hydrogen partial pressures (at least > 400 Pa as in the experiments here), prior steps in the generation of methane may be rate limiting (Valentine et al., 2004; Penning et al., 2005) such that CO\(_2\) in the growth medium is not in isotopic equilibrium with the metabolic intermediates. This is sometimes evidenced by relatively small carbon isotope fractionation factors between methane and CO\(_2\)—for example \(\alpha_{CH4-CO2}\) values range from 0.927 to 0.976 in pure-culture experiments (Valentine et al., 2004; Conrad, 2005).
As the model presented here assumes that MCR is the rate-limiting step and that all isotope effects are ‘normal,’ i.e., that products are always isotopically lighter than the reactants, it requires that $\alpha_{\text{CH}_4-\text{CO}_2}$ will be between 0.92 and 0.932. This indicates that for some pure-culture experiments, the model presented will be unlikely to fit the carbon isotope data. However, as we did not measure the $\delta^{13}$C values of the CO$_2$ in our experiments nor in most of the environmental samples, it is difficult to evaluate whether this is an issue for the data presented here. A larger range of $\alpha_{\text{CH}_4-\text{CO}_2}$ can be modeled if steps prior to MCR are allowed to be rate limiting in some conditions. Though conceptually simple, incorporation of this step introduces up to 9 new and unconstrained variables: the reversibility of the step, two forward (or reverse) and two equilibrium isotope effects for bulk hydrogen and carbon isotopic compositions, and up to two forward (or reverse) and equilibrium isotope effects for clumping reactions between $^{13}$C with D and D with D. In contrast to this model, Wang et al. (in press) present a model with 6 potentially rate-limiting steps, the diffusion of CO$_2$ into the cell, 4 hydrogenation steps, and the diffusion of CH$_4$ out of the cell. To reduce the number of unconstrained free variables in the model, they assume that CO$_2$ diffusion into the cell and CH$_4$ diffusion out of the cell are not isotopically discriminating steps, that CO$_2$ in the cell is in chemical equilibrium with CO$_2$ outside of the cell, that all hydrogenation steps for methane precursors (i.e., CO$_2$, R-CH, R-CH$_2$, R-CH$_3$, where R represents the molecule to which the carbon) have the same forward and reverse kinetic isotope effects for bulk carbon, bulk hydrogen, and clumped isotopic compositions (but with different values for bulk carbon-isotope vs. bulk hydrogen-isotope vs. clumped-isotope effects), and that all hydrogenation steps have the same forward vs. reverse reaction rate, i.e., are equally reversible or irreversible. They predict that $\alpha_{\text{CH}_4-\text{CO}_2}$ values will be between 0.96 and 0.932, but do not present or discuss $\delta^{13}$C measurements of CO$_2$ in their environmental samples vs. the prediction of the model. Future models could attempt to calculate or experimentally constrain the various isotope effects involved with all species intermediate to CH$_4$ and CO$_2$ and H$_2$. Additionally, a clear next step is to perform experiments and make measurements in natural systems in which the $\delta^{13}$C$_{\text{CO}_2}$ value is monitored along with $\delta$D$_{\text{H}_2}$O, $\delta$D$_{\text{CH}_4}$, $\delta$C$_{\text{CH}_4}$, and $\Delta_{18}$.

(2) The model implicitly requires that isotopic equilibrium between CH$_4$ and both CO$_2$ and H$_2$O is reached at the same time. However, it is possible that MCR could catalyze exchange reactions more rapidly between CH$_4$ and H$_2$O than slower back reactions to CO$_2$. In this case, isotopic equilibrium between CH$_4$ and H$_2$O and, as a consequence, internal isotopic (i.e., $\Delta_{18}$) equilibrium could be achieved without carbon-isotope equilibrium between CH$_4$ and CO$_2$. If so, a more complex model that allows different rates of isotopic exchange for H and C would be required, such as those used to describe the combined sulfur and oxygen isotope systematics of SO$_2^-$ during dissimilatory sulfate reduction (e.g., Brunner et al., 2005). This consideration raises a potentially interesting point related to possible effects on $\Delta_{18}$ values for anaerobic methane oxidation. It has recently been suggested, based on both experimental data and model constraints, that under low sulfate conditions (~1–2 mM sulfate) anaerobic methane oxidizers can nearly completely (80%) isotopically equilibrate methane with dissolved CO$_2$ (Yoshinaga et al., 2014). As discussed above, this process is thought to be catalyzed through operation of MCR in reverse (Hallam et al., 2004; Thauer, 2011), which itself, for the model of Yoshinaga et al. (2014), is up to 80% reversible. This then suggests that hydrogen-isotope-exchange reactions between CH$_4$ and H$_2$O should be at least as equilibrated if not more so than carbon isotopes between CH$_4$ and CO$_2$. Consequently, anaerobic methane oxidation could result in $\Delta_{18}$ values of residual methane that reflect the temperatures of methane oxidation at low sulfate levels. In such a scenario, the $\Delta_{18}$ values of gases from, for example, thermogenic systems, could potentially be reset to reflect lower temperatures of methane oxidation. This appears not to be the case in the Antrim Shale, indicating either limited amounts of anaerobic methane oxidation is occurring or that thermogenic gases are sorbed to shales or otherwise inaccessible to microbial communities living in fractures until active pumping in a well occurs.

