1 **Microbial methane from *in situ* biodegradation of coal and shale:**
2 **A review and reevaluation of hydrogen and carbon isotope signatures**

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12 **Abstract**

13 Stable carbon and hydrogen isotope signatures of methane, water, and inorganic carbon are
14 widely utilized in natural gas systems for distinguishing microbial and thermogenic methane and for
15 delineating methanogenic pathways (acetoclastic, hydrogenotrophic, and/or methylotrophic
16 methanogenesis). Recent studies of coal and shale gas systems have characterized *in situ* microbial
17 communities and provided stable isotope data ($\delta\text{D-CH}_4$, $\delta\text{D-H}_2\text{O}$, $\delta^{13}\text{C-CH}_4$, and $\delta^{13}\text{C-CO}_2$) from a wider
18 range of environments than available previously. Here we review the principal biogenic methane-yielding
19 pathways in coal beds and shales and the isotope effects imparted on methane, document the uncertainties
20 and inconsistencies in established isotopic fingerprinting techniques, and identify the knowledge gaps in

understanding the subsurface processes that govern H and C isotope signatures of biogenic methane. We also compare established isotopic interpretations with recent microbial community characterization techniques, which reveal additional inconsistencies in the interpretation of microbial metabolic pathways in coal beds and shales. Collectively, the re-assessed data show that widely-utilized isotopic fingerprinting techniques neglect important complications in coal beds and shales.

Isotopic fingerprinting techniques that combine $\delta^{13}\text{C}\text{-CH}_4$ with $\delta\text{D}\text{-CH}_4$ and/or $\delta^{13}\text{C}\text{-CO}_2$ have significant limitations: (1) The consistent $\sim 160\text{‰}$ offset between $\delta\text{D}\text{-H}_2\text{O}$ and $\delta\text{D}\text{-CH}_4$ could imply that hydrogenotrophic methanogenesis is the dominant metabolic pathway in microbial gas systems. However, hydrogen isotopes can equilibrate between methane precursors and coexisting water, yielding a similar apparent H isotope signal as hydrogenotrophic methanogenesis, regardless of the actual methane formation pathway. (2) Non-methanogenic processes such as sulfate reduction, Fe oxide reduction, inputs of thermogenic methane, anaerobic methane oxidation, and/or formation water interaction can cause the apparent carbon isotope fractionation between $\delta^{13}\text{C}\text{-CH}_4$ and $\delta^{13}\text{C}\text{-CO}_2$ ($\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$) to differ from the true methanogenic fractionation, complicating interpretation of methanogenic pathways. (3) Where little-fractionating non-methanogenic bacterial processes compete with highly-fractionating methanogenesis, the mass balance between CH_4 and CO_2 is affected. This has implications for $\delta^{13}\text{C}$ values and provides an alternative interpretation for net C isotope signatures than solely the pathways used by active methanogens. (4) While most of the reviewed values of $\delta\text{D}\text{-H}_2\text{O} - \delta\text{D}\text{-CH}_4$ and $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ are apparently consistent with hydrogenotrophic methanogenesis as the dominant pathway in coal beds and shales, recent microbial community characterization techniques suggest a possible role for acetoclastic or methylotrophic methanogenesis in some basins.

Keywords: stable isotopes; biogenic gas; carbon isotopes; hydrogen isotopes; coalbed methane

1 Introduction

1.1 Importance of distinguishing biogenic sources of methane

Microbial degradation of organic matter in the subsurface has been estimated to contribute to ~20% of natural gas resources worldwide, whereas ~80% of economically significant gas is thought to be thermogenic, produced from thermal cracking of organic matter (Rice and Claypool, 1981). Biogenic gas has been detected in conventional hydrocarbon systems and in unconventional settings, such as coal beds and shales. In an examination of conventional natural gas, Milkov (2011) estimated that 3-4% of recoverable gas is of primary microbial origin, derived from biodegradation of sedimentary organic matter, and another 5-11% is secondary, derived from biodegradation of thermogenic hydrocarbons. However, the estimates of Milkov (2011) did not address unconventional gas hosted in coal and shales. Coalbed methane is estimated to represent 9% of United States natural gas production (Gao et al., 2014). In coal beds and shales, the generated gas is largely retained by adsorption to water-saturated coal and shale, then released when the formation is depressurized, such as by pumping during production (Martini et al., 1998, 2008; Pashin, 2014). As with other gas resources, coal and shale gas can be thermogenic, secondary biogenic, primary biogenic, or a mixture of these sources. Here we focus on generation of microbial gas from *in situ* biodegradation of coal- and shale-derived organics, that is, primary biogenic gas. Primary biogenic gas depends on steady-state biodegradation of the coal and shale source materials, producing low molecular-weight intermediates and methane precursors, coupled to microbial methanogenesis that yields CH₄ and CO₂. While gas samples may record a long gas accumulation history, this linked (syntrophic) metabolism is in many cases thought to represent organisms and/or metabolic pathways that remain active in sedimentary basins.

Reliably identifying biogenic natural gas in the subsurface has numerous implications, such as assessing the origins of methane, evaluating natural gas resources, and understanding the environmental footprint of production practices: (1) Methanogenesis is the terminal step in organic matter biodegradation, yielding the greenhouse gases CH₄ and CO₂. The active methanogenic metabolic pathway(s) are linked to the history of organic matter deposition and to upstream biogeochemical

reactions that produce substrates needed by methanogens. Therefore, in a variety of environments, identifying methanogenic pathways is a significant part of understanding organic matter decomposition and fluxes of carbon compounds (e.g. Formolo, 2010; Hornibrook and Aravena, 2010). (2) In basins with complex thermal histories or hydrologic settings, mixed biogenic-thermogenic gas may be present, and elucidating this gas mixture is a long-standing problem (Scott et al., 1994; Martini et al., 1998, 2003, 2008; Cheung et al., 2010; McIntosh et al., 2010; Osborn and McIntosh, 2010; Golding et al., 2013; Stolper et al., 2015). (3) In systems with active microbial methanogenesis, there is interest in stimulating underlying processes to yield additional gas resources, which requires understanding of organic matter biodegradation and methane generation (Jones E.J.P. et al., 2008; 2010; Ulrich and Bower, 2008; Papendick et al., 2011; Strapoć et al., 2011b; Barnhart et al., 2013; Schlegel et al., 2013; Ritter et al., 2015). (4) As unconventional natural gas extraction from hydrocarbon-bearing formations grows in economic importance, tools and techniques are needed to assess potential environmental impacts of production practices (Vidic et al., 2013; Jackson et al., 2013; Brantley et al., 2014; Darrah et al., 2014; Vengosh et al., 2014). Formations containing biogenic gas are among the shallowest targets for hydraulic fracturing in the USA (e.g. Antrim Shale in Michigan Basin; Jackson et al., 2015), so the detection of biogenic gas in the near-surface environment is of significant interest.

1.2 Need for critical re-examination of C and H isotope applications to biogenic gas

Geochemical and isotopic fingerprints utilizing stable carbon and hydrogen isotopes have been applied for decades to (1) identify microbial vs. thermogenic gas and (2) to distinguish pathways of microbial methane generation in natural gas systems. These C and H isotope techniques remain important for addressing established and emerging problems. Isotopic fingerprinting techniques are especially important in field-based studies where the active metabolic pathway is not controlled experimentally and substrate concentrations alone do not point to an obvious dominant pathway. An improved understanding of the microbial controls on the C and H isotope composition of methane can provide insights on identifying *in situ* methanogenesis and quantifying inputs of microbial gas into groundwater and thermogenic-dominated gas systems. In addition, stable isotopic fingerprints and models have been used

to assess methane leakage from gas infrastructure into the atmosphere (e.g. Townsend-Small et al., 2012; Phillips et al., 2013) and to shallow aquifers (e.g. Osborn et al., 2011; Révész et al., 2010; Jackson et al., 2013).

Widely-used geochemical and isotopic evidence of the microbial origin of gas includes: (1) ratios of methane to ethane and propane ($C_1/(C_2+C_3)$), typically >1000 in samples of microbial gas (Bernard et al., 1976; Golding et al., 2013); (2) $\delta^{13}C\text{-CH}_4$ values generally less than approximately -55‰ expected for biogenic gas (although showing considerable overlap with thermogenic gas in practice; Bernard et al., 1976; Whiticar et al., 1986; Schoell, 1988; Whiticar, 1999; Hornibrook and Aravena, 2010; Golding et al., 2013); (3) correlation of $\delta D\text{-CH}_4$ and $\delta D\text{-H}_2O$ values as evidence of microbial methanogenesis and characteristic of metabolic pathways (Schoell, 1980; Whiticar et al., 1986; Whiticar, 1999; Golding et al., 2013); (4) plotting $\delta^{13}C\text{-CH}_4$ vs. $\delta D\text{-CH}_4$ and comparing to diagnostic fields for biogenic and thermogenic gas (Schoell, 1980; Whiticar et al., 1986; Whiticar, 1999); (5) characteristic values of $\delta^{13}C\text{-CO}_2 > 0\text{‰}$ in microbial gas systems (Scott et al., 1994; Martini et al., 2003, 2008; Golding et al., 2013); and (6) the difference between $\delta^{13}C\text{-CO}_2$ and $\delta^{13}C\text{-CH}_4$ values as diagnostic of methanogenic pathways (Whiticar et al., 1986; Whiticar, 1999; Conrad, 2005; Golding et al., 2013).

Many of the data previously utilized to define the isotopic signatures of methanogenic pathways were from recent aquatic sediments or related cultures, combined with the available data from biogenic gas basins (e.g. Whiticar et al., 1986). However, the nature and time scale of biodegradation likely differ between modern and fossil C sources due to the preferential removal of easily-metabolized carbohydrates during sediment aging, differences in rate-limiting steps (e.g. H_2 production), and distinctive environmental conditions such as sulfate availability (e.g. Miyajima et al., 1997; Nakagawa et al., 2002a; Strapoć et al., 2011b). Short-term seasonal hydrologic, temperature, and redox fluctuations (e.g. Blair et al., 1993; Blair and Aller, 1995; Avery et al., 1999), common in aquatic sediments, are not expected in gas-bearing formations. Moreover, methane oxidation, which would affect apparent isotopic signatures, is less likely to be significant in the persistently-anoxic deep subsurface than in shallow subsurface

sediments. Collectively, these factors point to the need for data from consistent carbon sources and environmental conditions.

Since the 1990s, unconventional gas exploration has greatly expanded, including microbial coalbed methane and shale gas, making available recently-published data sets of paired water and gas isotopes from microbial gas systems. In addition, culturing and molecular microbial investigations have improved documentation of organisms and pathways that appear to be active in coal and shale biodegradation. Here we review the expected H and C isotope effects of methanogenic pathways and competing non-methanogenic pathways in coal beds and shales; compare these data to expected patterns derived from recent sediments and cultures; and document inconsistent interpretations of metabolic pathways using isotopic and microbial techniques. Evidence from recent studies demonstrates challenges in applying C and H isotopes to interpreting the *in situ* biogeochemical structure in coal beds and shales. In addition, discrepancies are apparent between observed methanogen populations and the pathways inferred from H and C isotopes of field-collected water and gas samples (see Table 1 for examples). These challenges call for a re-assessment of what phenomena are actually recorded by C and H isotopes in methanogenic coal beds and shales.

1.3 Microbial methane formation in coal and shale: Pathways and environmental controls

1.3.1 Syntrophic metabolism in coal and shale biodegradation

Coals formed from land plants contain a distinct kerogen type from marine shales containing planktonic-derived organic matter (van Krevelen, 1993; Golding et al., 2013). Briefly, organic-rich marine shale is typically enriched in Type II kerogen and is capable of yielding oil or gas, whereas coal is dominated by Type III kerogen derived from higher land plants. These kerogen types in turn have distinct chemistry (O/C and H/C ratios) and maceral composition, which refers to physically-definable organic components with distinct aliphatic and aliphatic content, biodegradable functional groups, and perhaps also isotopic composition (Van Krevelen, 1993; Taylor et al., 1998; Strapoć et al., 2011b). Compared to shale, coal has higher total organic carbon content and a higher yield of low-molecular weight organic intermediate compounds (C_{LMW}) during biodegradation (Gao et al., 2013). While carbon sources differ

substantially between organic matter in most coals and shales, and specific biodegradation pathways and intermediate compounds could differ as well, coal and shale kerogen appear to biodegrade to yield dry natural gases with similar attributes (Golding et al., 2013).

