Combined carbon, hydrogen, and clumped isotope fractionations reveal differential reversibility of hydrogenotrophic methanogenesis in laboratory cultures

Jeemin H. Rhim[a,1,*] and Shuhei Ono[a]

[a] *Department of Earth, Atmospheric and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. jrhim@alum.mit.edu (J. H. Rhim), sono@mit.edu (S. Ono)*

[1] *Present address: Department of Earth Sciences, Dartmouth College, Hanover, NH 03755, USA*

[*] *Corresponding author: jrhim@alum.mit.edu*

Abstract

Stable isotope analysis has been widely used to aid the source identification of methane. However, the isotopic ($^{13}C/^{12}C$ and D/H) and isotopologue ($^{13}CH_3D$ and $^{12}CH_2D_2$) signatures of microbial methane in natural environments are often different from those in laboratory cultures in which methanogens are typically grown under optimal conditions. Growth phase and hydrogen (H_2) concentration have been proposed as factors controlling the isotopic compositions of methane, but their effects on the relationship among carbon, hydrogen and doubly-substituted "clumped" isotopologue systems have not been assessed in a quantitative framework. Here we experimentally investigate the bulk ($\delta^{13}C$ and δD) and clumped ($\Delta^{13}CH_3D$) isotopologue compositions of methane produced by hyperthermophilic hydrogenotrophic (CO_2-reducing) methanogens using batch and fed-batch systems at different growth phases and H_2 mixing ratios (*Methanocaldococcus bathoardescens* at 82 or 60 °C and on 80 or 25% H_2; *Methanothermobacter thermautotrophicus* ΔH at 65 °C and on 20, 5 or 1.6% H_2). We observed a large range (18 to 63‰) of carbon isotope fractionations, with larger values observed during later growth phase, consistent with previous observations. In contrast, hydrogen isotope fractionations remained relatively constant at –317 ± 25‰. Linear growth was observed for experiments with *M. bathoardescens*, suggesting that dissolution of gaseous H_2 into liquid media became the rate limit as cell density increased. Accordingly, the low (and undersaturated) dissolved H_2 concentrations can explain the increased carbon isotope fractionations during the later growth phase. The δD and $\Delta^{13}CH_3D$ values indicated departure from equilibrium throughout experiments. As the cell density increased and dissolved H_2 decreased, $\Delta^{13}CH_3D$ decreased (further departure from equilibrium), contrary to expectations from previous models. Our isotopologue flow network model reproduced the observed trends when the last H-addition step is less reversible relative to the first three H-addition steps (up to CH_3-CoM). In this differential reversibility model, carbon, hydrogen and clumped isotopologue fractionations are largely controlled by the reversibility of the first three H-addition steps under high H_2 concentrations; the last H-addition step becomes important under low H_2. The magnitude of depletion and decreasing trend in $\Delta^{13}CH_3D$ values were reproduced when a large (≥6‰) secondary clumped kinetic isotope effect was considered in the model. This study highlights the advantage of combined bulk and clumped isotope analyses and the importance of physiological factors (growth phase) and energy availability (dissolved H_2 concentration) when using isotope analyses to aid the source identification of methane.

1 Introduction

Methane (CH_4) is an important energy source, a potent greenhouse gas as well as a potential biosignature in subsurface and extraterrestrial environments. Stable carbon ($\delta^{13}C$) and hydrogen (δD) isotope ratios have been extensively used to apportion the relative contributions of different methanogenic pathways, e.g., acetoclastic *vs*. hydrogenotrophic methanogenesis (Welhan and Lupton, 1987; Whiticar, 1990; Sherwood Lollar et al., 2002; Flores et al., 2008; Sherwood Lollar et al., 2008; Pohlman et al., 2009; Baldassare et al., 2014). More recently, technological advances have allowed the precise measurements of the abundance of multiply-substituted or "clumped" isotopologues of methane (e.g., $^{13}CH_3D$ and $^{12}CH_2D_2$; Ono et al., 2014; Stolper et al., 2014; Young et al., 2016; Gonzalez et al., 2019). Methane clumped isotopologue abundance has in some cases served as an isotopic geothermometer and provided temperature estimates that are consistent with environmental temperatures (e.g., Stolper et al., 2015; Wang et al., 2015; Young et al., 2017).