(3) The model does not explicitly treat methylotrophic methanogenesis in which acetate or some other organic substrate (e.g. methanol or methylamine) is the precursor to methane. The $\Delta_{18}$ values of methane generated by this pathway, either in the lab or in nature, are unknown and will be important to constrain in future work. Nevertheless, we note that our pond sample likely contained acetoclastic methanogens, as they are commonly present in shallow, freshwater anoxic sediments (Whiticar et al., 1986), yet data from this sample are broadly consistent with the model. We speculate that – because acetoclastic methanogens also use MCR – our model may be more broadly generalizable.

Despite these model simplifications, the critical point is that clumped-isotope measurements from both culture-based and natural biogenic methane can be explained (Fig. 6) in the context of a simple, plausible, and testable (Fig. 7) model. Just as models to describe sulfate reduction in the context of isotopic measurements have evolved over the course of 40 years (Rees, 1973; Farquhar et al., 2003; Brunner and Bernasconi, 2005; Bradley et al., 2011; Wing and Halevy, 2014), we expect that more advanced and complex treatments of methanogenesis will address the issues presented above.
5.5. Implications for clumped-isotope thermometry of biogenic methane

Our model provides insight into the metabolic state of methanogens living in different environmental settings and is consistent with the, albeit limited, empirical data to-date (Fig. 6). For example, systems where low, environmentally plausible $\Delta_{18}$D temperatures have been observed are marine settings in the Santa Barbara and Santa Monica Basins, buried oil-degrading methanogenic communities in the Gulf of Mexico (Stolper et al., 2014b), and organisms buried in the Antrim Shale. In these, ‘deep biosphere’ systems and shallow marine settings, organic carbon has already been substantially degraded and even thermally altered before becoming available to methanogens and hydrogen partial pressures are likely very low (Burke, 1993). In such systems, growth and metabolic rates are slow compared to laboratory cultures of methanogens, and thus could allow internal isotopic equilibrium to be reached. For example, in the New Albany Shale, which is similar in age and depositional environment to the Antrim Shale, rates of methane generation are estimated based on accumulations of radiogenic $^4$He to have been 10$^{-8}$–10$^{-6}$ slower than equivalent laboratory manipulations (Schlegel et al., 2011). In the context of the model, MCR would be almost entirely reversible in such settings and thus would generate methane in internal isotopic equilibrium. Additionally, this insight provides independent justification for the assumption that the methanogens in the Antrim Shale generate methane at internal isotopic equilibrium.

Fast metabolic rates in which methane is generated rapidly and MCR is not completely reversible would be expected to generate methane out of isotopic equilibrium. This is also consistent with current observations: In laboratory cultures, large partial pressures of hydrogen and CO$_2$ are used to stimulate growth and generate methane rapidly. In the Caltech pond, plant litter is continually deposited at the sediment water interface that has undergone limited biodegradation, potentially stimulating rapid methanogenesis.

Based on these considerations, we predict that systems in nature where growth rates are slow, such as in marine sediments and deeply buried (many meters) continental systems, methane will form in internal isotopic equilibrium. Shallow sediments in terrestrial systems, on the other hand, where methane generation rates are high, will yield methane out of equilibrium. More broadly, this analysis indicates that $\Delta_{18}$D values of biogenic methane potentially reflect the metabolic state of methanogens and thus can be used to probe growth and metabolic rates of methanogenesis in the environment.

Additionally, our model potentially explains a long-standing discrepancy between the $\delta$D values of methane observed in culture experiments vs. in some natural settings. Specifically, in many natural settings, especially marine sediments, differences between $\delta$D$_{\text{H}_2\text{O},\text{liquid}}$ and $\delta$D$_{\text{H}_2\text{O},\text{gas}}$ are significantly less (>$100\%_\circ$; Schoell, 1980; Whiticar et al., 1986; Burke, 1993) than observed in cultures (e.g., Balabane et al., 1987; Valentine et al., 2004; Yoshioka et al., 2008; Hattori et al., 2012) and natural systems such as cow rumens (e.g., Bilek et al., 2001) and freshwater soils (e.g., Sugimoto and Wada, 1995). Explanations for this difference have included the incorporation of H from H$_2$ into methane (Burke, 1993) and variability of isotopic fractionation (Valentine et al., 2004). Our model and the $\Delta_{18}$D values suggest an alternative, though not necessarily exclusive, explanation: specifically that in marine settings and ‘deeply’ buried continental systems isotopic equilibrium between methane and local waters is reached. This equilibrium results in an $\sim$160–200$\%_\circ$ difference in $\delta$D between water and methane (Schoell, 1980; Whitticar et al., 1986). In contrast, in shallow, terrestrial systems, isotopic equilibrium is not reached and a larger kinetic isotope effect for D/H ratios of methane is expressed.

Finally, the model, as inspired by the work of Blair (1998), Valentine et al. (2004), and Penning et al. (2005) and building on the initial ideas of Stolper et al. (2014b), Stolper et al. (2014c), and Eiler et al. (2014b), demonstrates the importance of considering both kinetics and equilibrium thermodynamics in the consideration of the controls on the isotopic compositions of reactants and products not only in methanogenesis, but a range of other metabolisms. This is not a novel idea and has been long considered in the studies of dissimilatory sulfate reduction (Rees, 1973; Farquhar et al., 2003; Brunner and Bernasconi, 2005; Bradley et al., 2011; Wing and Halevy, 2014). However, the use of clumped-isotope measurements to study microbial metabolisms provides a new, distinct avenue for studying the flow of metabolites in the metabolism of organisms by providing a reference frame that is grounded in equilibrium thermodynamics and a fingerprint that remains with the molecule of interest even after separation from its formational environment.