Methanogenic biodegradation of coal and shale involves the conversion of geopolymers in the source rock organic matter to C_{LMW} . C_{LMW} is converted to methane precursors (including acetate, H_2 , acetate, formate, carbon monoxide, methanol, and other methylated compounds) through bacterially-mediated reactions (Formolo, 2010; Strapoć et al., 2011b). While abiotic mechanisms have been shown to generate H_2 in the deep subsurface, such as serpentinization and radiolytic H_2 production (Crespo-Medina et al., 2014; Dzaugis et al, 2016), these are assumed to be much less significant than organic-derived H_2 in carbon-rich formations such as coals and shales. The final step of biodegradation, mediated by methanogenic Archaea, yields methane. The bacteria-produced methane precursors serve as electron donors for methanogenic Archaea in a syntrophic association. CO_2 is an additional product of the overall biodegradation sequence, required by electron balance of C_{LMW} compounds. Methanogenesis is therefore the final step of a biodegradation process that can route carbon through multiple methanogenic or nonmethanogenic pathways (Figure 1). In addition, recent studies have shown that direct electron transfer is also possible from bacteria (*Geobacter*) to methanogens (*Methanosaeta*, *Methanosarcina*) without requiring intermediate H_2 production (Rotaru et al., 2013, 2014), although the potential environmental significance of this strategy is not known.

1.3.2 Methanogenesis: Pathways and environmental controls

Methanogenic Archaea are obligate anaerobes, requiring oxygen-free conditions for growth. Methanogenesis is further governed by thermodynamic constraints that limit its free energy yield relative to more energetically favorable anaerobic processes. Substrates such as H_2 , formate, and acetate, known as competitive substrates (Whiticar, 1999), are aggressively scavenged by heterotrophic bacteria that mediate non-methanogenic pathways such as nitrate, iron, and sulfate reduction, which yield more free energy per mole of substrate than methanogenesis (Table 2). In waters free of alternative electron acceptors such as sulfate, two methanogenic pathways are of the best-documented environmental

significance in utilizing the competitive substrates: (1) predominant reduction of the methyl group to methane and oxidation of the carboxyl group to CO₂ (referred to as acetoclastic methanogenesis or acetate fermentation); and (2) reduction of CO₂ to methane using H₂ as an electron source, referred to as hydrogenotrophic methanogenesis or CO₂ reduction (Table 2; Whiticar et al., 1986; Whiticar, 1999; Formolo, 2010). CO₂ is orders of magnitude more abundant than H₂, so H₂ limits the extent of hydrogenotrophic methanogenesis in natural systems. At near-neutral pH and sufficiently low salinity for the growth of bacteria and methanogenic archaea, environmental conditions such as sulfate availability are a primary control on whether methanogenesis or CO₂-yielding heterotrophic bacterial metabolism will utilize the competitive substrates.

Beyond the relatively well-documented acetoclastic and hydrogenotrophic pathways, recent studies have identified the importance of methanogenesis utilizing a range of methylated compounds including methanol and methylamines, such as by the obligate methylotroph *Methanobus* (Table 2; Whitman et al., 2014). Utilization of methylated compounds, possibly derived from coal kerogen demethoxylation (Strapoć et al., 2011b), has been suggested as a methanogenic pathway in some coal and shale gas systems (Waldron et al., 2007; Doerfert et al., 2009; Strapoć et al., 2011a,b; Wawrik et al., 2012; Barnhart et al., 2013, Ashby et al., 2015). Alternative methylated substrates are referred to as noncompetitive substrates because they are not effectively utilized by denitrification, iron reduction, sulfate reduction, and/or other heterotrophic bacterial pathways (Oremland and Polcin, 1982; Whitman et al., 2014). In the presence of sulfate, or perhaps microbially-reducible Fe oxides, methylotrophs can utilize methylated compounds in methanogenesis when bacteria outcompete methanogens for the competitive substrates. The overall environmental significance of methylotrophic methanogenesis is less well understood than acetoclastic and hydrogenotrophic methanogenesis, but is argued to be less significant globally than acetoclastic and hydrogenotrophic methanogenesis (Formolo, 2010). Finally, beyond the three ideal methanogenic reactions discussed here (Table 2), other combinations of substrates could yield methane, perhaps in specialized environments (e.g. CH₃OH + H₂ = CH₄ + H₂O; Whitman et al., 2014). However, in this study we assume that CO₂ is the electron acceptor in hydrogenotrophic

methanogenesis, given the high abundance of CO₂ in the subsurface and the relative lack of studies documenting the environmental availability and significance of methanol and other methylated compounds.

Temperature and salinity are also potentially important environmental conditions affecting microbial growth and methanogenic biodegradation. For example, temperatures above ~80°C apparently inhibit the growth of methanogenic organisms (e.g. Schlegel et al., 2011a; Head et al., 2014). In addition, major freshwater recharge events during a basin's history can trigger pulses of methanogenesis, and economically-significant accumulations of microbial gas are associated with the freshwater flushing of previously saline coal and shale formations (Scott et al., 1994; Martini et al., 1996, 1998; McIntosh et al., 2002, 2004; Faiz and Hendry, 2006; Schlegel et al., 2011b; Pashin et al., 2014). Variations in salinity may also affect methanogenic Archaea because the energy required to exclude salts from microbial cells reduces the favorability of methanogenesis. It has been argued that acetate is efficiently scavenged by methanogens at total salinity of up to ~2 M, whereas hydrogenotrophic methanogenesis can persist at higher salinity (Waldron et al., 2007; McIntosh et al., 2010; Schlegel et al., 2011a, 2013). For example, a recently-cultivated CO₂-reducing methanogen can grow at 3.4 M salinity (Zhilina et al., 2013). While several studies, noted above, inferred that hydrogenotrophic methanogenesis is more salt-tolerant than acetoclastic methanogenesis, recent *in situ* microbial activity measurements imply that both pathways are of similar salt tolerance, occurring at up to approximately 2.0-2.5 M total salinity. Methylotrophic methanogenesis using noncompetitive methylated substrates is thought to be more salt-tolerant than hydrogenotrophic or acetoclastic methanogenesis (up to ~4 M total salinity; Oren, 2011). Intriguingly and in contrast to previous findings, one recent culturing study using coal bed microbes found increasing relative abundance of acetoclastic methanogens with increasing salinity in the range 1.1-3.0 M (Kirk et al., 2015). In addition, recent microcosm experiments also imply that the salinity range of methanogenesis is linked to temperature. At lower temperatures seen in many shallow biogenic coal and shale gas formations (many basins $\leq 30^{\circ}\text{C}$; Figure S1), hydrogenotrophic methanogenesis is more salt-tolerant than

acetoclastic methanogenesis, whereas at 60°C, both pathways become less salt-tolerant (Head et al., 2014).

At lower salinity, far below the overall limits of growth for methanogens, environmental conditions can favor specific metabolic strategies. Sulfate can inhibit methanogens from using the competitive substrates altogether, as described above, and sulfate can influence the nature of methanogenesis when it does become favorable. It has been argued that where sulfate is present, such as in marine sediments, sulfate-reducing bacteria rapidly scavenge any available acetate, and that hydrogenotrophic methanogenesis is dominant after sulfate reducers deplete the available sulfate. Therefore, hydrogenotrophic methanogenesis has been characterized as a marine pathway, whereas acetoclastic methanogenesis is thought to dominate in freshwater sediments because of the initial lack of sulfate (Whiticar et al., 1986; Whiticar, 1999; Hornibrook et al., 2000; Formolo, 2010). Methylo-trophic methanogenesis is also argued to be a marine pathway due to the abundance of sulfate in seawater (Oremland and Polcin, 1982; King, 1984; Whiticar, 1999; Zhuang et al., 2016). Others have argued that widespread microbial iron oxide reduction could compete with methanogens for organic substrates when Fe-reducing bacteria can access microbially-reducible Fe oxides (Table 2; Lovley et al., 1989; Révész et al., 1995; Krüger et al., 2002; Whitman et al., 2014). Other electron acceptors (e.g. nitrate) can be reduced more favorably than methanogenesis can occur (Table 2), but nitrate is not hypothesized to be abundant in coal beds and shales isolated from nitrate sources on the surface. Where it occurs in anoxic freshwater environments, Fe oxide reduction could diminish the apparent distinction in the methanogenic pathways that occur between marine and nonmarine environments.

1.4 Recent microbial community characterization: A complement to isotopic techniques

Microbial community characterization, including nucleic acid analysis of water and solids and culturing techniques, has differentiated methanogenic coal bed and shale consortia containing CO₂ reducers, acetoclastic methanogens, and methylo-trophic methanogens. Briefly and as reviewed in detail by Strapoć et al. (2011b), methods for examining the microbial community include enrichment, imaging, and molecular approaches. Enrichment (culturing) approaches include measuring methane production

under controlled substrate or environmental conditions and analysis of marker compounds that indicate a specific pathway is active (metabolite profiling). Imaging approaches include electron microscopy, fluorescent *in situ* hybridization (FISH) to identify labeled rRNA and thereby identify bacteria and Archaea, and secondary ion mass spectrometry (SIMS) for locating isotopic labels within organisms. Molecular techniques of analyzing uncultured material are especially important because many environmentally-relevant microorganisms cannot be cultured. These methods include phylogenetic analysis of 16S rRNA and metagenomics, a profiling technique that identifies genes associated with specific metabolic pathways. In studies of coal beds, microbial analysis on water is more widely practiced than on solids, largely due to ease of availability of formation water samples (Strapoć et al., 2011b). The two approaches may yield different results. For example, Klein et al. (2008) documented differences in methanogens between coal waters and coal surfaces, and Penner et al. (2010) reported that enrichment cultures, but not the source coal, yielded detectable methanogenic Archaea by nucleic acid analysis.

Culturing, molecular, and isotopic analyses of field-collected samples have yielded inconsistent results, summarized for several biogenic gas systems in Table 1. Overall, recent microbial studies in hydrologically diverse basins suggest that acetoclastic and/or methylotrophic methanogenesis are of plausible but unconfirmed environmental significance, in addition to hydrogenotrophic methanogenesis, which has been thought to dominate methanogenesis in fossil organic matter such as coal (e.g. Schoell, 1988). Culturing approaches could differ from analysis of field-collected samples in the time scale of observation, substrate availability, the survival of obligate anaerobes during handling, and/or the accelerated growth rates of organisms in the laboratory compared to the subsurface. Given that subsurface microbes may be persistently starved, drawing down H₂ concentrations to levels that yield just enough energy for cell growth (Hoehler et al., 1998), and may reproduce slowly (e.g. Biddle et al., 2006), natural methanogens in the subsurface could be specialized to low energy availability, competition, or syntrophic interactions in a way that is difficult to simulate experimentally (Hoehler et al., 2010). Experimental factors apply not only to the methanogenic Archaea, but also to the bacterial members of the biodegrading consortium, which have been shown to affect methane yield in enrichments (e.g. Wawrik et al., 2012).

The overall degradation rates in laboratory incubations may exceed natural biodegradation rates by orders of magnitude (Larter et al., 2003; Schlegel et al., 2011b). Together, these factors may complicate replication of natural steady-state conditions in the laboratory (e.g. Gray et al., 2009). Moreover, the presence of an organism in a sample does not necessarily prove that it is active in the natural environment, nor does presence alone confirm which specific substrates and pathways a versatile organism is using. Recent efforts to colonize sample material in the natural subsurface may better approximate the actual rates and nutrient availability of coal beds (Barnhart et al., 2013).