While both bulk and clumped isotopic compositions of methane can help identify the source(s) of methane, some factors complicate the interpretation of the isotopic signatures. For example, overlapping isotopic signatures in $\delta^{13}C$ and δD often lead to ambiguous source identifications (e.g., Schoell, 1988; Whiticar, 1990, 1999; Pohlman et al., 2009; Etiope and Sherwood Lollar, 2013), and some microbial methane samples from surface environments have yielded unreasonably high temperature estimates for clumped isotopologue equilibrium (e.g., Stolper et al., 2015; Wang et al., 2015; Douglas et al., 2017; Young et al., 2017). In particular, there are significant discrepancies between the bulk and clumped isotopic signatures observed in natural samples of microbial methane and those produced by laboratory cultures that presumably use the same metabolic pathway (Stolper et al., 2015; Wang et al., 2015; Okumura et al., 2016; Young et al., 2017; Gruen et al., 2018). In general, the $\delta^{13}C$ and δD values of microbial methane

in marine environments, where hydrogenotrophic methanogenesis is thought to be a primary methanogenic pathway, tend to indicate isotopic equilibrium with CO_2 and H_2O. In contrast, the $\delta^{13}C$ and δD values observed in laboratory cultures often indicate kinetic isotope effect (i.e., departure from equilibrium). Similarly, the $\Delta^{13}CH_3D$ values, representing the relative abundance of $^{13}CH_3D$ clumped isotopologues, measured from microbial methane in marine and deep subsurface sediments indicate internal isotopic equilibrium whereas those from pure cultures carry strong kinetic isotope signatures (Stolper et al., 2015; Wang et al., 2015; Douglas et al., 2017; Young et al., 2017; Gruen et al., 2018).

Previous studies that investigated the factors controlling isotope fractionation during microbial methanogenesis shed some light on the cause of the observed discrepancy. Multiple studies have investigated the changes in the carbon isotope fractionation factor ($^{13}\alpha$) during hydrogenotrophic methanogenesis and have identified growth phase and/or hydrogen partial pressure (pH_2) as important controlling factors (Games and Hayes, 1978; Fuchs et al., 1979a; Belyaev et al., 1983; Balabane et al., 1987; Krzycki et al., 1987; Botz et al., 1996; Valentine et al., 2004; Penning et al., 2005; Londry et al., 2008; Yoshioka et al., 2008; Hattori et al., 2012; Okumura et al., 2016; Topçuoğlu et al., 2019; Nguyen et al., 2020). In general, carbon isotope fractionation increases at low pH_2 such that the apparent magnitude of fractionation is close to that expected at CH_4-CO_2 equilibrium. These observations corroborate the differential reversibility hypothesis, which predicts that the variation in carbon isotope fractionation is a result of the changes in reversibility in the enzymatic steps of the hydrogenotrophic methanogenesis pathway (Valentine et al., 2004).

The effect of pH_2 on hydrogen isotope fractionation has been investigated by comparing pure cultures grown on high concentrations of H_2 against cocultures containing hydrogenotrophic

methanogens and syntrophic heterotrophic bacteria (e.g., *Syntropothermus lipocalidus* str. TGB-C1 and *Methenothermobacter thermautotrophicus* str. ΔH) (Yoshioka et al., 2008; Hattori et al., 2012; Okumura et al., 2016). Cocultures provide a means to create relatively low H_2 conditions (e.g., 6.8 to 64.9 Pa; Okumura et al., 2016) compared to high pH_2 conditions (>10^5 Pa) often used for pure cultures grown on H_2. So far, to the best of our knowledge, no experiment has observed the magnitude of hydrogen isotope fractionation expected at CH_4-H_2O equilibrium (*ca*. –178‰ at 25 °C; Horita and Wesolowski, 1994; Gropp, Iron and Halevy, 2021) that is often observed in natural samples of microbial methane. If the differential reversibility hypothesis can be applied to hydrogen isotope system, higher reversibility and near-equilibrium hydrogen isotope fractionation are expected at lower H_2 environments. This would suggest that the H_2 concentrations tested in experiments so far were not low enough to produce near-equilibrium hydrogen isotope signatures.

Laboratory experiments with methane clumped isotope data have only been conducted in batch cultures under high pH_2 conditions (e.g., Stolper et al., 2015; Wang et al., 2015; Young et al., 2017; Gruen et al., 2018). Isotope models relating the dissolved H_2 concentration and $\Delta^{13}CH_3D$ values have been proposed and predict changes in $\Delta^{13}CH_3D$ values toward equilibrium (i.e., increase in $\Delta^{13}CH_3D$ values toward 6‰ at 25 °C) at low H_2 concentrations (e.g., Stolper et al., 2015; Wang et al., 2015), consistent with the overall concept of the differential reversibility hypothesis. However, direct investigations of $\Delta^{13}CH_3D$ values produced at different growth phases or H_2 concentrations are needed to validate whether the differential reversibility model can be applied to clumped isotopologue systematics.