6. CONCLUSIONS

We have demonstrated that clumped-isotope measurements of methane can be useful for quantifying the amount of biogenic vs. thermogenic methane in natural settings. In the Antrim Shale, significant quantities of both biogenic and thermogenic methane are present in different wells, with $\Delta_{18}$D-based temperatures correlating with other measured parameters such as C$_{1}/$C$_{2.4}$ ratios, $\delta$D$_{\text{H}_2\text{O},\text{liquid}}$ and $\delta$D$_{\text{H}_2\text{O},\text{gas}}$. The mixing model results show that, in contrast to previous indications, biogenic and thermogenic methane are approximately equal in importance in the northern margin of the Michigan basin. Additionally, the Antrim Shale results support previous findings that biogenic gases in natural systems frequently yield low $\Delta_{18}$D temperatures consistent with their formation environments. This was also observed in samples from offshore seeps of biogenic gas. However, a pond sample and pure-culture experiments yield $\Delta_{18}$D temperatures that are strongly inconsistent with the known environmental or experimental temperatures. We present a metabolically based model that explicitly considers the reversibility of enzymes in methanogenesis. This model predicts that when rates of methanogenesis are slow, environmentally consistent temperatures result from the internal equilibration of methane with its metabolic precursors while the unrealistic temperatures result from the
expression of kinetic isotope effects during rapid methane generation. Our model indicates that in many natural settings where methanogen growth rates are slow, such as in sedimentary basins and marine sediments, methane can be produced in or near isotopic equilibrium. By contrast, in settings that allow for high rates of methane generation such as ponds and near-surface environments, the methane is generated out of internal isotopic equilibrium. This potentially explains why methane from different environments can have drastically different D/H fractionations relative to water, and indicates that methanogens in many natural settings live near thermodynamic equilibrium, eking out a slow living.

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APPENDIX A.

A.1. Derivation of isotopically enabled methanogenesis model

In this section we derive a model to describe the concentrations of various isotopologues of methane generated during microbial methanogenesis in order to calculate δDCH4, δ13CH4, and Δ18 values. This model is based on the quantitative framework of Rees (1973) originally used to describe isotope fractionations of sulfur during dissimilatory sulfate reduction. We only deal with hydrogenotrophic methanogenesis here and do not incorporate acetoclastic or thiotrophic methanogenesis.

The generation of methane by methanogens involves many steps with 6 distinct metabolic intermediates between CO2 + H2 and CH4 (Thauer, 1998). A full description of the isotopic fractionation factors of each individual step in both the forward and backward direction is desirable, and ultimately necessary for a complete description of the isotopic ratios observed for methane in nature. However, the individual fractionation factors for 13C or D in the singly substituted isotopologues are not known for most steps, let alone for 13CH3D and 12CH2D2 (Scheller et al., 2013). We deal with this complexity by making a few simplifying assumptions regarding the metabolism of methanogens. We assume that the final step of methanogenesis in which a hydrogen atom is added to a methyl group attached to coenzyme M (CH3-S-CoM) is the rate-limiting step, and that this step proceeds sufficiently slowly compared to the previous steps that the precursor metabolites are in isotopic equilibrium (Fig. A3). This follows arguments made in Valentine et al. (2004) based on the ‘differential reversibility’ hypothesis put forward there and discussed in the text above. We additionally assume, following Botz et al. (1996), that only a small proportion of CO2 is used for biomass generation (<10%; Fuchs et al., 1979) as compared to methane production and therefore can be, to first order, neglected in the model. We note though that M. barkeri belongs to an order of methanogens that have significantly (~2-3x) higher biomass yields than other orders (Thauer et al., 2008). This organism gave the most negative Δ18 value and may indicate that incorporation of biomass yields is important for understanding the isotopic composition of methane.

We can thus, in a simplified way, describe the overall generation of methane during methanogenesis with two reactions. The first reaction between CO2 and CH3-S-CoM:

\[ \text{CO}_2 + 3\text{H}_2 + \text{H-S-CoM} \rightleftharpoons \text{CH}_3\text{S-CoM} + 2\text{H}_2\text{O} \]  

(A1.1)

where all species are in isotopic equilibrium (Fig. A3). S-CoM represents coenzyme M. The two-headed arrows indicate that the different species are at chemical and isotopic equilibrium. The rate-limiting step (where reduced H is donated from a reduced hydrogen pool) is then:

\[ \text{CH}_3\text{S-CoM} + 2\text{H} \rightarrow \text{CH}_4 + \text{H-S-CoM}. \]  

(A1.2)

In this derivation, the consequences of allowing equation A1.2 to proceed in the reverse direction will be explored.
A.2. $^{13}$CH$_4$ Derivation

The set of equations that describe the dependence of the $^{13}$CH$_4$/^{12}$CH$_4$ ratio on the degree of reversibility of equation (A1.2) are derived here for the following reactions:

\[ ^{12}$CH$_3$-S-CoM + H $\xrightleftharpoons[12CH_4 k_{rev}]{12CH_4 k_f} ^{12}$CH$_4 \]  
(A2.1)

and

\[ ^{13}$CH$_3$-S-CoM + H $\xrightleftharpoons[13CH_4 k_{rev}]{13CH_4 k_f} ^{13}$CH$_4 \]  
(A2.2)

Here, for clarity, we have left off the additional reaction in which H-S-CoM is regenerated as written in Eq. (A1.2). The rate constants describing each reaction are given next to the arrows for each step. The final step of the reaction in which methane leaves the system can be envisioned as diffusion or escape of methane out of the cell or environment such that it can no longer interact with the enzymes of the methanogens. It is assumed that all reactions follow the kinetic forms discussed in Rees (1973). Equations will only be developed for the system at steady state such that the concentrations of all metabolic intermediates are time invariant.