As a complement to direct cultivation and/or observation of methanogens, isotopic analysis of field-collected water and gas samples offers the potential to: (1) document the signatures of microbial process under *in situ* conditions of steady-state substrate availability and actual biodegradation rates, which may be difficult to reconstruct in the laboratory; (2) integrate spatial and temporal complexity because field-collected samples may represent a lengthy gas accumulation history; and (3) incorporate vertical heterogeneity across long open intervals in wells.

2 What factors can control $\delta^{13}\text{C}$ and δD of microbial methane?

Methanogenesis, the final step of C_{org} biodegradation, imparts large isotopic effects on carbon and hydrogen atoms, yielding methane with more negative δD and $\delta^{13}\text{C}$ values than the source organic matter. Carbon isotope signatures of methane result mainly from kinetic fractionation during methanogenesis as ^{12}C -bearing substrates are utilized at a higher rate constant than ^{13}C -bearing substrates, leaving the residual (unreacted) substrate enriched in ^{13}C (Whiticar et al., 1986; Blair et al., 1987; Blair and Carter, 1992; Whiticar, 1999; Conrad, 2005; Penger et al, 2012). Hydrogen isotope effects are controlled by (1) the hydrogen source (derived from organic matter or water, which varies by methanogenic pathway) and (2) fractionation during incorporation of H atoms into methane. Previous investigators have argued that, in general, methane from hydrogenotrophic methanogenesis exhibits more positive δD values and more negative $\delta^{13}\text{C}$ values than methane from acetoclastic methanogenesis (Whiticar et al., 1986; Whiticar, 1999).

A large range of $\delta^{13}\text{C}$ values has been reported for microbial methane in natural systems, from < -100‰ to approximately -40‰ (Figure 2). This is substantially ^{13}C -depleted relative to bulk organic matter with its $\delta^{13}\text{C}$ value near -25‰. $\delta^{13}\text{C}$ of biogenic methane can significantly overlap the expected $\delta^{13}\text{C}$ range of thermogenic methane, especially at $\delta^{13}\text{C}\text{-CH}_4$ values between approximately -55‰ and -40‰ (e.g. Schoell, 1988; McIntosh et al., 2002; Hornibrook and Aravena, 2010; Golding et al., 2013). To identify a gas sample as biogenic or thermogenic, $\delta^{13}\text{C}\text{-CH}_4$ is often combined with gas composition, specifically the ratio of methane to C_{2+} hydrocarbons (e.g. $\text{C}_1/(\text{C}_2+\text{C}_3)$; Figure 2). Thermogenic gas tends to exhibit $\text{C}_1/(\text{C}_2+\text{C}_3)$ ratios <100, whereas pure microbial gas exhibits $\text{C}_1/(\text{C}_2+\text{C}_3)$ ratios >1000 (Bernard et al., 1976; Golding et al., 2013). This approach is more reliable than examining $\delta^{13}\text{C}\text{-CH}_4$ alone. While the large methanogenic fractionation is likely the largest influence on the ^{13}C depletion of biogenic methane, isotope effects related to (1) the biodegradable source organic matter in coal and shale or (2) the production and consumption of intermediate compounds may influence the ultimate C and H isotope signatures of biogenic methane. These possible factors are discussed below.

2.1 Conversion of coal and shale to methane precursors: Influences on product isotope ratios

Microbial gas forms most abundantly in source organic material that has not been affected by deep burial temperatures during a basin's thermal history. This thermally-immature organic matter is characterized by low vitrinite reflectance, R_0 , with highest biogenic gas production from organic matter with R_0 of 0.3-0.8% (Gao et al., 2014). Higher-maturity coal ($R_0 > 0.8\%$) can still be biodegraded, although at a lower rate (Strapoć et al., 2011b). Within thermally-immature organic matter, specific functional groups or their attachment points are significantly more biodegradable than carbon-carbon bonds. For example, methoxy groups in immature organic matter have been shown to biodegrade into methane precursors in the laboratory (Liu and Suflita, 1993; Hanselmann et al., 1995). With their high oxygen content, methoxy groups are considered more biodegradable than the bulk coal and are hypothesized to be a potential source of methylated compounds (Strapoć et al., 2011a,b). A second location for preferential biodegradation is heteroatoms, the non-carbon atoms incorporated into organic molecules. Heteroatoms are not uniformly distributed in coal, but rather heteroatom content varies among

the vitrinite, liptinite, and inertite maceral groups (Strapoć et al., 2011b; Furmann et al., 2013). In addition to the compounds and functional groups that may be bioavailable or water-soluble in the solids, aromatic-rich (Allan and Larter, 1983), solvent-soluble oil-like compounds can be generated in coals at moderate to higher thermal maturity (Wilkins and George, 2002). Small quantities of oil have been observed in some coals and produced waters associated with biogenic coalbed methane (e.g. Strapoć et al., 2008b; Pashin et al., 2014). While the retention of oil in coal is long-debated, it appears that oil is retained in coal macromolecules rather than being expelled through a porosity network, as occurs with shale source rocks. Therefore, coal is not considered a major source rock for oil; instead, oil is thermally cracked to gas at higher maturity levels (Pepper and Corvi, 1995; Wilkins and George, 2002). It is possible that diffusion of reactive compounds to the coal cleat (fracture) environment, where water and nutrients are available, may provide a path of potential bioavailability of this solvent-soluble material (Pant et al., 2015). However, the potential bioavailability of coal-associated oil is poorly understood given its water-insoluble nature (Furmann et al., 2013).

Although only a small fraction of the bulk coal or shale is easily biodegraded (likely <10%; Strapoć et al., 2011b), the $\delta^{13}\text{C}$ and δD values of bulk source material provide a starting point for estimating $\delta^{13}\text{C}$ of intermediate compounds during biodegradation. Type II kerogen (generally related to shale; section 1.3) exhibits bulk $\delta^{13}\text{C}$ of -32‰ to -27‰ and type III kerogen (generally related to coal) exhibits $\delta^{13}\text{C}$ of -28‰ to -26‰. Bulk coal $\delta^{13}\text{C}$ values range from -27‰ to -22‰ (Whiticar, 1996). Coal and shale maceral groups exhibit small, overlapping but systematic variations from the bulk $\delta^{13}\text{C}$ value. In coal, $\delta^{13}\text{C}$ of maceral groups exhibits <3‰ of documented variability, with overall $\delta^{13}\text{C}$ of liptinite $\leq$ vitrinite $\leq$ inertinite (Whiticar, 1996; Rimmer et al., 2006). In shale, $\delta^{13}\text{C}$ of amorphinite $\leq$ alginite (Mastalerz et al., 2012), in which amorphinite and alginite are both members of the liptinite maceral group that dominates shale organic matter (Taylor et al., 1998). The slightly ^{13}C -depleted liptinite macerals in coal are generally thought to yield more biogenic gas than the more abundant, aromatic-rich vitrinite maceral group (Scott, 1999; Faiz and Hendry, 2006; Strapoć et al., 2011b; Papendick et al., 2011). Overall, however, it should be noted that both aromatic and aliphatic compounds may be

biodegraded in coals (Furmann et al., 2013; Gao et al., 2013). While thermal maturation of coal affects its bioavailability, heating has no significant effect on bulk or maceral-specific $\delta^{13}\text{C}$ (Whiticar, 1996; Lis et al., 2006).

Due to the low matrix permeability of coals, cleats help in creating permeable pathways for microbial activity. Further, as degradation rates will be heavily influenced by mass transport factors, and fractures can also dominate mass transport processes (Pant et al, 2015), more brittle macerals such as vitrinite, which more readily fracture, may potentially enhance microbial access and degradation rates over more hydrogen-rich but ductile macerals. Thus, the true bioavailability and reactivity of physically-defined macerals in bulk kerogens and coals depends on physical as well as chemical factors and remains poorly studied and understood. Still, with apparent differences in biodegradability and $\delta^{13}\text{C}$ among maceral groups, it might be hypothesized that $\delta^{13}\text{C}$ of low-molecular weight intermediates ($\delta^{13}\text{C}$ -LMW) could differ by up to a few per mil from the bulk coal, based essentially on the initial organic composition and biodegradable fractions in coal or shale.

The hydrogen isotope composition of organic matter follows similar patterns as carbon isotopes, with larger expected variations than seen in carbon isotopes because of the large relative mass difference between hydrogen-1 and deuterium. Bulk kerogen and coal exhibit δD in the range -175‰ to -75‰. Maceral groups exhibit a large range of δD values, with >40‰ separation between the D-enriched inertinite, the moderate vitrinite, and the D-depleted liptinite maceral groups (Whiticar, 1996). Likewise in shale kerogen, ~15‰ variability in δD has been observed between the more aliphatic, D-enriched alginite and the more aromatic, D-depleted amorphinite macerals (Mastalerz et al., 2012). Unlike carbon isotopes that show little or no isotopic enrichment with thermal maturation, discussed above, organic matter becomes D-enriched with increasing maturity (Schimmelmann et al., 2006). Because organic matter and water are the two expected sources for hydrogen in methane, it would be ideal for isotopic tracing if organic matter and the coexisting water occupied distinct ranges of δD . It is noteworthy that the D-depleted waters seen in inland, high-elevation or high-latitude basins with δD as negative as -170‰ (e.g. Aravena et al, 2003; Bates et al., 2011) can have δD values overlapping the expected range of

organic matter. In contrast, the more D-enriched waters seen in coastal, lower elevation, and/or lower-latitude settings are likely D-enriched relative to the organic matter, allowing a clear distinction between these two H sources (e.g. Whiticar, 1999; Sessions et al., 2004; Schimmelmann et al., 2006).

Bacterially-mediated fermentation and oxidation of soluble organic source material yields low molecular weight intermediate organic compounds, C_{LMW} (section 1.3.1; Figure 1). As discussed in Section S1, the identity, concentrations, and isotopic composition of specific intermediates during coal and shale biodegradation is little-known, made especially challenging by their short residence times and low steady-state concentrations (Whiticar, 1999). It is expected that large compound-specific fractionations affect compounds such as acetate that are produced and consumed relatively late in the biodegradation sequence, rather than higher molecular weight compounds in which any ^{13}C -substitution is diluted by the other C atoms in the molecule (Hayes, 2001; Meckenstock et al., 2004). Modest intramolecular carbon isotope fractionation during biological synthesis has been shown by measurements made on acetate (Blair et al., 1987; Hayes, 2001; Galimov, 2006). Acetate has been shown to have intramolecular ^{13}C depletion in the methyl carbon and ^{13}C enrichment in the carboxyl carbon. A few intramolecular $\delta^{13}C$ measurements have been reported for field-based samples; for example, Blair and Carter (1992) reported 7‰ intramolecular variation in acetate in a sulfate-reducing marine sediment unaffected by methanogenic fractionation, and Conrad and Klose (2011) estimated 10-15‰ intramolecular variation in newly-synthesized acetate in rice field soil.

Collectively, intermediate biodegradation steps, while not thoroughly documented, can produce modest differences of $\delta^{13}C$ between the bulk organic matter and newly-synthesized methanogenic substrates (prior to their consumption by methanogenic Archaea). Therefore, $\delta^{13}C$ measurements of bulk C_{org} are an approximation of the carbon ultimately routed through methanogenesis. In general, the C_{LMW} and substrates used by methanogens are thought to have $\delta^{13}C$ within a few per mil of the bulk organic matter (e.g. Blair and Carter, 1992; Révész et al., 1995; Whiticar, 1999; Jones D.M. et al., 2008; Heuer et al; 2009; Conrad and Klose, 2011; Conrad et al., 2011; Table S1). In aggregate, intermediate isotopic effects are thought to be much smaller than methanogenesis, which yields methane with $\delta^{13}C$ tens of per

mil more negative than the substrates utilized. As with carbon isotopes, the δD of intermediate compounds is not thought to differ greatly from the source organic matter that was biodegraded (Whiticar, 1999).

2.2 Possible implications of autotrophic acetogenesis and syntrophic acetate oxidation for C isotope signatures of biogenic gas

While two biochemically distinct pathways of methanogenesis are adapted to utilize acetate and H_2+CO_2 (section 1.3), acetate and H_2 can also undergo interconversion by acetogenesis and syntrophic acetate oxidation. These anaerobic pathways are depicted on Figure 1 and reviewed in Section S2. Briefly, acetogenesis (section S2.1) involves the formation of acetate from CO_2 and H_2 , and is thermodynamically favorable in high- H_2 conditions (Table 2).