In this study, we cultured two different species of methanogens, *Methanocaldococcus bathoardescens* and *Methanothermobacter thermautotrophicus* str. ΔH, in batch and fed-batch systems under a pH_2 range from 1.6 kPa to 80 kPa and simultaneously measured $\delta^{13}C$ and δD of

the substrates (CO_2 and H_2O) as well as the $\delta^{13}C$, δD and $\Delta^{13}CH_3D$ of the product (CH_4). We present isotopologue flow network model results along with the estimated dissolved H_2 concentrations and measured isotopologue ratios to explain the observed fractionation trends by the effects of differential reversibility at the last H-addition step. We propose the $\delta^{13}C$, δD and $\Delta^{13}CH_3D$ trajectories expected for a wide range of dissolved H_2 concentrations encompassing both natural environments and experimental conditions (10^{-9} to 10^{-2} M H_2) that can be applied for future investigations of these isotope signatures for source identifications of methane.

2 Materials and Methods

2.1 Organisms

Cultures of *Methanocaldococcus bathoardescens* were provided by James F. Holden (University of Massachusetts, Amherst). Culture medium for *M. bathoardescens* was prepared following the "282 mod" recipe (Ver Eecke *et al.*, 2012) containing (L^{-1}): 0.34 g KCl, 4.00 g $MgCl_2 \cdot 6H_2O$, 0.14 g KH_2PO_4, 3.45 g $MgSO_4 \cdot 7H_2O$, 18 g NaCl, 0.25 g NH_4Cl, 0.14 g $CaCl_2 \cdot 2H_2O$, 2.0 mL $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (0.1% w/v), 10.0 mL trace element solution SL-10 (DSMZ 320), 5.00 g $NaHCO_3$, 10.0 mL vitamins solution (DSMZ 141). Resazurin was omitted, and 2 mM cysteine and 1 mM sulfide were used as reducing agents instead of dithiothreitol.

Methanothermobacter thermautotrophicus str ΔH (DSM 1053) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The seed culture was transferred to and maintained in mineral medium modified from DSMZ 1523 containing (L^{-1}): 0.50 g KH_2PO_4, 0.40 g $MgSO_4 \cdot 7H_2O$, 0.40 g NaCl, 0.40 g NH_4Cl, 0.05 g $CaCl_2 \cdot 2H_2O$, 1.00 mL trace element solution SL-10 (DSMZ 320), 4.00 g $NaHCO_3$, 1.00 mL vitamins solution (DSMZ 503). The medium was reduced with 1 mM sulfide.

2.2 Culture conditions

Table 1 summarizes the conditions for culture experiments conducted in this study. *M. bathoardescens* was grown at 82 or 60 °C and on 80 or 25% H_2, and *M. thermautotrophicus* ΔH was grown at 65 °C and on 20, 5 or 1.6% H_2.

2.2.1 Batch cultures

M. bathoardescens batch culture series was prepared in 100 mL of "282mod" medium described above in 200 mL bottles with 2 bars absolute pressure of H_2/CO_2 (80:20). Each bottle was inoculated with 2% (v/v) of pre-culture in exponential growth phase. Cultures were incubated at 82 °C. At given timepoints, culture headspace was sampled for gas chromatography and $\delta^{13}C_{CO2}$ analysis. Medium was sampled for cell counts with a counting chamber (CTL-HEMM-GLDR, LW Scientific; depth = 0.1 mm) and phase-contrast light microscope. Immediately after sampling, the entire remaining culture was sacrificed by adding 5 mL of 1 M sodium hydroxide. The headspace of a killed culture was used for methane purification and subsequent isotopologue analysis.

2.2.2 Fed-batch cultures

All fed-batch culturing experiments were carried out using a 2-L glass bioreactor (Ace Glass) equipped with a fritted gas dispersion tube (Ace Glass), pH meter (ML-05990-40; Cole-Parmer), temperature monitor/controller, liquid sampling port and a condenser leading to the gas outlet (Figure 1). Both the culturing apparatus and 1.7 L of medium were sterilized by autoclaving at 121 °C for 20 minutes. A set of mass flow controllers was used to control the flow rates of H_2, CO_2 and He (or N_2) to achieve desired mixing ratios of H_2, xH_2, (80, 25, 20, 5 and 1.6%) in the influent gas (Table 1). A column filled with copper was placed between the gas tanks and the

156 reactor and heated to 450 °C to remove trace amounts of oxygen in the incoming gas mixture
157 (Wolfe, 2011). After the reactor was heated to desired temperatures (82, 65 or 60 °C), vitamin
158 solution was added and the pH was adjusted to 6.0 and 7.0 (for *M. bathoardescens* and *M.*
159 *thermautotrophicus*, respectively) while bubbling with the gas mixture (20% CO_2). Cysteine (2
160 mM) and sulfide (2 mM) or cysteine (2 mM) and titanium citrate (0.1 mM) were added as reducing
161 agents before adding a 2% (v/v) of inoculum. Effluent gas from the reactor was passed through a
162 condenser (12 °C) which is followed by an additional column filled with $CaCl_2$ for water removal,
163 and directly connected to an on-line gas chromatography system or a gas sampling bag (Cali-5-
164 Bond™, Calibrated Instruments, Inc., McHenry, MD, USA) (Figure 1).