With this in mind, for the reaction in (A2.1),

\[ \frac{d[^{13}$CH$_4]}{dt} = 0 \]

\[ = ^{12}$CH$_3 k_f[^{12}$CH$_3$-S-CoM][H] \]

\[ -^{12}$CH$_4 k_{rev} - ^{12}$CH$_4 k_{net}[^{12}$CH$_4], \]  
(A2.3)

such that, at steady state,

\[ ^{12}$CH$_4 = \frac{^{12}$CH$_3 k_f[^{12}$CH$_3$-S-CoM][H]}{^{12}$CH$_4 k_{rev} + ^{12}$CH$_4 k_{net}}. \]  
(A2.4)

Brackets denote concentrations of the species. In writing the equations in this form, we assume there exists some pool of hydrogen (and deuterium) that is used for all reactions. Multiple enzymes donate H to methane precursors, and thus this assumption is not strictly correct (Thauer, 1998; Valentine et al., 2004). Here, we assume that all pools of hydrogen are isotopically equilibrated with water. This is generally assumed and supported by a variety of experiments and observations (Daniels et al., 1980; Schoell, 1980; Balabane et al., 1987; Sugimoto and Wada, 1995; Whiticar, 1999; Valentine et al., 2004; Yoshioka et al., 2008). However, we note this assumption has recently been questioned by Kawagucci et al. (2014). Their results suggest, as proposed by Burke (1993), that the isotopic composition of hydrogen from H$_2$ independent of the isotope composition of water, partially controls the isotopic
composition of the resultant methane. Regardless, we will proceed with the assumption that all hydrogen in methane is ultimately derived from water. Similarly, for Eq. (A2.2) we have,

\[ ^{13}\text{CH}_4 = \frac{^{13}\text{CH}_3 k_{\text{rev}}^{13}\text{CH}_2\text{S-CoM}[H]}{^{13}\text{CH}_3 k_{\text{rev}} + ^{13}\text{CH}_3 k_{\text{net}}} \]  

(A2.5)

An important approximation is used in Eqs. (A2.3)–(A2.5): a small proportion of methyl groups have deuterium added instead of a hydrogen (as written). These deuterium addition reactions are ignored as they represent <0.1% of all methane groups generated for each reaction, which we consider an acceptable approximation.

Finally, we introduce here a 'degree of reversibility' term for Eq. (A2.1). This term, 'r', introduced by Rees (1973), compares the rate of back reaction of methane into the metabolic intermediates vs. the forward generation rate of methane. In the form of an equation,

\[ r = \frac{^{12}\text{CH}_3 k_{\text{rev}}^{12}\text{CH}_2\text{S-CoM}[H]}{^{12}\text{CH}_3 k_{\text{rev}} + ^{12}\text{CH}_3 k_{\text{net}}} \]  

(A2.6)

In this framework, r can vary from 0 to 1. Combination of (A2.6) with (A2.4) results in:

\[ r = \frac{^{12}\text{CH}_3 k_{\text{rev}}}{^{12}\text{CH}_3 k_{\text{rev}} + ^{12}\text{CH}_3 k_{\text{net}}} \]  

(A2.7)

Division of (A2.5) by (A2.4) yields

\[ \frac{[^{13}\text{CH}_4]}{[^{12}\text{CH}_4]} = \frac{^{13}\text{CH}_3 k_{\text{rev}}^{13}\text{CH}_2\text{S-CoM}[H]}{^{12}\text{CH}_3 k_{\text{rev}}^{12}\text{CH}_2\text{S-CoM}[H]} \times \frac{^{12}\text{CH}_3 k_{\text{rev}} + ^{12}\text{CH}_3 k_{\text{net}}}{^{13}\text{CH}_3 k_{\text{rev}} + ^{13}\text{CH}_3 k_{\text{net}}} \]  

(A2.8)

This can be rewritten using the following notations:

\[ \frac{^{13}\text{CH}_3 R}{^{12}\text{CH}_3 R} = \frac{[^{13}\text{CH}_4]}{[^{12}\text{CH}_4]} \text{ and } \frac{^{13}\text{CH}_3 R}{[^{12}\text{CH}_3 R]} = \frac{[^{13}\text{CH}_4]}{[^{12}\text{CH}_4]} \]  

(A2.9a and b)

and

\[ k_j = \frac{k_j}{^{13}\text{CH}_3 k_{\text{rev}}} \]  

(A2.10)