Recent investigations of acetogenesis in substrate-limited environments show potential, but unproven, implications for coal beds and shales with their low availability of substrates. If acetogenesis were a significant part of carbon flow in coal beds, there could be considerable impacts on the interpretation of carbon isotope signatures. Unlike acetate production by fermentation reactions, acetogenesis imparts a large isotopic fractionation, yielding acetate with $\delta^{13}C$ values more negative than the source CO_2 (Gelwicks et al., 1989; Heuer et al., 2009; Blaser et al., 2013). Therefore, acetogenesis, if occurring, has the potential to influence $\delta^{13}C-CH_4$ if the acetate produced is subsequently converted to methane (Alperin et al., 1992; Conrad, 2005). For example, autotrophic acetogenesis followed by acetoclastic methanogenesis would yield methane with more negative $\delta^{13}C$ values than would bacterial fermentation yielding acetate that was utilized by methanogens. In the above scenario, methane from acetate might be misidentified as being from hydrogenotrophic methanogenesis due to its very negative $\delta^{13}C$ value. Isotopic modeling approaches do not typically incorporate the possible effects of acetogenesis on C isotope signatures of methane (e.g. Hornibrook and Aravena, 2010).

Syntrophic acetate oxidation (section S2.2) converts acetate to CO_2+H_2 , and is the reverse stoichiometry of acetogenesis. This reaction, which yields H_2 , is thermodynamically favorable when only H_2 concentrations are kept low, requiring a syntrophic relationship between acetate-utilizing and H_2 -

utilizing microbes. Unlike autotrophic acetogenesis or acetoclastic methanogenesis, syntrophic acetate oxidation is not thought to be highly-fractionating and would not impart a large effect on the isotopic composition of the residual unreacted acetate or the yielded CO₂ (Blair and Carter, 1992; Jones D.M. et al., 2008; Conrad and Klose, 2011). The overall isotope effect of syntrophic acetate oxidation depends on the terminal H₂-consuming pathway to which it is linked: hydrogenotrophic methanogenesis or heterotrophic bacterial metabolism such as sulfate reduction (Figure 1). Syntrophic acetate oxidation linked to hydrogenotrophic methanogenesis could yield methane with the apparent isotopic signature of hydrogenotrophic methanogenesis, even though acetate production was an important step in methane formation.

3 Reevaluation of isotopic fingerprinting approaches

Apparent geochemical and isotopic signatures from gas samples and associated waters are re-evaluated here, utilizing data from recently published studies of microbial gas derived from coal and shale biodegradation. The previously published data selected for this analysis include stable hydrogen and carbon isotopic ratios of methane ($\delta\text{D-CH}_4$, $\delta^{13}\text{C-CH}_4$), coexisting water ($\delta\text{D-H}_2\text{O}$), CO₂ gas ($\delta^{13}\text{C-CO}_2$), and where available, temperature and salinity (Table 3). To our knowledge, the data utilized in this review are from gas accumulations in the source formation itself, inferred to be derived from *in situ* methanogenesis. By focusing on methane generated and retained in water-saturated formations (Rice, 1993; Ayers, 2002; Curtis, 2002; Brown, 2011), this analysis disregards possible isotope effects associated with the migration of gas from source rocks to reservoirs in conventional gas systems. Select data sets of mixed thermogenic and biogenic gas were also incorporated into this analysis from representative coal and shale formations to further illustrate the potential overlap between thermogenic and biogenic gas isotopic signatures and their effects on interpreting the origins of the biogenic fraction.

3.1 H isotope fingerprinting of methanogenic pathways

3.1.1 Rationale for hydrogen isotope fingerprinting in coal and shale gas systems

Hydrogen isotope ratios of methane and the coexisting groundwater are often used to infer the biogenic nature of methane and methanogenic pathways. In methane derived from acetate, three of the

four hydrogen atoms are transferred from the acetate methyl group and therefore from an organic source (Pine and Barker, 1956; Schoell, 1980; Woltemate et al., 1984; Whiticar, 1999), whereas in hydrogenotrophic methanogenesis all four hydrogen atoms are derived from the water molecule (Fuchs et al., 1979; Daniels et al., 1980; Schoell, 1980; Whiticar, 1999). H_2 equilibrates rapidly with water (Valentine et al., 2004), and the net isotope effect associated with incorporation of four hydrogen atoms from H_2 into methane during hydrogenotrophic methanogenesis is $\sim 160\%$ (Schoell, 1980; Whiticar et al., 1986). We discuss hydrogen isotope effects in terms of the separation of δD between water and methane ($\delta D-H_2O - \delta D-CH_4$ or $\Delta\delta D$) because of the large net methane-water hydrogen isotope effects associated with methanogenesis. Where such large isotope offsets occur, the α or ϵ expressions are inexact approximations of the actual fractionation factor (Schimmelmann et al., 2006).

Methane derived from acetoclastic methanogenesis is thought to exhibit more negative $\delta D-CH_4$ values than methane from hydrogenotrophic methanogenesis because: (1) the δD value of source organic matter is thought to be more negative than the coexisting water (section 2.1); and (2) the isotope effect associated with incorporating the fourth H atom from water during acetoclastic methanogenesis is thought to be large ($\geq 300\%$; Whiticar, 1999). Whiticar (1999) also argued that methanogenesis using noncompetitive methylated substrates (e.g. methanol; section 1.3) should have a similar H isotope signature as when acetate was utilized. However, few studies have examined the fractionations imparted upon methylated substrates. It is possible to assign slopes to hydrogenotrophic and acetoclastic methanogenesis on a plot of $\delta D-H_2O$ vs. $\delta D-CH_4$ as shown in Figure 3a. The hydrogen isotope fingerprinting technique was originally applied using observed data exhibiting $\delta D-H_2O$ values near and moderately D-depleted relative to seawater in which there was little to no overlap of δD between organic matter and the coexisting water (-85 to 10% ; Schoell, 1980; Whiticar et al., 1986).

Empirical relationships have guided hydrogen isotope fingerprinting techniques, but whether, when, and how water hydrogen actually is incorporated into methane are not well understood. The fourth hydrogen atom during acetoclastic methanogenesis (not derived from a methyl group) illustrates an unresolved problem with the H isotope application. This H atom must be derived from water, an

incorporation associated with a 160‰ effect in the case of hydrogenotrophic methanogenesis. A mass balance of organic δD values (three atoms) and water δD (one atom) suggests that the fourth hydrogen would have an unreasonably negative δD value. Either this fourth hydrogen atom is subject to exceedingly large fractionation while being incorporated into methane, or perhaps another isotope effect is imparted on the three hydrogen atoms that were bonded to carbon in the precursor methyl group (Whiticar, 1999).

3.1.2 The influence of water on the isotopic composition of methane in coal and shale gas systems

Empirical and experimental evidence indicates that the isotopic composition of biogenic methane is correlated with the δD value of water. In a global review of field data from freshwater wetlands exhibiting δD -H₂O values from -130 to 10‰, Waldron et al. (1999) argued that δD -CH₄ is primarily controlled by δD -H₂O and suggested a relationship from field data of $0.675(\delta D) - 284$ ‰ (Figure 3a). This field relationship differed from laboratory incubations, and it was concluded that δD -CH₄ values were governed by water hydrogen isotopic composition and environmental factors such as migration effects, but not primarily by methanogenic pathways (Waldron et al., 1999). Other experiments also suggested that δD -CH₄ is fairly insensitive to methanogenic pathway but primarily records water sources. For example, hydrogen isotopes in a wetland sediment implied a mixture of hydrogenotrophic and acetoclastic methanogenesis, inconsistent with ¹⁴C tracers indicating that acetate was not converted to methane (Lansdown et al., 1992).

The data presented in Figure 3a (including some data sets discussed by Golding et al. (2013)) exhibit an apparent systematic pattern: δD -H₂O plotted against δD -CH₄ lies generally near the line representing hydrogenotrophic methanogenesis with slope of 1 and offset of ~160‰. The median difference between δD -H₂O and δD -CH₄ (Figure 3a) of all wells in this study is 170 ± 13 ‰ ($n=284$) or 168 ± 12 ‰ when only considering samples in the “Microbial gas” field on Figure 2 ($n=149$; both $\pm 1\sigma$). This pattern is consistent with the trends that Schoell (1980) and Whiticar et al. (1986) documented within a smaller range of δD -H₂O values. The apparent dominance of hydrogenotrophic methanogenesis in coal beds and shales (Figure 3a) contrasts with previous reviews of aquatic sediments, in which H

isotopes of many gas samples imply mixed microbial pathways, plotting between the hypothetical hydrogenotrophic methanogenesis and acetate fractionation lines (Whiticar et al., 1986; Whiticar, 1999; Waldron et al., 1999). While H isotopes almost uniformly imply that hydrogenotrophic methanogenesis is dominant in coal beds and shales, other isotopic and microbiological evidence suggests a more complex assemblage of pathways. Nucleic acid analysis and culturing of native subsurface microbes suggests that acetoclastic or methylotrophic methanogenesis could be significant in some of these coals and shales. In addition, carbon isotopes, discussed in a subsequent section, are not fully consistent with the apparent H isotope interpretation (Table 1).

While it might be inferred from H isotopes that hydrogenotrophic methanogenesis dominates in biogenic coal and shale gas as reviewed above, we argue that pervasive hydrogen isotope transfer from water during biodegradation is reflected in the methane produced. It must be emphasized that direct H isotope exchange between water and hydrocarbons is more associated with thermal maturation than biodegradation, requiring temperature and time exceeding the expected conditions of biogenic gas systems. Organic H undergoes very slow isotope exchange with water, estimated to require 10^4 - 10^8 years at 50-100°C, and methane is among the least exchangeable of all documented organic compounds (Sessions et al., 2004; Schimmelmann et al., 2006). At the even lower temperatures and shorter time scales of biogenic gas systems, direct methane-water hydrogen exchange can be neglected completely. At the temperatures of biogenic gas systems, where intermediate compounds are produced enzymatically (Strapoć et al., 2011b), hydrogen transfer from water seems to occur as methane precursors are synthesized or used. H transfer has been documented in a small number of incubation studies implying enzymatic incorporation of water into methane precursors during methanogenesis. de Graaf et al. (1996) observed loss of deuterium label from acetate in a sediment incubation and inferred that this transfer of deuterium was mediated by carbon monoxide dehydrogenase or methyl group dehydrogenation enzymes. Similarly, de Graaf and Cappenburg (1996) documented loss of deuterium label from formate to water in methanogenic sediments, attributing this to the formate dehydrogenase enzyme. Neither study was

conducted at *in situ* substrate availability conditions, so the actual environmental significance of these phenomena was not confirmed.

While few studies have systematically examined the mechanisms or environmental importance of water hydrogen incorporation into biogenic methane, as noted above, recent clumped isotope studies have added valuable new insights on hydrogen transfer, its time scale of occurrence, and its environmental significance. The clumped isotope approach examines specific substitutions of deuterium within methane, for example, methane of mass 18, $^{13}\text{CH}_3\text{D}$. These measurements provide important evidence of how hydrogen isotopes equilibrate at natural substrate availability conditions in biogenic gas systems and on what time scale. Clumped isotope measurements of methane from Powder River Basin coal beds (Wang et al., 2015) and the Michigan Basin (Antrim Shale; Stolper et al., 2015) confirm hydrogen isotope equilibrium with water in slowly-generated and fairly long-lived biogenic gas. The co-occurrence of ^{13}C and D in a methane molecule relative to a random distribution of isotopologues, reported as $\Delta^{13}\text{CH}_3\text{D}$, is correlated with $\delta\text{D-H}_2\text{O} - \delta\text{D-CH}_4$ when water and methane are in equilibrium at $<50^\circ\text{C}$. Relatively high $\Delta^{13}\text{CH}_3\text{D}$ values, seen in biogenic coal and shale gas, fit model calculations of slow methanogenesis at nanomolar H_2 concentrations, in which methane acquired an isotopic signature that is equilibrated with water (Wang et al., 2015).