165 2.3 Analytical procedures

166 2.3.1 Gas chromatography

167 Mixing ratios of headspace gases were measured using a gas chromatograph (GC-2014, Shimadzu,
168 Columbia, MD, USA), equipped with a packed column (Carboxen-1000, 5' by 1/8", Supelco,
169 Bellefonte, PA, USA) with argon carrier gas at 140 °C. A thermal conductivity and a methanizer-
170 flame ionization detector were used to quantify the mixing ratios of H_2, N_2, CH_4 and CO_2. The
171 following compositions of commercial and in-house standard gases were used for calibration: 7%
172 CO, 15% CO_2, 4% O_2, 4.5% CH_4 balanced in N_2 (Supelco; P/N 501743); 4% CH_4, 20% CO_2, 2%
173 CO balanced in N_2 (MESA International Technologies, Inc.); 80% H_2, 20% CO_2 (Airgas). The
174 accuracy of GC analyses was ±5% of measured values. Headspace samples from experiments B.82
175 and F.82.80 were analyzed via manual syringe injection, and all other experiments were measured
176 on-line GC using a gas sampling valve with a 500µL injection loop (VC-SL500CW, VICI Valco,
177 Houston, TX, USA) (Figure 1).

2.3.2 $\delta^{13}C_{CO2}$ analysis

The carbon isotopic composition of headspace CO_2 ($\delta^{13}C_{CO2}$) was measured using an isotope-ratio mass spectrometer (IRMS; MAT 253, Thermo-Fisher). CO_2 from subsamples of the headspace gas collected from serum bottles (batch cultures) or at the exhaust (fed-batch cultures) was purified by cryogenically separating water and CO_2 from H_2, N_2 and CH_4 into a cold trap at –196 °C and warming up the trap to –80 °C and freezing the eluted CO_2 in a sample vial. Typical analytical precision for $\delta^{13}C$ analysis is ±0.2‰.

2.3.3 δD_{H2O} analysis

The hydrogen isotopic composition of media water (δD_{H2O}) was measured using cavity ring-down spectrometry (L-1102i WS-CRDS, Picarro, Sunnyvale, CA, USA) at the University of Massachusetts Amherst. Samples were vaporized at 110 °C. International reference standards (IAEA, Vienna, Austria) were used to calibrate the instrument to the VSMOW-VSLAP scale and working standards were used with each analytical run. Long-term averages of internal laboratory standard analytical results yield an instrumental precision of 0.5‰.

2.3.4 Methane isotopologue analysis

Methane samples were purified following the preparative GC method described by Wang et al. (2015). For batch culture experiment (B.82), the entire headspace of each killed serum bottle was replaced with helium during the sample preparation. For fed-batch experiments, multi-layer foil sampling bags (Cali-5-Bond™, Calibrated Instruments, Inc., McHenry, MD, USA) used to collect downstream headspace gas at the exhaust were directly connected to the sample preparation system. The relative abundances of methane isotopologues $^{12}CH_4$, $^{13}CH_4$, $^{12}CH_3D$ and $^{13}CH_3D$ were

199 measured using a tunable infrared laser direct absorption spectroscopy (TILDAS) described
200 previously (Ono et al., 2014; Wang et al., 2015).

201 2.3.5 Isotope notation and calculation of isotope fractionation factors

202 Bulk isotope values are reported using standard delta notation:

$$\delta^{13}C = \frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{VPDB}} - 1 \qquad \text{(Eqn. 1)}$$

$$\delta D = \frac{(D/H)_{sample}}{(D/H)_{VSMOW}} - 1 \qquad \text{(Eqn. 2)}$$

203 where VPDB and VSMOW are Vienna Pee Dee Belemnite and Vienna Standard Mean Ocean
204 Water, respectively. The factor of 1000 was omitted from (Eqn. 1) and (Eqn. 2), following the
205 IUPAC recommendations (Coplen, 2011). Natural gas samples with published $\delta^{13}C$ and δD values
206 (NGS-1 and NGS-3) were used for the calibration of $\delta^{13}C$ and δD values of methane analyzed via
207 TILDAS (Wang et al., 2015). Experimental samples were considered to contain methane
208 isotopologues at or sufficiently close to their natural abundances, hence the following
209 approximations are valid within analytical uncertainty: $^{13}C/^{12}C \approx [^{13}CH_4]/[^{12}CH_4]$ and $D/H \approx ¼$
210 $[^{12}CH_3D]/[^{12}CH_4]$. The abundance of $^{13}CH_3D$ clumped isotopologue is reported as $\Delta^{13}CH_3D$, a
211 metric representing the deviation of the abundance of $^{13}CH_3D$ from a random distribution of
212 isotopes among isotopologues $^{12}CH_4$, $^{13}CH_4$, $^{12}CH_3D$ and $^{13}CH_3D$ (Ono et al., 2014; Wang et al.,
213 2015):