'Unsubstituted' refers to an isotopologue without $^{13}$C or D: i.e., $^{13}$CH$_2$S-CoM or $^{13}$CH$_4$. Substituting Eqs. (A2.7), (A2.9) and (A2.10) into (A2.8) and assuming that there is no isotopic fractionation during the net removal of methane (i.e., $^{13}$CH$_4\times$ net = 1) gives

\[ \frac{^{13}\text{CH}_3 R}{^{12}\text{CH}_3 R} = \frac{^{13}\text{CH}_3 R}{^{12}\text{CH}_3 R} \times \frac{1}{1 + r^{13}\text{CH}_3 k_{\text{rev}}} \]  

(A2.11)

It is inconvenient to describe $^{13}$CH$_3$D in terms of $^{13}$CH$_3$R because $^{13}$CH$_3$R is not a measurable quantity. This term can be eliminated because it is assumed that in the system, at all times, all metabolites from CO$_2$ and H$_2$ through CH$_2$S-CoM are in isotopic equilibrium. Thus we can write the following isotope-exchange reaction:

\[ ^{13}\text{CO}_2 + ^{13}\text{CH}_2\text{S-CoM} \leftrightarrow ^{13}\text{CO}_2 + ^{13}\text{CH}_2\text{S-CoM} \]  

(A2.12)

The equilibrium constant for this reaction is:

\[ K_{eq}^{^{13}\text{CH}_3 R} = \frac{^{13}\text{CH}_3 R}{^{12}\text{CO}_2 R} \]  

(A2.13)

where

\[ ^{12}\text{CO}_2 R = \frac{[^{12}\text{CO}_2]}{[^{12}\text{CO}_2]} \]  

(A2.14)

Substituting (A2.13) into (A2.11) yields:

\[ \frac{^{13}\text{CH}_3 R}{^{12}\text{CH}_3 R} = \frac{^{13}\text{CH}_3 R}{^{12}\text{CH}_3 R} \times K_{eq}^{^{13}\text{CH}_2\text{S-CoM}} \times ^{13}\text{CH}_3 k_j \]  

\[ \times \left( \frac{1}{1 + r^{13}\text{CH}_3 k_{\text{rev}}} \right) \]  

(A2.15)

We define a new term such that:

\[ ^{13}\text{CH}_3 k_j' = \frac{K_{eq}^{^{13}\text{CH}_3 R}}{^{12}\text{CH}_3 R} \times ^{13}\text{CH}_3 k_j \]  

(A2.16)

$^{13}$CH$_3 k_j'$ is thus not strictly a ratio of rate constants (as kinetic isotope effects are usually defined), but instead incorporates the equilibrium fractionation between CO$_2$ and CH$_2$S-CoM and the rate-limiting step of hydrogen addition to CH$_2$S-CoM. Such 'combined,' multi-step fractionation factors are commonly described in models of this sort as often only the product and reactants are measurable (Hayes, 2001). Regardless, substitution of (A2.16) into (A2.15) yields:

\[ \frac{^{13}\text{CH}_3 R}{^{12}\text{CH}_3 R} = \frac{^{13}\text{CH}_3 R}{^{12}\text{CH}_3 R} \times \frac{^{13}\text{CH}_3 k_j'}{^{13}\text{CH}_3 k_j} \times \left( \frac{1}{1 + r^{13}\text{CH}_3 k_{\text{rev}}} \right) \]  

(A2.17)

When $r = 1$, CH$_3$ and CO$_2$ are in isotopic equilibrium (by definition). And we have:

\[ \frac{^{13}\text{CH}_3 R_{eq}}{^{12}\text{CH}_3 R_{eq}} = \frac{^{13}\text{CH}_3 R_{eq}}{^{12}\text{CH}_3 R_{eq}} \]  

(A2.18)

where 'eq' signifies a value at isotopic equilibrium. We can write the following isotope-exchange reaction as well:

\[ ^{13}\text{CO}_2 + ^{13}\text{CH}_4 \leftrightarrow ^{13}\text{CO}_2 + ^{13}\text{CH}_4 \]  

(A2.19)

This reaction has the following equilibrium constant,

\[ K_{eq}^{^{13}\text{CH}_3 R} = \frac{^{13}\text{CH}_3 R_{eq}}{^{12}\text{CO}_2 R_{eq}} \]  

(A2.20)

\[ ^{13}\text{CO}_2 R_{eq}^{^{13}\text{CH}_3 R} = \frac{^{13}\text{CH}_3 R_{eq}}{^{12}\text{CO}_2 R_{eq}} \]  

Combination of (A2.20) with (A2.18) yields:

\[ ^{13}\text{CO}_2 R_{eq} = ^{13}\text{CH}_3 R_{eq} \times ^{13}\text{CH}_3 k_j' \]  

(A2.21)

This equilibrium constant is calculable using the theory of, for example, Richet et al. (1977). Thus, Eq. (A2.21) removes a degree of freedom from Eq. (A2.17).