Together, the equilibrium relationship between $\Delta^{13}\text{CH}_3\text{D}$ and $\delta\text{D-H}_2\text{O} - \delta\text{D-CH}_4$ indicates that the water-methane isotope effect inherited no kinetic fractionation signature from slow methanogenesis. These well-equilibrated gases exhibit $\delta\text{D-H}_2\text{O} - \delta\text{D-CH}_4$ of $\sim 160\text{‰}$ (Wang et al., 2015). In contrast to the slowly-formed coal and shale gas, methane from some cultures and aquatic sediments formed too rapidly for H isotopes to equilibrate with the coexisting water (Stolper et al., 2014, 2015; Wang et al., 2015). These non-equilibrated, rapidly-formed gases exhibit $\delta\text{D-H}_2\text{O} - \delta\text{D-CH}_4 > 160\text{‰}$. In cultures and recent sediments, disequilibrium between $\Delta^{13}\text{CH}_3\text{D}$ and $\delta\text{D-H}_2\text{O} - \delta\text{D-CH}_4$ shows that gases carry inherited kinetic isotope effects of the methanogenic pathway and is consistent with rapid methanogenesis at H_2 concentrations orders of magnitude higher than the coal and shale formations discussed here (Wang et al., 2015). Here the clumped isotope data illustrate the different sensitivity to water hydrogen transfer

between various biodegradation rates and therefore depositional settings. Low-H₂ settings such as coals and shales are evidently the most challenging environments for extracting metabolic pathway information from hydrogen isotopes due to their high sensitivity to water δ D.

The rate-dependence of H isotope equilibration argues that wetland or culture methanogenic fractionation data should be applied to slowly biodegraded coal beds and shales carefully, if at all. Also, the dependence of δ D-CH₄ on water hydrogen calls into question the use of δ D-CH₄ to simply fingerprint methane formation pathways in the case of slowly-biodegraded fossil carbon sources. Nonetheless, hydrogen isotopes can be applied to establish: (1) that methane is of microbial rather than thermal origin; and (2) that methanogenesis occurred in contact with the coexisting water, rather than having migrated from an external source formation. In some cases the association of δ^2 H-CH₄ with isotopically distinct water (e.g. Pleistocene water; Martini et al., 1996) can also constrain the timing of methanogenesis.

3.1.3 Implications of hydrogen transfer for combined C-H fingerprinting of biogenic gas

A widely-used application for delineating methanogenic pathways combines δ D-CH₄ with δ^{13} C-CH₄. Diagnostic C-H isotopic fields described by Schoell (1980), Whiticar et al. (1986), and Whiticar (1999) and argued to represent hydrogenotrophic methanogenesis, methyl-type fermentation (acetoclastic/methylotrophic methanogenesis), and thermogenic gas (Figure 3b) have been widely applied to coal and shale gas systems (e.g. Thielemann et al., 2004; Zhang et al., 2005; Barker and Dallegge, 2006; Faiz and Hendry, 2006; Strapoć et al., 2007, 2011a,b; Butland and Moore, 2008; Warwick et al., 2008; Flores et al., 2008; Kotarba and Pluta, 2009; Cheung et al., 2010; Kinnon et al., 2010; Dawson et al., 2012; Ni et al., 2012; Susilawati et al., 2013; Pashin et al., 2014; Baublys et al., 2015). One advantage to this approach is that no water analysis is required. However, values of δ D-CH₄ are complicated by the water-dependence of hydrogen isotope signatures, as detailed above.

The environmental behavior of H isotopes is especially critical for applying the C-H fields to inland, higher-latitude, higher-elevation coal and shale basins, exhibiting δ D-H₂O values as negative as -170‰ and δ D-CH₄ values as negative as -330‰ (e.g. Powder River Basin, × symbols; Elk Valley, □ symbols; Williston Basin, ◇ symbols; Figure 3a-b). These D-depleted gases typically plot in the

“methyl-type fermentation” (acetoclastic/methylotrophic methanogenesis) field of Whiticar (1999) as shown on Figure 3b (× symbols). Contradicting the diagnostic C-H fields, the ~160 ‰ difference of δD values between methane and water at these sites (section 3.1.2; Figure 3a) implies that hydrogenotrophic methanogenesis dominates. Golding et al. (2013) noted the apparent inadequacy of the C-H diagnostic fields in the case of these inland, high-elevation, high-latitude basins, and proposed that the global freshwater wetland hydrogen isotope trend of Waldron et al. (1999; section 3.1.2; Figure 3a) with its slope of 0.675, could replace the relationship of Whiticar (1999), with its slope of 0.25, to represent acetoclastic methanogenesis. Making this substitution to represent the hypothesized net effects of acetoclastic methanogenesis, Golding et al. (2013) argued that, if acetoclastic methanogenesis were occurring, the resulting methane would reasonably plot in the “methyl-type fermentation” field across a range of water isotopic compositions. Therefore, Golding et al. (2013) advocated the continued use of C-H fields combined with direct comparison of $\delta D-H_2O$ and $\delta D-CH_4$ (e.g. Figure 3a), and concluded that coal and shale gas formations are dominated by hydrogenotrophic methanogenesis.

Yet as we argue above (section 3.1.2), H isotope signatures of slowly-formed methane in coal beds and shales biogenic methane show evidence of equilibration with water hydrogen. When isotopic equilibration with water is considered (uncertainty along the X-axis in Figure 3b), the C-H fields do not adequately separate hydrogenotrophic and acetoclastic methanogenesis. Beyond applications that do provide significant constraints on thermal or migration effects, noted in section 3.1.2, hydrogen isotopes apparently contain little reliable information for metabolic pathway identification in coal beds and shales, while they may retain their fidelity in faster biodegradation settings or less-limited substrate availability conditions.

3.2 Carbon isotope fingerprinting techniques using methane and coexisting inorganic carbon

As discussed in section 2.1, the large isotopic fractionation during methanogenesis is thought to be the dominant control on the resulting $\delta^{13}C-CH_4$ value. In some data sets reviewed here, $\delta^{13}C-CH_4$ is consistent with an unambiguous microbial origin. For example, the thermally immature Powder River Basin is dominated by dry microbial gas exhibiting $\delta^{13}C-CH_4$ values < -50‰ (× symbols in Figure 2). In

other data sets reviewed here showing evidence of both thermogenic and biogenic components (e.g. Illinois Basin shale, ● symbols; Michigan Basin shale, ■ symbols; and San Juan Basin coalbed methane, △ symbols; Figure 2), $\delta^{13}\text{C-CH}_4$ values are apparently insensitive to variations in the $\text{C}_1/(\text{C}_2+\text{C}_3)$ ratio (vertical trends on Figure 2), implying that mixing calculations would not reveal the $\delta^{13}\text{C}$ value of the biogenic fraction.

In addition to yielding very negative values of $\delta^{13}\text{C-CH}_4$ values, the highly-fractionating nature of methanogenesis also causes the CO_2 yielded from biodegradation to be ^{13}C -enriched, yielding distinctive values of $\delta^{13}\text{C-CO}_2 > 0\text{‰}$. ^{13}C -enriched CO_2 and dissolved inorganic carbon (DIC) can be seen in cases of pure microbial gas or in mixtures with thermogenic gas (e.g. Scott et al., 1994; Martini et al., 2008; Osborn and McIntosh, 2010; Bates et al., 2011). In this section, we illustrate factors that can cause carbon isotope signatures of methane and coexisting inorganic carbon to vary from the true methanogenic signal in field-collected samples.

3.2.1 Apparent fractionation factors in field-collected samples: Application of $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$

In an ideal closed methanogenic system, methane would exhibit more negative values of $\delta^{13}\text{C}$ than the source organic carbon. ^{13}C -enriched CO_2 would also be present, representing a mass balance of the biodegraded C_{LMW} . The separation between $\delta^{13}\text{C-CH}_4$ and $\delta^{13}\text{C-CO}_2$ is often reported as $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ (e.g. Whiticar et al., 1986):

$$\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4} = (\delta^{13}\text{C-CO}_2 + 1000)/(\delta^{13}\text{C-CH}_4 + 1000) \quad (1)$$

When applied to field-collected samples representing multiple biogeochemical pathways, $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ is a net apparent fractionation and not strictly a substrate-to-product fractionation (Conrad, 2005). As discussed in section 2, hydrogenotrophic methanogenesis is thought to impart larger carbon isotope fractionation than acetoclastic methanogenesis. In recent sediments, Whiticar (1999) estimated a $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ range of 1.039-1.058 for acetoclastic methanogenesis and 1.049-1.095 for hydrogenotrophic methanogenesis. Methylophilic cultures using methanol, trimethylamine, and dimethyl sulfide (section 1.3) have been reported to impart larger substrate-to-methane fractionations than acetoclastic

methanogenesis (Whiticar, 1999; Conrad, 2005; Penger et al., 2012). Therefore, methylotrophic methanogenesis in the field could exhibit $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ overlapping with that of hydrogenotrophic methanogenesis (Penger et al., 2012).

Overall, comparison of apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ calculated from recent field-based studies indicates: (1) the majority of apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ values fall within the range inferred to result from solely hydrogenotrophic methanogenesis (>1.058); but (2) lower, acetoclastic-like values of apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ are reported in several basins reviewed here. For example, systematic variation in apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ has been documented along gradients of inferred recharge and/or nutrient availability in the Powder River Basin (Flores et al., 2008; Bates et al., 2011). In the Powder River Basin data (× symbols in Figure 4a), the shallow basin-edge environment exhibits apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ values lower than the range expected for hydrogenotrophic methanogenesis, while the deeper portion of the basin exhibits apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ >1.058 , fully consistent with hydrogenotrophic methanogenesis. This gradient has been interpreted to represent variations of dominant metabolic pathway between acetoclastic methanogenesis and hydrogenotrophic methanogenesis (Gorody, 1999; Flores et al., 2008). In some cases, the lower, acetoclastic-like values of apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ are associated with more negative values of $\delta^{13}\text{C-CH}_4$ normally expected for hydrogenotrophic methanogenesis, the more fractionating pathway (note × symbols at more negative values of $\delta^{13}\text{C-CH}_4$ and lower values of $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ in Figure 4a).

Four types of observations imply that the estimated ranges of apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ do not prove that a specific metabolic pathway truly dominates a field-collected sample: (1) Microbial community characterization suggests that acetoclastic and/or methylotrophic methanogenesis may occur in formations showing apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ values consistent with hydrogenotrophic methanogenesis (section 1.4; Table 1). (2) Steady-state substrate concentrations of coal beds and shales are difficult to simulate in the laboratory (section 1.4), and therefore realistic reference values of $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ for the substrate-poor coal bed environment can be difficult to define. When large initial substrate concentrations are used, culturing can underestimate $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ as substrates are consumed and not replaced (e.g. Whiticar et al., 1986; Conrad, 2005). Likewise, recent sediments can experience high substrate availability not seen in slowly-

biodegraded material (e.g. Miyajima et al., 1997; Nakagawa et al., 2002a,b). $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ values from cultures or recent sediments (such as the ranges of Whiticar (1999), quoted above) should be applied with caution to slowly-biodegraded coal beds and shales. (3) Hypothesized shifts of apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ with temperature and salinity are not seen in field-based studies reviewed here (see Section S3 for discussion), likely overwhelmed by other influences on apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$. (4) Common nonmethanogenic processes impart effects on apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ in field-collected samples, obscuring the methanogenic signature. These phenomena are discussed below.