$$\Delta^{13}CH_3D = \frac{[^{13}CH_3D][^{12}CH_4]}{[^{13}CH_4][^{12}CH_3D]} - 1 \qquad \text{(Eqn. 3)}$$

The value of $\Delta^{13}CH_3D$ was calibrated by equilibrating methane at 250 °C using Pt catalyst (Ono et al., 2014).

The isotope fractionation factor (α) is defined as the ratio of relative abundances of isotopes between a substrate and its product. For a batch experiment (B.82), isotope fractionation factors are calculated assuming an irreversible closed system isotope effect, based on the conventional Rayleigh equation (Mariotti et al., 1981). For the reduction of CO_2 to CH_4:

$$({}^{13}\alpha - 1) \cdot \ln f = \ln \frac{\delta^{13}C + 1}{\delta^{13}C_0 + 1} \quad \text{(Eqn. 4)}$$

where f is the fraction of CO_2 remaining; ${}^{13}\alpha$ is the kinetic isotope fractionation factor for ${}^{13}C/{}^{12}C$; and $\delta^{13}C_0$ is the initial isotopic compositions of CO_2.

The bulk isotope fractionation factors for fed-batch experiments (F.82.80, F.60.80, F.82.25, F.65.20 and F.65.5) were calculated as follows:

$${}^{13}\alpha_{CH4/CO2} = \frac{\delta^{13}C_{CH4} + 1}{\delta^{13}C_{CO2} + 1} \quad \text{(Eqn. 5)}$$

$${}^{2}\alpha_{CH4/H2O} = \frac{\delta D_{CH4} + 1}{\delta D_{H2O} + 1} \quad \text{(Eqn. 6)}$$

The equilibrium fractionation factors for carbon (${}^{13}\alpha_{eq}$; Horita, 2001), hydrogen (${}^{2}\alpha_{eq}$; Horibe and Craig, 1995) and clumped ($\Delta^{13}CH_3D_{eq}$; Ono et al., 2014) isotope systems were calculated based on experimental and/or theoretical calibrations.

2.3.6 Data processing

Methanogenesis reaction,

$$CO_{2(g)} + 4\ H_{2(g)} \rightarrow CH_{4(g)} + 2\ H_2O_{(l)} \qquad \text{(Eqn. 7)}$$

consumes 5 molecules of gas and produce 1 molecule of gas and water that is mostly in liquid phase. Because of this decrease in volume, the flow rate of effluent gas (Q_{out}) does not equal to the flow rate of the influent gas (Q_{in}: 200 mL/min) and can be lower by up to 25% for our experimental conditions. Based on the stoichiometry of the reaction (Eqn. 7), the flow rate of effluent gas was calculated from the mixing ratios of H_2 and CH_4:

$$Q_{out} = \frac{x_{H2}^{in}}{4x_{CH4}^{out} + x_{H2}^{out}} \cdot Q_{in} \qquad \text{(Eqn. 8)}$$

where x_{H2}^{in} and x_{H2}^{out} are H_2 mixing ratios of the influent and effluent gases, respectively, and x_{CH4}^{out} is CH_4 mixing ratio of the effluent gas measured by the GC.

The total methane production rate (*MPR*; mol/hr) was calculated from GC measurements of x_{CH4}^{out} and Q_{out} calculated above:

$$MPR = \frac{P}{RT} \cdot Q_{\text{out}} \cdot x_{CH4}^{out} \qquad \text{(Eqn. 9)}$$

where R is gas constant (8.314 m^3·Pa/mol/K), and T and P are temperature (K) and headspace pressure ($\approx 10^5$ Pa) during measurements. Cell-specific *MPR* (cs*MPR*; mol/cell/hr) was calculated by dividing *MPR* by the total number of cells in the reactor:

$$\text{cs}MPR = \frac{MPR}{N_{\text{c}} \cdot V_{\text{liq}}} \qquad \text{(Eqn. 10)}$$

where N_c is cell density (cells/m^3), V_{liq} is the volume of media (m^3).

2.3.7 Estimating dissolved H_2 concentrations in the liquid medium

For our experiments, the concentration of dissolved H_2, [H_2], is lower than what is expected from the saturation gas solubility and pH_2 in the influent gas. This is because of 1) high water vapor

pressure in the reactor headspace during hyperthermophilic experiments and 2) the slow kinetics of H_2 dissolution from gas phase to liquid media. We considered the following to estimate [H_2].