A.3. $^{12}$CH$_3$D derivation

The set of equations that describe the dependence of the $^{12}$CH$_3$D/$^{12}$CH$_4$ ratio on the degree of reversibility of Eq.
(A1.2) is derived in this section. The derivations, although similar to Section A2, have a fundamental difference related to the fact that $^{12}\text{CH}_3\text{D}$ can be made two different ways:

\[
^{12}\text{CH}_3\text{-S-CoM} + \text{D} \xrightarrow{\Delta_{\text{D-k}}^{12}} ^{12}\text{CH}_3\text{D} \to \tag{A3.1}\]

and

\[
^{12}\text{CH}_2\text{D-S-CoM} + \text{H} \xrightarrow{\Delta_{\text{H-k}}^{12}} ^{12}\text{CH}_3\text{D} \to \tag{A3.2}\]

Following Section A2, we can write the steady state concentration of $^{12}\text{CH}_3\text{D}$ as

\[
^{[12]}\text{CH}_3\text{D} = \frac{^{12}\text{H}_k[^{12}\text{CH}_3\text{D-S-CoM}][\text{H}]}{^{12}\text{H}_k[^{12}\text{CH}_3\text{D-S-CoM}] + ^{12}\text{D}_k[^{12}\text{CH}_3\text{D-S-CoM}][\text{D}])
\]

Division of (A3.3) by (A2.4) yields:

\[
^{[12]}\text{CH}_3\text{D} = \frac{^{12}\text{H}_k[^{12}\text{CH}_3\text{D-S-CoM}][\text{H}]}{^{12}\text{H}_k[^{12}\text{CH}_3\text{D-S-CoM}] + ^{12}\text{D}_k[^{12}\text{CH}_3\text{D-S-CoM}][\text{D}]}
\]

We define the following ratios:

\[
^{12}\text{CH}_3\text{D}_R = \frac{^{12}\text{CH}_3\text{D}}{[^{12}\text{CH}_4]}, \quad ^{12}\text{CH}_3\text{D}_{\text{D}} = \frac{^{12}\text{CH}_3\text{D-S-CoM}}{[^{12}\text{CH}_3\text{S-CoM}]}, \quad \text{and} \quad ^{12}\text{D}_R = \frac{[\text{D}]}{[\text{H}]}
\]

Substituting (A3.5a, b and c) into (A3.4) and utilization of the algebra and simplifications introduced in Section A2 yields:

\[
^{12}\text{CH}_3\text{D}_R = \frac{^{12}\text{D}_k \times ^{12}\text{H}_k \times ^{12}\text{CH}_3\text{D}_R}{1 + ^{12}\text{D}_k^{12}\text{H}_k^{12}\text{D}_R} \tag{A3.6}
\]

At metabolic equilibrium, the following isotope-exchange reaction is in isotopic equilibrium:

\[
^{12}\text{CH}_3\text{S-CoM} + \text{D} \xrightarrow{\Delta} ^{12}\text{CH}_2\text{D-S-CoM} + \text{H} \tag{A3.7}
\]

The equilibrium constant for this reaction is:

\[
^{12}\text{CH}_3\text{D}_R = \frac{K_{\text{eq}}}{^{12}\text{CH}_3\text{D}_{\text{D}}^{12}\text{CH}_3\text{S-CoM}} \tag{A3.8}
\]

Substituting (A3.8) into (A3.6) gives

\[
^{12}\text{CH}_3\text{D}_R = ^{12}\text{D}_R \times \frac{^{12}\text{D}_k \times ^{12}\text{H}_k \times ^{12}\text{CH}_3\text{D}_R}{1 + ^{12}\text{D}_k^{12}\text{H}_k^{12}\text{D}_R} \tag{A3.9}
\]

The fact that there are two pathways for making $^{12}\text{CH}_3\text{D}$ may have implications for understanding what controls the $\Delta-D$ value of methane as well as its clumped isotopic composition. This is because these two paths could have very different isotope effects as one is a primary isotope effect, the addition of D, and the other is a secondary effect, the addition of H to an already deuterium-containing molecule. This could, perhaps, be recast as a mixing problem instead of a kinetic problem in which two distinct pools of $^{12}\text{CH}_3\text{D}$ (or $^{13}\text{CH}_3\text{D}$) are created at different rates and with different isotope effects. However, as there are few constraints on the sizes of these isotope effects (Scheller et al., 2013), we will simplify the problem by combing them into a single, amalgamated isotope effect:

\[
^{12}\text{CH}_3\text{D}_k^{12} = ^{12}\text{D}_k + ^{12}\text{H}_k \times ^{12}\text{CH}_3\text{D}_{\text{D}}^{12}\text{CH}_3\text{S-CoM} \tag{A3.10a}
\]

and

\[
^{12}\text{CH}_3\text{D}_k^{12} = ^{12}\text{D}_k + ^{12}\text{H}_k \times ^{12}\text{CH}_3\text{D}_{\text{D}}^{12}\text{CH}_3\text{S-CoM} \tag{A3.10b}
\]

This yields the following simplified equation:

\[
^{12}\text{CH}_3\text{D}_R = ^{12}\text{H}_R \times \left( \frac{^{12}\text{CH}_3\text{D}_k^{12}}{1 + ^{12}\text{H}_R^{12}\text{D}_R} \right) \tag{A3.11}
\]

This greatly simplifies the equation, but it is critical to note that this form of the equation masks the true and interesting complexity of the reactions taking place. At equilibrium, $r = 1$, and

\[
^{12}\text{CH}_3\text{D}_R \to ^{12}\text{CH}_3\text{D}_R \times \left( \frac{^{12}\text{CH}_3\text{D}_k^{12}}{1 + ^{12}\text{H}_R^{12}\text{D}_R} \right) \tag{A3.12}
\]

Additionally at equilibrium, following Section A2, we can write the following isotope-exchange reaction:

\[
^{12}\text{CH}_3 + \text{D} \xrightarrow{\Delta} ^{12}\text{CH}_2\text{D} + \text{H} \tag{A3.13}
\]