3.2.2 Non-methanogenic anaerobic processes: Implications for $\delta^{13}\text{C-CH}_4$ and apparent

$\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$

While $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ is utilized to fingerprint methanogenic pathways, nonmethanogenic processes can also produce or consume methane and CO_2 and affect $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$. Therefore, in field-collected samples, eq. 1 yields a net apparent value of $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ that may overlook additional subsurface complexity encoded in C isotope signatures. Nonmethanogenic processes affecting methane or CO_2 and therefore $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ include: (1) bacterial processes such as sulfate reduction and iron reduction, which produce CO_2 (but not CH_4) with much smaller fractionation on CO_2 than methanogenesis; (2) methane oxidation, which converts CH_4 to CO_2 with isotopic effects on both (Whiticar and Faber, 1986); (3) mixing of microbial gas with thermogenic gas with its distinct isotopic composition (Whiticar et al., 1986); and (4) formation water interaction, which can cause gas loss and mineral reactions with isotopic effects on CH_4 and CO_2 . These are discussed below.

When alternative electron acceptors such as sulfate are present, heterotrophic bacteria are more efficient than methanogenic Archaea at utilizing the competitive substrates including H_2 and acetate (section 1.3.2). By transferring electrons to inorganic oxidants (Table 2), biodegradation yields more CO_2 and less CH_4 than the ideal, methanogenic stoichiometric ratio in eq. S1, predicted for C_{LMW} with given H/C and O/C ratios. The CO_2 -yielding bacterial processes are little-fractionating on carbon (section 2.2), yielding microbial CO_2 of isotopic composition close to that of C_{LMW} and also affecting the isotopic difference between $\delta^{13}\text{C-CH}_4$ and $\delta^{13}\text{C-CO}_2$. Therefore, where a sample includes a blend of sulfate-

reducing and methanogenic conditions, the apparent value of $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ would be lower than the true methanogenic value (trending downward on Figure 4b). This effect can be seen in shallow methanogenic environments apparently affected by nonmethanogenic processes during the gas generation history, where values of apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ as low as 1.044 occur (Powder River Basin, $\times$ symbols; Figure 4a). Similarly, the majority of coalbed methane wells in the Illinois Basin exhibit apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4} > 1.058$, but a few exhibit apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ as low as 1.032. These lower values could be consistent with a combination of methanogenic and non-methanogenic pathways ($+$ symbols in Figure 4a). Elsewhere, however, evidence of sulfate reduction, such as sulfate concentrations $> 1\text{mM}$ or ^{34}S -enriched sulfate, is not always matched by systematic carbon isotope shifts. For example, New Albany Shale (Illinois Basin) waters exhibit variations in sulfate ($< 1\text{-}22\text{ mM}$), and ^{34}S -enriched sulfate is present, but $\delta^{13}\text{C-CH}_4$, $\delta^{13}\text{C-CO}_2$, and apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ fall within narrow, methanogenic ranges (based on matched gas and water samples in McIntosh et al., 2002; $\bullet$ symbols in Figure 4a). Therefore, apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ does not always record sulfate reduction supported by independent evidence.

Above we discussed the case of sulfate, iron, or other electron acceptors competing with active methanogenesis, causing methane to form at lower than stoichiometric abundance and imparting effects on apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$. In addition, already-formed methane can be affected by biogeochemical or hydrodynamic processes. Methanotrophs are capable of exploiting the small energy yield associated with methane oxidation, either alone or in consortium with sulfate-reducing bacteria (Boetius et al., 2000; Alperin and Hoehler, 2010; Milucka et al., 2012). The inputs of oxygen required for aerobic methane oxidation in the deep subsurface are implausibly large (e.g. Head et al., 2003; Martini et al., 2003), so anaerobic methane oxidation is somewhat more realistic, driven by electron acceptors such as sulfate. To date, anaerobic methane oxidation has been documented mainly in marine sediments and methane seeps, where sulfate is ubiquitous and migration and diffusion bring methane into contact with oxidants. The methane pool in subsurface microbial gas systems is larger and less subject to redox fluctuations than other environments where methane oxidation contributes substantially to the amount of methane present. Overall, methane oxidation is less likely to consume a large proportion of accumulated methane in coal

beds and shales than the shallow subsurface. Still, if it occurs, methane oxidation could impart an effect on apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ in methane-bearing coal beds or shales perhaps where pumping brings methane in contact with oxidants such as sulfate. The residual un-oxidized methane exhibits more positive $\delta^{13}\text{C-CH}_4$ after methane oxidation. Methane oxidation, if occurring, would therefore shift the residual gas to more positive values of $\delta^{13}\text{C-CH}_4$ and lower values of apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ (e.g. Whiticar et al., 1986; Whiticar and Faber, 1986; Révész et al., 1995; Whiticar, 1999), trending down and to the right on Figure 4b.

Mixtures of biogenic and thermogenic gas could affect the apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ of a gas sample because biogenic and thermogenic gas occupy different ranges of $\delta^{13}\text{C-CH}_4$ and $\delta^{13}\text{C-CO}_2$. Briefly and in general, thermogenic gas typically exhibits more positive $\delta^{13}\text{C-CH}_4$ and more negative $\delta^{13}\text{C-CO}_2$ than biogenic gas. As thermal maturity increases, both CH_4 and CO_2 become ^{13}C -enriched, and $\text{C}_1/(\text{C}_2+\text{C}_3)$ decreases with the thermal production of C_{2+} hydrocarbons. $\delta^{13}\text{C}$ of low-maturity thermogenic CH_4 may overlap with biogenic CH_4 near -50‰, whereas high-maturity CH_4 is significantly ^{13}C -enriched, reaching values of approximately -25‰. Thermogenic CO_2 in coal is likely to fall in the range of -25 to -10‰, that is, slightly ^{13}C enriched relative to the source coal but not as ^{13}C -enriched as microbial CO_2 from methanogenesis which can exceed 0 ‰ (Hornibrook and Aravena, 2010; Golding et al., 2013). Given these expected trends, thermogenic-biogenic mixing would cause a systematic offset to lower values of apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ as argued by Whiticar et al. (1986), trending down and to the right on Figure 4b.

As groundwater flows through coal and shale formations, methane and CO_2 can be lost through dissolution and advective removal from the formation, rather than being fully retained on the solids. While $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ assumes that CH_4 and CO_2 are fully retained, leaky retention (a partially open system) can impart isotopic effects on the remaining gas. CO_2 is more strongly adsorbed to coals than CH_4 (Weniger et al., 2010), but CO_2 is also more soluble in groundwater than CH_4 (Stumm and Morgan, 1996; Brown, 2011). The ^{13}C -substituted gases, being more strongly adsorbed, are preferentially retained in the formation, and the ^{12}C -bearing gases are preferentially desorbed if pressure is insufficient to fully retain CH_4 and CO_2 (Strapoć et al., 2006). It has been argued that CO_2 is overall more prone to be lost than CH_4 in coal beds (Strapoć et al., 2007). Since CO_2 and methane differ in their solubility and tendency to

adsorb to the solids, their relative retention may be formation-specific (i.e. gas generation rate vs. groundwater flow rate). Groundwater dissolution of CO₂ is also implied by the low abundances of gas-phase CO₂ in typical biogenic coal and shale gas samples (<10%; McIntosh et al., 2002; Martini et al., 2003; Strapoć et al., 2008a; Bates et al., 2011), whereas stoichiometry suggests that microbial CO₂ abundance should be on the same order as CH₄ abundance (eq. S1). Dissolution of microbial CO₂ results in high dissolved inorganic carbon (DIC) concentrations in formation water (e.g. Révész et al., 1995; McIntosh et al., 2002; Martini et al., 2003; Bates et al., 2011). At near-neutral pH and 25°C, DIC is ¹³C-enriched by approximately 8‰ relative to gas-phase CO₂ (Salomons and Mook, 1980; Clark and Fritz, 1997), which would reduce apparent α¹³C_{CO₂-CH₄} values in the gas phase. Depending on local conditions, mineral reactions with formation water can further affect the isotopic composition of produced CO₂. Dissolution of carbonate minerals (e.g. Thorstensen et al., 1979) would dilute the methanogenic signal in DIC and gas-phase CO₂, whereas carbonate precipitation (e.g. Budai et al., 2002; Pitman et al., 2003) would not substantially alter the δ¹³C of the DIC that remained (Clark and Fritz, 1997). At the time scales of groundwater flow in coal and shale basins (likely >10³⁻⁴ yr; Bates et al., 2011; Schlegel et al., 2011b), groundwater is capable of removing microbial CO₂ (and to a lesser degree CH₄) from the formation. While few field-based studies have attempted to quantify the combined isotopic effects of hydrodynamic loss, it appears that the carbon isotopic effects are small, up to a few per mil on CH₄ and CO₂, suggested by model calculations (Kinnon et al., 2010), desorption studies (Strapoć et al., 2006), and repeat sampling of wells that shows little change of δ¹³C-CH₄ and δ¹³C-CO₂ during production histories of wells that coincide with formation depressurization and gas desorption (Illinois Basin: Strapoć et al., 2008a; Antrim Shale: Kirk et al., 2012; repeat sampling of Powder River Basin wells: Flores et al., 2008; Bates et al., 2011; Vinson et al., 2012). Together, the comparative retention between CH₄ and CO₂, and between ¹²C and ¹³C-bearing compounds, implies that water interaction and subsequent transfers of DIC in formation water, could impart a small increase or decrease on the apparent α¹³C_{CO₂-CH₄} value of the gas that was retained in the formation. From the limited available data, it seems that these desorption or groundwater

effects are smaller than the methanogenic fractionation - perhaps up to 0.01 shift in apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ (Figure 4b).

3.2.3 Pathway-independent mass balance: Implications for $\delta^{13}\text{C}$ and apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$

As detailed above, nonmethanogenic processes can modify values of apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$, a fingerprinting technique that assumes that all C_{LMW} is routed through methanogenesis. In this section, we apply an open-system steady-state mass balance model that requires no assumptions about methanogenesis being the dominant biodegradation pathway. The model could apply to systems dominated by primary biogenic gas where $\delta^{13}\text{C}\text{-CH}_4$ and/or $\delta^{13}\text{C}\text{-CO}_2$ are seen to vary within a basin, and without major thermogenic inputs or secondary biodegradation of thermogenic hydrocarbons. The mass balance model tests the sensitivity of $\delta^{13}\text{C}$ values to highly-fractionating methanogenesis vs. little-fractionating nonmethanogenic processes.

The open-system mass balance model assumes that any available C_{LMW} is consumed efficiently, without accumulating. The identity of source C_{LMW} governs the relative yield of methane and CO_2 (eq. S1), and in turn $\delta^{13}\text{C}\text{-CH}_4$ and $\delta^{13}\text{C}\text{-CO}_2$ values, because of mass and electron balance. The simple branchpoint model shown in Figure 5 (e.g. Hayes, 2001) depicts carbon flow through methanogenesis and nonmethanogenic pathways (oxidation to CO_2). Hypothetically, the yield of CO_2 and methane and their $\delta^{13}\text{C}$ values could be determined for each methanogenic pathway (upper diagrams in Figure 5). In field-based samples, however, the C fluxes through each methanogenic pathway are not usually known (ϕ_{11} vs. ϕ_{21} vs. ϕ_{31} in Figure 5). An alternative pathway-independent approach utilizes mass balance between methane and CO_2 yielded by all active metabolic pathways, represented by a single branchpoint (methane production represented by ϕ_1 in Figure 5). Using the pathway-independent model (lower diagram on Figure 5), f approximates the proportion of C_{LMW} that is converted to methane by all combined pathways:

$$f \approx \frac{\phi_1}{\phi_0 + \phi_1} \approx \frac{\phi_{11} + \phi_{21}}{\phi_{10} + \phi_{11} + \phi_{12} + \phi_{20}} \quad (2)$$

Assuming that C_{LMW} is produced and subsequently converted to CH_4 or CO_2 (C_{LMW} does not accumulate in groundwater; sections 2.1 and S1), and assuming that CH_4 and CO_2 are retained in the formation

(section 3.2.2), the $\delta^{13}\text{C}$ values of methane and coexisting inorganic carbon are governed by mass balance (Blair, 1998; Hayes, 2001; Brown, 2011):

$$\delta^{13}\text{C-LMW} \approx f(\delta^{13}\text{C-CH}_4) + (1-f)(\delta^{13}\text{C-CO}_2) \quad (3)$$

The minimum value of f is 0, implying that no methanogenesis occurred and that C_{LMW} is converted only to CO_2 , not to CH_4 . The maximum value of f , f_{max} , occurs when all C_{LMW} is routed through methanogenesis. f_{max} is a property of the organic compounds biodegraded into methanogenic substrates (e.g. Brown, 2011). For a given carbon source, f_{max} is controlled by the mass and electron balance in equation S1 and represents the proportion of C_{LMW} converted to CH_4 if non-methanogenic pathways (e.g. sulfate reduction) do not also consume C_{LMW} . By assuming that bulk organic source material has equivalent properties to the biodegradable portion, Brown (2011) predicted increasing f_{max} values with increasing coal rank or source maturity. Estimated f_{max} values ranged from 0.3-0.5 for peat and lignite to 0.75-0.78 for $\geq \text{C}_5$ alkanes in oil (Brown, 2011). Specific compounds and compound classes identified in coal bed and shale waters may approximate C_{LMW} , although the intermediate compounds converted to methanogenic substrates are poorly understood (section S1). A suite of possible C_{LMW} compounds, reported in produced waters from five coal and shale gas systems by Orem et al. (2014), was converted into estimated f_{max} values based on their H/C and O/C ratios. If these compounds were biodegraded, f_{max} values would range from 0.48 to 0.77 (median 0.62; Figure S3; see Section S4 for further discussion).