Water vapor pressure at saturation (pH_2O_{sat}) can be as high as 0.51 bars at 82 °C, 0.25 bars at 65 °C, and 0.20 bars at 60 °C (Haynes et al., 2016). If headspace gas was saturated with water vapor, pH_2 in the gas headspace for our fed-batch reactor can be lower by a factor of two compared to dry gas mixing ratios measured by GC. To consider the water vapor pressure in headspace (and bubbles), we estimated the pH_2O-corrected $pH_{2,VP}$ as:

$$pH_{2,VP} = xH_2 \cdot (p_{reactor} - pH_2O_{sat}) \qquad \text{(Eqn. 11)}$$

where $p_{reactor}$ is the total pressure of reactor, pH_2O_{sat} is the saturation water vapor pressure calculated as a function of temperature. The total pressure of reactor was assumed to be 1 bar. Headspace pressures were measured without inoculation and did not exceed over 1.05 bars.

In addition, the mass transport limit of H_2 from gas to dissolved phases can result in significant discrepancies between actual [H_2] and the [H_2] values expected at saturation with the gas phase H_2 (e.g., Pauss et al., 1990; Jud et al., 1997). The mass balance of H_2 for the liquid phase can be expressed as:

$$\frac{d[H_2]}{dt} = -(H_2 \text{ consumption rate}) + k_L a \cdot \left(K_H pH_{2,VP} - [H_2]\right) \qquad \text{(Eqn. 12)}$$

where H_2 consumption rate is 4 times the *MPR*, and k_La is the global mass transfer coefficient (e.g., hr^{-1}), which quantifies the rate of mass transfer for the whole reactor under a specific experimental condition. K_H is the Henry's law constant for H_2 (mol/L/Pa), calculated as a function of temperature and salinity following Chabab et al. (2020). The steady state solution for (Eqn. 12) is:

$$[\mathrm{H_2}] = \mathrm{K_H} \cdot pH_{2,\mathrm{VP}} - \frac{4 \cdot MPR}{k_L a} \quad \text{(Eqn. 13)}$$

The values of k_La were estimated based on the following equation:

$$k_L a = \frac{D}{\delta \cdot V_{\mathrm{liq}}} \cdot a \quad \text{(Eqn. 14)}$$

where D is the diffusivity coefficient for H_2 (m^2/hr); a is the sum of the surface area at the headspace-medium interface (A_h) and total surface area of bubbles (A_b) (m^2); and δ is the thickness of the diffusion layer (m). Using the δ values of 1 μm and 0.5 μm and other parameters specific to the experimental setup of this study (Table 2), k_La values of 380 and 760 hr^{-1} were used in (Eqn. 13) to calculate [H_2]. The parameter k_La is unique to a specific experimental condition and therefore varies between studies; however, the k_La range of 380 to 760 hr^{-1} falls reasonably within the ranges reported previously (e.g., 0.16 h^{-1}, Pauss et al., 1990; 220–1540 h^{-1}, Jud et al., 1997).

Following are brief justifications of the values used in Table 2. D is a typical diffusion coefficient for H_2 at 25 °C, 5.0×10^{-5} cm^2 sec^{-1} (Macpherson and Unwin, 1997). A_b is a function of the geometry and the number of bubbles, which is determined by the relationship between the size and residence time of bubbles and Q_{gas}. The upward velocity of bubbles for small bubbles (radius < 0.1 cm) was calculated following Park et al. (2017). The residence time and total volume of bubbles can be calculated for a known travel distance (i.e., height of the medium) and, from this, the total area of bubbles, A_b, was calculated. Finally, the surface area at the headspace-medium interface (A_h) is calculated from the reactor dimension.

2.3.8 Isotopologue flow network model

To examine the isotopologue data in this study with respect to the modeled [H_2] values, we applied the isotopologue flow network model adapted from Wang et al. (2015). The model calculates the expected isotopologue compositions of CH_4 as well as the intermediate carbon-containing species. During hydrogenotrophic methanogenesis (Eqn. 7), CO_2 is reduced to CH_4 via seven reactions and six intermediate carbon species. Following Wang et al. (2015) and Cao et al. (2019), we reduced the number of intermediate species to three by treating species with the same redox state as the same pool (Figure 2A). Two sets of input parameters required for the model are reversibilities (φ) for enzymatic reactions in the methanogenesis pathway and kinetic isotope effect (KIE) intrinsic to enzymatic reactions.