The equilibrium constant for this reaction is

\[
\frac{^{12}\text{CH}_3\text{D}_R}{^{12}\text{CH}_3\text{D}_{\text{D}}^{12}\text{CH}_3\text{S-CoM}} = \frac{K_{\text{eq}}}{^{12}\text{CH}_3\text{D}_{\text{D}}^{12}\text{CH}_3\text{S-CoM}} \tag{A3.14}
\]

Problematically, $^{12}\text{CH}_3\text{D}_R$ is not measurable. We will presume, for simplicity, as discussed above, that the hydrogen and deuterium pool accessible to methane and methane precursors is in rapid equilibrium with the hydrogen and deuterium in water and that there is a constant offset between $^{12}\text{H}_R$ and $^{12}\text{H}_R^{12}\text{D}_R$ (the D/H ratio of liquid $\text{H}_2\text{O}$). For simplicity, we will assume that they are equal. Any actual offset in this will be absorbed in $^{12}\text{CH}_3\text{D}_k^{12}$, and thus mathematically is unimportant. However, if the actual kinetic isotope effects of each individual step for methanogenesis are known, then using the exact value of the D/H ratio of the hydrogen donor to the methane precursor will be important. Regardless, for the derivation here, we assume,

\[
^{12}\text{CH}_3\text{D}_R = \frac{^{12}\text{H}_R^{12}\text{D}_R}{^{12}\text{H}_R^{12}\text{D}_R} = \frac{^{12}\text{H}_R^{12}\text{D}_R}{^{12}\text{H}_R^{12}\text{D}_R} = \frac{^{12}\text{H}_R^{12}\text{D}_R}{^{12}\text{H}_R^{12}\text{D}_R} \tag{A3.15}
\]

Inserting (A3.15) into (A3.14) yields:

\[
^{12}\text{CH}_3\text{D}_R \to ^{12}\text{CH}_3\text{D}_R \times \left( \frac{^{12}\text{CH}_3\text{D}_k^{12}}{1 + ^{12}\text{H}_R^{12}\text{D}_R} \right) \tag{A3.16}
\]

The ratio of $^{12}\text{CH}_3\text{D}_R$ is a calculable function of temperature (Richet et al., 1977; Horibe and Craig, 1995) and thus removes a degree of freedom from the model.
A.4. $^{13}$CH₃D derivation

This section derives the $^{13}$CH₃D/$^{12}$CH₄ ratio as a function of the degree of reversibility of Eq. (A1.2). Following Sections A2 and A3, with similar symbology and assumptions, the following equation is found:

$$^{13}\mathrm{CH}_3D/^{12}\mathrm{CH}_4 = \frac{^{13}\mathrm{CH}_3D \times D/R + ^{13}\mathrm{H}_2 + ^{13}\mathrm{K}_f \times ^{13}\mathrm{CH}_3D \times R}{1 + R^{(13-D)_{K_{rev}} + ^{13}\mathrm{H}_{K_{rev}} - 1}}. \quad (A4.1)$$

where

$$^{13}\mathrm{CH}_3D/^{12}\mathrm{CH}_4 = \frac{[^{13}\mathrm{CH}_3D]}{[^{12}\mathrm{CH}_4]} \quad \text{and} \quad ^{13}\mathrm{CH}_3D/^{12}\mathrm{CH}_2S-CoM = \frac{[^{13}\mathrm{CH}_3D-S-CoM]}{[^{12}\mathrm{CH}_2S-CoM]} \quad (A4.2a \text{ and } b).$$

Using the constraints from metabolic equilibrium, the following isotope-exchange reaction can be written:

$$^{13}\mathrm{CH}_2S-CoM + D = ^{13}\mathrm{CH}_2D-S-CoM + H \quad (A4.3)$$

such that:

$$^{13}\mathrm{CH}_2D-S-CoM/^{13}\mathrm{CH}_2S-CoM = ^K_{eq} \quad (A4.4)$$

Incorporating (A4.4) and (A2.13) into (A4.1) yields,

$$^{13}\mathrm{CH}_3D/^{12}\mathrm{CH}_4 = D/R \times ^{13}\mathrm{CH}_2S-CoM \left( \frac{^{13}\mathrm{CH}_3D \times K_{eq} + ^{13}\mathrm{H}_2 + ^{13}\mathrm{K}_f \times ^{13}\mathrm{CH}_3D \times K_{eq} \times ^{13}\mathrm{CH}_2S-CoM - ^{13}\mathrm{CH}_2D-S-CoM}{1 + R^{(13-D)_{K_{rev}} + ^{13}\mathrm{H}_{K_{rev}} - 1}} \right). \quad (A4.5)$$

As in Section 3, we define the following 'combined' fractionation factors:

$$^{13}\mathrm{CH}_3D/^{12}\mathrm{CH}_4 = ^{13}\mathrm{K}_f \times ^{13}\mathrm{CH}_3D/^{12}\mathrm{CH}_4 \quad (A4.6a)$$

and

$$^{13}\mathrm{K}_f \times ^{13}\mathrm{H}_2 + ^{13}\mathrm{K}_f \times ^{13}\mathrm{CH}_3D/^{12}\mathrm{CH}_4 \quad (A4.6b)$$

Substituting (A4.6) into (A4.5) gives

$$^{13}\mathrm{CH}_3D/^{12}\mathrm{CH}_4 = D/R \times ^{13}\mathrm{CH}_2S-CoM \left( \frac{^{13}\mathrm{CH}_3D \times ^{13}\mathrm{K}_f}{1 + R^{(13-D)_{K_{rev}} - 1}} \right). \quad (A4.7)$$