If $f = f_{\text{max}}$, the CH_4/CO_2 ratio would equal the ideal methanogenic CH_4/CO_2 ratio shown in equation S1 (Section S1). However, in the subsurface, not all C_{LMW} need be routed through methanogenesis. That is, f could be less than f_{max} , depending on the competition between methanogenesis and non-methanogenic processes such as sulfate reduction and Fe oxide reduction (section 3.2.2). Carbon uptake into microbial biomass is also possible, but is thought to be minor in comparison to the products CO_2 and CH_4 (e.g. Sugimoto and Wada, 1993; Conrad, 2005). If some C_{LMW} is converted to CO_2 through non-methanogenic processes, the CH_4/CO_2 ratio of products (ϕ_1 / ϕ_0 ; eq. 2) would be lower than the ideal methanogenic ratio implied by the carbon source (eq. S1). The relative yield of methane and CO_2 can affect the $\delta^{13}\text{C}$ value of both products by mass balance. As less methane is yielded relative to CO_2 , the

$\delta^{13}\text{C}$ values of methane and CO_2 become more negative as the majority of substrates are routed through little-fractionating pathways yielding CO_2 , and $\delta^{13}\text{C}\text{-CO}_2$ approaches $\delta^{13}\text{C}\text{-LMW}$ (eq. 3). Mass balance can explain observations of very ^{13}C -depleted methane. For example, near the sulfate reduction-methanogenesis boundary (f near 0), a small quantity of methane with $\delta^{13}\text{C}\text{-CH}_4 < -100\text{‰}$; Sackett et al., 1979; Heuer et al., 2009; Pantano, 2012) coexists with CO_2 exhibiting $\delta^{13}\text{C}$ little modified from the inferred C_{LMW} isotopic signature (Figure S4).

The basins examined in this study exhibit large variations of apparent f , assuming $\delta^{13}\text{C}\text{-LMW} \approx -25\text{‰}$. For example, Upper Silesian gases exhibit relatively low values of f (median 0.21, range 0.02-0.32; symbols in Figure 4d), the Cherokee and Forest City Basins exhibit moderate f values (median 0.47, range 0.30-0.51; symbols), and the dry Michigan Basin shale gases exhibit higher values of f (median 0.63, range 0.55-0.66; symbols). Large variation of apparent f occurs within the Powder River Basin (median 0.52, range 0.01-0.59; symbols in Figure 4d) and Illinois Basin coalbed gas (median 0.46, ranging from a non-meaningful negative value to 0.56; symbols). Lower values of f could occur where nonmethanogenic processes (e.g. sulfate or iron reduction) are significant at competing with methanogenesis for substrates and/or where subsequent gas inputs, transport, or gas loss (section 3.2.2) have affected carbon isotope values. In four biogenic gas systems where both f and f_{max} have been estimated, f approaches, but on average does not exceed, its hypothetical maximum value f_{max} (section S4; Figure S5).

Because C_{LMW} does not accumulate as argued above, open-system modeling of intermediates and methane precursors follows different mass balance assumptions than modeling the consumption of an initial pool of source organic material (that is, closed-system consumption of the whole coal or shale). These assumptions are discussed in Section S5. To evaluate the possible sensitivity of $\delta^{13}\text{C}\text{-CH}_4$ to the degree of coal or shale consumption, a closed-system Rayleigh-type model was applied to hypothetical coal biodegradation. Briefly and as discussed in Section S5, the results of these calculations suggest that the accumulated CH_4 and CO_2 would not experience large changes in $\delta^{13}\text{C}$ as the bioavailable compounds

in coal and shale are progressively consumed and not replaced. This is essentially due to the small fractionation expected to occur during C_{LMW} production and the subsequent full conversion of available C_{LMW} to methane and CO_2 (Figure S6-S7).

In summary, interpreting f requires no assumption of a dominant methanogenic pathway (acetoclastic, hydrogenotrophic, or methylotrophic methanogenesis), which was implied by variations in apparent $\alpha^{13}C_{CO_2-CH_4}$ (section 3.2.1-3.2.2). Instead, variations in $\delta^{13}C-CH_4$ and $\delta^{13}C-CO_2$ can be governed by the favorability or extent of methanogenesis relative to non-methanogenic pathways. The large difference in fractionation between methanogenesis and the nonmethanogenic pathways could overwhelm the relatively modest differences among the methanogenic pathways themselves (section 3.2.1). Therefore, the combined use of $\delta^{13}C-CH_4$ and $\delta^{13}C-CO_2$ could provide less pathway-specific diagnostic information about active methanogens in field-based studies than previously argued.

Collectively, complications in applying C and H isotopes to biogenic coal and shale gas point to needed improvements, possibly including: (1) Characterization of source organic matter involved in methanogenic biodegradation can better constrain expected values of $\delta^{13}C-CO_2$ and $\delta^{13}C-CH_4$; (2) Models that consider controls on biodegradation (such as substrate availability) and site-specific environmental conditions (such as sulfate or perhaps Fe oxide availability) can strengthen interpretation of pathway-independent tracers such as $\delta^{13}C-CO_2$ and $\delta^{13}C-CH_4$; (3) Application of pathway-specific tracers such as compound-specific and intramolecular isotopic analysis of methane precursors (e.g. acetate, methanol) may provide pathway-specific evidence of a substrate's utilization in methanogenesis; (4) Finally, emerging clumped isotope techniques can improve constraints on biogenic-thermogenic mixing, confirm the role of water hydrogen in microbial methane formation, and underline the significance of biodegradation rate differences between coal beds and aquatic sediments, complementing conventional stable isotopic applications.

4 Conclusions

Hydrogen and carbon isotope signatures have been applied routinely to microbial gas systems in the terrestrial subsurface to distinguish metabolic pathways (hydrogenotrophic methanogenesis vs.

acetoclastic methanogenesis) and to identify thermogenic-microbial gas mixtures. More recent studies point to a possible role for methylotrophic methanogenesis using substrates such as methanol, but the environmental significance and isotopic signatures of this pathway are not currently well understood. Interpretations based on established isotopic techniques neglect complications that are evident in a growing body of data from microbial gas systems worldwide:

(1) Hydrogen isotope equilibration between organic methane precursors and water suggests that $\delta\text{D-CH}_4$ is ineffective for diagnosing methanogenic pathways in coal beds and shales, either by evaluating the difference between $\delta\text{D-CH}_4$ and $\delta\text{D-H}_2\text{O}$ or by plotting $\delta\text{D-CH}_4$ vs. $\delta^{13}\text{C-CH}_4$ with respect to diagnostic fields. However, hydrogen isotopes can still provide supporting evidence that methane is of microbial origin and in some cases constrain the timing of methane formation.

(2) The net apparent carbon fractionation (expressed as $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$) is influenced by competing effects including: (a) substrate conversion to CO_2 by competitive bacteria including sulfate reducers, (b) mixing of thermogenic and microbial gas, (c) anaerobic methane oxidation, and/or (d) formation water interactions. Therefore, shifts in net apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ need not record a shift of methanogenic pathways and the methanogenic fractionation could be overwhelmed by nonmethanogenic processes affecting field-collected samples.

(3) $\delta^{13}\text{C}$ values of accumulated CH_4 and CO_2 are influenced by the nature of source organic matter and the extent of methanogenesis relative to non-methanogenic processes such as sulfate reduction. Therefore, approaches that consider the mass balance of source carbon compounds during biodegradation are more likely to be successful than single-fingerprint techniques.

Many of the expected carbon and hydrogen isotope signatures in coal beds and shales were previously derived from recent sediments or cultures, while differences in biodegradation rates, substrate availability, and perhaps other factors suggest that these data should be applied to slowly-biodegraded carbon sources with caution. The patterns reviewed here have implications for the biogeochemical structure and active pathways for coal biodegradation and methanogenesis in slowly-biodegraded coal beds and shales. Conventional isotope fingerprints, especially values of apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$ and the

separation between $\delta D-H_2O$ and $\delta D-CH_4$, suggest that hydrogenotrophic methanogenesis is the dominant methanogenic pathway in most coal beds and shales. Complementary analysis of microbial communities suggests that a greater variety of methanogenic pathways strategies is plausible, but the environmental significance of these additional pathways beyond hydrogenotrophic methanogenesis is not well understood. Finally, we argue that nonmethanogenic processes (e.g. sulfate reduction) can have a large influence on C isotope signatures in some environments by routing carbon through a mix of highly fractionating and little-fractionating pathways. Therefore, C isotopic signatures could depend on a combination of substrate availability (the identity of easily-metabolized organic compounds) and/or subsurface environmental conditions that promote nonmethanogenic pathways (such as availability of sulfate or easily-reduced Fe oxides).

Acknowledgments

This research was supported by the American Chemical Society - Petroleum Research Fund (grants 51001-ND2 to JM and 47750-B2 to AM), U.S. Geological Survey, Carbon Management Canada, NSF EAR (grant 1322805 to JM), NSF Earth Sciences Postdoctoral Fellowship 1249916 (to DV), and the Institute for Sustainability and Energy at Northwestern University (to NB). SL acknowledges Canada Research Chairs and NSERC. We acknowledge Michael Lawson, Kinga Révész, Allan Kolker, and one anonymous *Chemical Geology* reviewer for their constructive reviews and Editor-in-Chief Michael Böttcher for editorial handling. We also thank Denise Akob, Matthew Kirk, and Tracy Quan for helpful discussions. (Needs to be exact wording): Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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Figure Captions

Figure 1. Simplified schematic representation of methanogenic biodegradation modified from Schink (2005), Formolo (2010), Strapoć et al. (2011b), and Gieg et al. (2014). Pathways associated with large carbon isotope fractionations are shown by solid arrows. Note that CH₄ is produced by highly-fractionating methanogenesis, whereas CO₂ can be produced by a combination of highly-fractionating and little-fractionating processes when nitrate, iron(III), and/or sulfate are present. The accumulated CH₄ and CO₂ can be a mixture of multiple pathways with differing isotopic effects.

Figure 2. Plot of $\delta^{13}\text{C-CH}_4$ vs. $\text{C}_1/(\text{C}_2+\text{C}_3)$ hydrocarbon ratio in coal and shale gas systems. Fields assigned to microbial and biogenic gas are from Bernard et al. (1976). Data sources are as described in Table 3.

Figure 3. (a) Hydrogen isotope ratios of methane and the host groundwater. Note that most data lie near the expected line inferred for hydrogenotrophic methanogenesis (Schoell, 1980; Whiticar et al., 1986; Whiticar, 1999). (b) Plot of C and H isotope ratios of methane from gas accumulations containing microbial methane. In panel (b), fields are slightly modified from Whiticar (1999). The field marked Acetoclastic/Methylotrophic includes all methyl-converting methanogenesis (“Methyl-type fermentation”) as described by Whiticar (1999). Data sources are as described in Table 3.