The first set of input parameters, metabolic reversibility (φ), is the ratio of backward to forward fluxes for an enzymatically-mediated reaction (Rees, 1973; Hayes, 2001). Previous models have used a range of φ values between 0 and 1, where φ = 0 is fully kinetic (non-equilibrium) and φ = 1 is fully reversible (equilibrium). Stolper et al., (2015) assumed φ = 1 for all except for the last H-addition step, which was varied. Wang et al. (2015) implemented a gradual and uniform departure from equilibrium (φ = 1→0) for all H-addition steps. Cao et al. (2019) tested binary cases, where φ is either 0 or 1 for each H-addition step. More recently, Gropp et al. (2021) re-evaluated these three models with calculated equilibrium fractionation factors and concluded that models in Wang et al. (2015) and Cao et al. (2019) can produce a range of carbon isotope fractionation observed in the natural environment with certain combinations of φ. This highlights the importance of φ, both in its degree of equilibrium at a given step and in its overall variation among the four H-addition steps, in determining the outputs of isotopologue flow network models. In this work, we parameterized φ values for the four H-addition steps as a function of H_2 by assuming Michaelis–Menten kinetics, as described in Wang et al. (2015):

$$\varphi_n = 1 - \frac{[\mathrm{H_2}]}{K_m + [\mathrm{H_2}]} \quad \text{(Eqn. 15)}$$

where n=1 to 4, representing the four H-addition steps (Figure 2A), and K_m is the effective half-saturation constant. According to the model for the energy conservation of hydrogenotrophic methanogenesis, the last step (reduction of methyl-CoM to CH_4) is exergonic and expected to be less reversible compared to other steps (Thauer *et al.*, 2008; Thauer, 2011; Ono *et al*., *in revision*).

We modeled differential reversibility by changing the K_m values (Eqn. 15) and compared the following three cases:

1) equilibrium end-member scenario with a high K_m value of 10^4 M for all four reactions,
2) uniform reversibility scenario with a K_m value of 5×10^{-5} M for all four reactions, and
3) differential reversibility scenario with a K_m value of 5×10^{-5} M for the first three reactions and a K_m value of 10^{-8} M (less reversible) for the last reaction.

The K_m value of 5×10^{-5} M approximates the experimentally determined K_m values for hyperthermophilic methanogens (66 μM for three *Methanocaldococcus* species; Ver Eecke *et al.*, 2012). The lower K_m value of 10^{-8} M makes the last reversible step largely unidirectional down to a low $[H_2]$ of ~10^{-8} M. For reference, the minimum threshold pH_2 estimated for pure cultures is 6.5 Pa (*ca*. 5×10^{-8} M at 25 °C), and the theoretical pH_2 at thermodynamic equilibrium for hydrogenotrophic methanogenesis is 0.1 Pa (*ca*. 1×10^{-9} M at 25 °C) assuming $[CO_2]/[CH_4] = 1$ (Lovley, 1985; Thauer et al., 2008).

The values of KIEs are the second set of required input parameters for the model. KIEs are experimentally determined only for the last reaction catalyzed by methyl-coenzyme M reductase (Scheller et al., 2013). KIEs for other reactions are chosen to reproduce the kinetic end-member solution and maintain the consistency with equilibrium fractionations (Appendix A; Table S1). Equilibrium fractionation factors (α^{eq}) estimated by quantum mechanical calculation (Gropp et al.,

2021; Ono et al., 2021) constrain the model solution for the equilibrium end-member scenario (Figure 2B). We use α^{eq} values estimated at 82 °C (experimental temperature for B.82 and F.82.80) by Gropp et al. (2021) for fractionations between intermediates and $H_2O_{(g)}$ or $CO_{2,(g)}$. Then, α^{eq} values against $H_2O_{(l)}$ were calculated from those against $H_2O_{(g)}$, using experimentally derived α values between $H_2O_{(l)}$ and $H_2O_{(g)}$ (Horita and Wesolowski, 1994).

3 Results

3.1 Batch culture experiment

The trends observed with increasing $\delta^{13}C_{CH4}$ values and decreasing δD_{CH4} and $\Delta^{13}CH_3D$ values (Table 3) were consistent with those previously reported for a batch culture experiment with *M. bathoardescens* (Gruen et al., 2018). The carbon, hydrogen and clumped isotope fractionation factors ($^{13}\alpha$, $^{2}\alpha$ and γ, respectively) calculated following Gruen et al. (2018) were comparable to those reported previously: $^{13}\alpha$ of 0.98 (this study) compared to 0.97; $^{2}\alpha$ from 0.64 to 0.59 (this study) compared to 0.69 to 0.57; and γ of 1.0005 (this study) compared to 1.0020 and 1.0032 (Supplementary Material, Figure S1D, S1E and S1F).