At r = 1, the following homogenous-phase equilibrium reaction occurs:

$$^{12}\mathrm{CH}_2D + ^{13}\mathrm{CH}_4 \leftrightarrow ^{12}\mathrm{CH}_4 + ^{13}\mathrm{CH}_3D. \quad (A4.8)$$

The equilibrium constant for (A4.8) is

$$K_{eq} = \left( \frac{[^{12}\mathrm{CH}_2D]}{[^{13}\mathrm{CH}_3D]} \right) \left( \frac{[^{13}\mathrm{CH}_3D]}{[^{12}\mathrm{CH}_4]} \right), \quad (A4.9)$$

which is a known function of temperature (Bottinga, 1969; Ma et al., 2008; Cao and Liu, 2012; Stolper et al., 2014a; Ono et al., 2014a; Webb and Miller, 2014). Following some algebraic manipulations, we can write:

$$K_{eq} = \left( \frac{[^{12}\mathrm{CH}_2D]}{[^{13}\mathrm{CH}_3D]} \times \frac{[^{13}\mathrm{CH}_3D]}{[^{12}\mathrm{CH}_4]} \right) = \frac{^{13}\mathrm{CH}_3D}{^{12}\mathrm{CH}_2D}, \quad (A4.10)$$

which removes a degree of freedom from the model.

A.5. $^{12}$CH₂D₂ derivation

This section derives the $^{12}$CH₂D₂/$^{12}$CH₄ ratio as a function of the degree of reversibility of Eq. (A1.2). Following Sections A2–A4, with similar symbology and assumptions, the following equation is found:

$$^{12}\mathrm{CH}_2D_2/^{12}\mathrm{CH}_4 = \left( \frac{^{12}\mathrm{CH}_2D_2 \times D/R + ^{12}\mathrm{HD}_2 + ^{12}\mathrm{K}_f \times ^{12}\mathrm{CH}_2D_2 \times R}{1 + R^{(12-D)_{K_{rev}} + ^{12}\mathrm{H}_{K_{rev}} - 1}} \right) \quad (A5.1)$$

where,

$$^{12}\mathrm{CH}_2D_2/^{12}\mathrm{CH}_4 = \frac{[^{12}\mathrm{CH}_2D_2-S-CoM]}{[^{12}\mathrm{CH}_2S-CoM]} \quad (A5.2).$$

Using constraints from metabolic equilibrium, we write

$$^{12}\mathrm{CH}_2D-S-CoM + D = ^{12}\mathrm{CH}_2D_2-S-CoM + H \quad (A5.3)$$

with the following equilibrium constant:

$$^{12}\mathrm{CH}_2D-S-CoM/^{12}\mathrm{CH}_2S-CoM = ^K_{eq} \quad (A5.4)$$

Combination of (A5.4) with (A3.8) yields

$$^{12}\mathrm{CH}_2D_2/^{12}\mathrm{CH}_4 = \left( \frac{D}{R} \right)^2 \times \frac{^{12}\mathrm{CH}_2D_2-S-CoM}{^{12}\mathrm{CH}_2S-CoM} \quad (A5.5)$$

Combining (A5.5) and (A3.8) with (A5.1) yields

$$^{12}\mathrm{CH}_2D_2/^{12}\mathrm{CH}_4 = \left( \frac{D}{R} \right)^2 \times \left( \frac{^{12}\mathrm{CH}_2D_2 \times K_{eq} + ^{12}\mathrm{HD}_2 \times K_{eq} \times ^{12}\mathrm{CH}_2D_2 \times D_{\alpha}^{12}\mathrm{CH}_2D-S-CoM}{1 + R^{(12-D)_{K_{rev}} + ^{12}\mathrm{H}_{K_{rev}} - 1}} \right). \quad (A5.6)$$
Rewriting with the following ‘combined’ fractionation factors:

\[
{^{12}CH_2D_2}K_f = \frac{H_2D + ^{12}CH_2D_2}{^{12}CH_2D_2} K_f \times \frac{H_2D}{H_2} \times \frac{K_{eq}}{K_{eq}}
\]

and

\[
{^{12}CH_2D_2}K_{rev} = \frac{H_2D}{H_2} D_2 \times \frac{H_2D}{H_2} ^{12}CH_2D_2 \times K_{eq}
\]

this equation becomes

\[
{^{12}CH_2D_2}R = \left( {^{12}CH_2D_2}K_f \right) \times \left( 1 \right)
\]

At \( r = 1 \), the following equilibrium reaction occurs:

\[
{^{12}CH_3D} + ^{12}CH_2D_2 \rightarrow ^{12}CH_4 + ^{12}CH_2D_2
\]

with the equilibrium constant

\[
K_{eq} = \frac{[^{12}CH_3D][^{12}CH_2D_2]}{[^{12}CH_3D][^{12}CH_4]}
\]

which, as discussed in Section A4, is a function of temperature and thus removes a degree of freedom from the model.

With some algebraic manipulation, this can be rewritten for convenience as

\[
K_{eq} = \left( {^{12}CH_2D_2}K_f \right) \times \left( \frac{H_2D}{H_2} \right)^2 \times \frac{^{12}CH_2D_2}{^{12}CH_2D_2}
\]

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