Figure 4. Plots showing the separation between $\delta^{13}\text{C-CH}_4$ vs. $\delta^{13}\text{C-CO}_2$ and its interpretation. In (a), contours represent apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$. Panel (b) depicts effects of nonmethanogenic processes on apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$. Note that nonmethanogenic substrate utilization can yield lower apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$. See section 3.2.2 for discussion. Panel (c) shows the trajectory of methanogenesis from $f = 0$ to $f = f_{\text{max}}$ (contours). Panel (d) shows the same data in (a), plotted relative to contours of apparent f , assuming $\delta^{13}\text{C-LMW} = -25\text{‰}$. Note samples at lower extents of methanogenesis (lower values of $\delta^{13}\text{C-CH}_4$, and $\delta^{13}\text{C-CO}_2$, and f) that exhibit lower apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$. Data sources are as described in Table 3.

Figure 5. Simplified steady-state branchpoint isotope models in which methanogenesis is inferred to be a more-fractionating step than the production of methane precursors (modified from Hayes, 2001). In the pathway-specific models, ϕ represents the carbon flux through each reaction. In the pathway-independent model, ϕ_0 and ϕ_1 are the net production of CO_2 and CH_4 , respectively, by all pathways that consume C_{LMW} .

Table 1. Paired isotopic and microbiological evidence for methanogenic pathways. Underlined text represents apparent evidence of hydrogenotrophic methanogenesis; **bold** text represents apparent evidence of acetoclastic methanogenesis; double underlined text represents apparent evidence of methylotrophic methanogenesis.

NOTE TO EDITORIAL STAFF: This table continues on the next page.

Basin	Molecular, culturing, or <i>in situ</i> microbiological evidence	Apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$	H isotope evidence	Interpretation of C-H isotope fields (Figure 3a)
Coalbed methane Cook Inlet	Nucleic acid analysis indicates methanogens dominated by <u>obligate methylotrophic <i>Methanobrevibacter</i></u> ; and <i>Methanosarcina</i> (Dawson et al., 2012); <u>Cultures from wells with highest proportion of methyl/methanol utilizers yielded most CH_4</u> (Strapoć et al., 2011a)			Plots in acetoclastic/methylotrophic field (Dawson et al., 2012)
Elk Valley	Enrichment cultures dominated by <i>Methanosarcina</i> spp., capable of utilizing <u>CO_2/H_2</u> and acetate (Penner et al., 2010)		<u>median $\delta\text{D-H}_2\text{O} - \delta\text{D-CH}_4 = 165\text{‰}$</u> (range 135- 415 ‰) (Aravena et al., 2003)	Acetoclastic/methylotrophic methanogenesis
Forest City Basin & Cherokee Basin	Both <u>hydrogenotrophs</u> and acetoclastic methanogens grew in cultures (McIntosh et al., 2008; Kirk et al., 2015)	<u>median 1.070</u> (range 1.059- <u>1.078</u>)	<u>median $\delta\text{D-H}_2\text{O} - \delta\text{D-CH}_4 = 181\text{‰}$</u> (range 169-221‰) (McIntosh et al., 2008; Kirk et al., 2015)	hydrogenotrophic methanogenesis
Gulf Coast (Wilcox coal)	<u>Strain isolated from CBM water converted methanol, methylamines to methane in enrichment cultures</u> ; no growth on acetate or H_2/CO_2 (Doerfert et al., 2009)	<u>median 1.061</u> (range 1.024 - <u>1.066</u>)	<u>median $\delta\text{D-H}_2\text{O} - \delta\text{D-CH}_4 = 164\text{‰}$</u> (range 162-167‰) (Warwick et al., 2008; McIntosh et al., 2010)	Thermogenic-biogenic mixture with hydrogenotrophic methanogenesis
Illinois Basin	<u>Coal bed water consortium dominated by methanogens utilizing H_2+CO_2; CO_2 reducers grew in enrichment cultures</u> (Strapoć et al., 2008b)	<u>median 1.066</u> (range 1.032 - <u>1.075</u>)	<u>median $\delta\text{D-H}_2\text{O} - \delta\text{D-CH}_4 = 174\text{‰}$</u> (range 162-212‰) (Strapoć et al., 2008a; Schlegel et al., 2011a)	hydrogenotrophic methanogenesis
Powder River Basin	Consortium from well water produced methane from acetate and methanol but not from CO_2/H_2 in enrichment cultures (Green et al., 2008); Conversion of added acetate to CH_4 (Ulrich and Bower, 2008); nucleic acid pyrosequences of methanogens in coal slurry dominated by <u>H_2-trophic</u> and <u>methylotrophic</u> methanogens (Barnhart et al., 2013)	<u>median 1.071</u> (range 1.044 - <u>1.078</u>)	<u>median $\delta\text{D-H}_2\text{O} - \delta\text{D-CH}_4 = 165\text{‰}$</u> (range 143-261‰)	Acetoclastic/methylotrophic methanogenesis
San Juan Basin	Nucleic acid analysis of waters indicated methanogens that can utilize acetate , <u>H_2</u> , <u>methanol</u> . Enrichment cultures yielded CH_4 from lactate, <u>H_2</u> , formate, <u>alcohols</u> , but not from acetate (Wawrik et al., 2012)	<u>median 1.063</u> (range 1.056- <u>1.068</u>)	(Gorody, 1999; Flores et al., 2008; Bates et al., 2011)	Thermogenic-biogenic mixture with hydrogenotrophic methanogenesis
South Sumatra Basin	Enrichment cultures using coal were dominated by acetoclastic <i>Methanosaeta</i> (Susilawati et al., 2015)	<u>1.072-1.076</u>	(Zhou et al., 2005)	Plots in hydrogenotrophic and mix/transition fields

Basin	Molecular, culturing, or <i>in situ</i> microbiological evidence	Apparent $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4}$	H isotope evidence	Interpretation of C-H isotope fields (Figure 3a)
(Susilawati et al., 2013)				
<i>Shale gas</i> Michigan Basin	<u>H₂-trophic</u> , acetoclastic , and <u>methyilotrophic</u> methanogens detected in nucleic acid analysis of well water; <u>hydrogenotrophs more widely documented</u> (Waldron et al., 2007; Formolo et al., 2008; Kirk et al., 2012)	<u>median</u> $\alpha^{13}\text{C}_{\text{CO}_2\text{-CH}_4} \approx$ <u>1.074 (range</u> <u>1.063-1.080)</u>	<u>median $\delta\text{D-H}_2\text{O} -$</u> <u>$\delta\text{D-CH}_4 = 173 \text{ ‰}$</u> <u>(range 143-261‰)</u>	Thermogenic-biogenic mixture with hydrogenotrophic methanogenesis
(Martini et al., 1998, 2003)				

Table 2. Representative anaerobic methanogenic and nonmethanogenic pathways and free energy yields per mole substrate consumed.

Reaction	Equation ^a	ΔG^0 (kJ/mol) ^b
Denitrification	$\text{CH}_2\text{O} + 0.8\text{NO}_3^- + 0.8\text{H}^+ = 0.4\text{N}_2 + \text{CO}_2 + 1.4\text{H}_2\text{O}$	-462.9
Mn oxide reduction	$\text{CH}_2\text{O} + 2\text{MnO}_2 + 4\text{H}^+ = 2\text{Mn}^{2+} + \text{CO}_2 + 3\text{H}_2\text{O}$	-457.3
Fe oxide reduction	$\text{CH}_2\text{O} + 4\text{Fe}(\text{OH})_3 + 8\text{H}^+ = 4\text{Fe}^{2+} + \text{CO}_2 + 11\text{H}_2\text{O}$	-348.3
Sulfate reduction	$\text{CH}_2\text{O} + 0.5\text{SO}_4^{2-} + 0.5\text{H}^+ = 0.5\text{HS}^- + \text{CO}_2 + \text{H}_2\text{O}$	-194.8
Methanogenesis (generic)	$\text{CH}_2\text{O} = 0.5\text{CH}_4 + 0.5\text{CO}_2$	-70.5
Methylotrophic methanogenesis	$\text{CH}_3\text{OH} = 0.75\text{CH}_4 + 0.25\text{CO}_2 + 0.5\text{H}_2\text{O}$	-68.7
Hydrogenotrophic methanogenesis	$\text{H}_2 + 0.25\text{CO}_2 = 0.25\text{CH}_4 + 0.5\text{H}_2\text{O}$	-43.9
Acetoclastic methanogenesis	$\text{CH}_3\text{COOH} = \text{CH}_4 + \text{CO}_2$	-31.1
Acetogenesis	$\text{H}_2 + 0.5\text{CO}_2 = 0.25\text{CH}_3\text{COOH} + 0.5\text{H}_2\text{O}$	-36.1

^a CH_2O denotes generic organic matter with carbon oxidation state of 0. More reduced carbon sources may change free energy yields significantly (Stumm and Morgan, 1996).

^b Calculated as kJ / mol substrate using the dominant species at pH 7 (acetate as CH_3COO^- , inorganic C as HCO_3^-). Thermodynamic constants are from Stumm and Morgan (1996), except acetate which is from Shock and Helgeson (1990).

Table 3. Sources of data from field-based studies of microbial gas.

Basin	Symbol	Reference	mol % CH ₄	mol % CO ₂	C ₁ /(C ₂ +C ₃)	δ ¹³ C-CH ₄	δ ¹³ C-CO ₂	δD-CH ₄	δD-H ₂ O	Chloride conc.	Temperature	Notes
<i>Coalbed methane</i>												
Alberta Basin (Canada)	*	Cheung et al. (2010)	X	X	X	X	X		X	X	X	Horseshoe Canyon / Belly River Group
Black Warrior Basin (USA)	⊗	Pashin et al. (2014)	X	X	X	X		X		X		
Cook Inlet (USA)	▼	Dawson et al. (2012)				X		X		X		
Elk Valley (Canada)	□	Aravena et al. (2003)				X		X	X			
Forest City & Cherokee Basins (USA)	○	McIntosh et al. (2008)	X	X	X	X	X	X	X	X	X	Repeat samples were averaged
		Kirk et al. (2015)	X	X	X	X	X	X	X	X	X	
Gulf Coast (USA)	◆	Warwick et al. (2008)	X	X	X	X	X	X	X	X	X	
		McIntosh et al. (2010)	X	X	X	X	X	X	X	X	X	
Illinois Basin (USA)	+	McIntosh et al. (2002)	X	X	X	X	X	X	X	X	X	Data from figures
		Strapoć et al. (2008a)			X	X	X	X	X	X	X	
		Schlegel et al. (2011a)	X	X	X	X	X	X	X	X	X	
Powder River Basin (USA)	×	Gorody (1999)				X	X	X				
		Flores et al. (2008)	X	X	X	X	X	X	X			
		Bates et al. (2011)	X	X	X	X	X	X	X	X	X	
San Juan Basin (USA)	△	Rice et al. (1989)	X	X	X	X						
		Zhou et al. (2005)	X	X	X	X	X	X	X			
Upper Silesian (Poland)	▣	Kotarba and Pluta (2009)			X	X	X	X	X	X		
		Weniger et al. (2012)	X	X	X	X	X					
Williston Basin (USA)	◇	Pantano (2012)	X	X	X	X	X	X	X	X	X	
Surat Basin (Australia)	⊠	Baublys et al. (2015)				X	X	X	X	X	X	
South Sumatra Basin (Indonesia)	▽	Susilawati et al. (2013)	X	X	X	X	X	X				
<i>Shale gas</i>												
Illinois Basin (USA)	●	McIntosh et al. (2002)	X	X	X	X	X	X	X	X		
		Schlegel et al. (2011a)	X	X	X	X	X	X	X	X	X	
Michigan Basin (USA)	■	Martini et al. (1998, 2003)	X	X	X	X	X	X	X	X	X	

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Specific methanogenic pathways

Pathway-independent model

Arrow definitions

- Highly-fractionating on C
- - - - -→ Little-fractionating on C
-→ Net apparent fractionation