3.2 Fed-batch culture experiments (*M. bathoardescens*)

The growth of *M. bathoardescens* in fed-batch experiments were characterized by linear increase in cell density (Supplementary Material, Figure S2A, S2D and S2G), consistent with previous observations with *M. bathoardescens* in fed-batch experiments (Ver Eecke et al., 2012). For all experiments, $^{13}\alpha$ decreased toward or went below that expected at CH_4-CO_2 equilibrium(Figure 3A4–3E4). $^{2}\alpha$ increased toward that expected at CH_4-H_2O equilibrium; however, the final $^{2}\alpha$ values were still lower than that expected at equilibrium (Figure 3A5–3E5; Figure 4B). $\Delta^{13}CH_3D$ values

were low (range from –4.1 to 2.1‰) compared to equilibrium, indicating strong kinetic fractionations, and decreased over time (Figure 3A6–3E6; Figure 4C). Detailed observations for each experiment are described below.

During Experiment F.82.80, *M. bathoardescens* was grown at 82 °C and on 80% H_2. $^{13}\alpha$ decreased from 0.957 to 0.944 (Figure 3A4; Figure 4A), and $^{2}\alpha$ increased from 0.674 to 0.708 (Figure 3A5; Figure 4B). $\Delta^{13}CH_3D$ values ranged from 1.25 ± 0.48‰ to –0.29 ± 0.47‰ (Figure 3A6; Figure 4C). These $\Delta^{13}CH_3D$ values are lower than those expected at equilibrium (4.1‰ at 82 °C). Notably, the changes in $\Delta^{13}CH_3D$ were in the direction away from the values expected at equilibrium, unlike $^{13}\alpha$ and $^{2}\alpha$ values that changed toward equilibrium values.

During Experiment F.60.80, *M. bathoardescens* was grown at a suboptimal temperature of 60 °C and on 80% H_2. $^{13}\alpha$ decreased from 0.982 to 0.964 (Figure 3B4; Figure 4A), and $^{2}\alpha$ increased from 0.672 to 0.730 (Figure 3B5; Figure 4B). $\Delta^{13}CH_3D$ values ranged from 1.52 ± 0.58‰ to 0.06 ± 0.17‰ (Figure 3B6; Figure 4C). These $\Delta^{13}CH_3D$ values are lower than those expected at equilibrium (4.6‰ at 60 °C) and also in the direction away from the values expected at equilibrium. During Experiment F.82.25**,** *M. bathoardescens* was grown at 82 °C and on a lower xH_2 of 25% H_2. Comparable to the observations in F.82.80, $^{13}\alpha$ decreased from 0.963 to 0.941 during F.82.25 (Figure 3C4; Figure 4A), and $^{2}\alpha$ increased from 0.668 to 0.699 (Figure 3C5; Figure 4B). $\Delta^{13}CH_3D$ values ranged from 1.15 ± 0.42‰ to –0.78 ± 0.73‰ (Figure 3C6; Figure 4C). The changes in $\Delta^{13}CH_3D$ values during F.82.25 were also in the direction away from the values expected at equilibrium, similar to the trends observed during F.82.80 and F.60.80.

The changes in bulk isotope values ($\delta^{13}C_{CO2}$, $\delta^{13}C_{CH4}$, δD_{H2O} and δD_{CH4}) during F.82.80, F.60.80 and F.82.25 are reported in Table 4 and Supplementary Material, Figure S3. Note that, for

F.82.25, the absolute $\delta^{13}C_{CH4}$ values for F.82.25 are higher compared to those of F.82.80 because different sources of CO_2 were used for the experiments.

3.3 Fed-batch culture experiments (*M. thermautotrophicus*)

The growth patterns of *M. thermautotrophicus* in fed-batch experiments were characterized by distinct periods of exponential growth during the first 26 and 12 hours for F.65.20 (65 °C, 20% H_2) and F.65.5 (65 °C, 5% H_2), respectively (Supplementary Material, Figure S4A and S4D). For F.65.5, xH_2 in the supply gas was decreased from 5% to 1.6% after 55 hours. After decreasing the xH_2 to 1.6%, the cell density remained relatively constant for the remainder of the experiment, and xCH_4 and cs*MPR* decreased (Supplementary Material, Figure S4F; Table 5).

$^{13}\alpha$ decreased from 0.966 to 0.950 during F.65.20 and from 0.957 to 0.938 during F.65.5 (Figure 3D4 and 3E4; Figure 4A). $^{2}\alpha$ slightly increased during F.65.20 and did not change significantly during F.65.5 (Figure 3D5 and 3E5; Figure 4B). $\Delta^{13}CH_3D$ values decreased over time, moving away from that expected at equilibrium, as was observed in *M. bathoardescens* experiments (Figure 3D6 and 3E6; Figure 4B). Notably, the magnitudes of depletion (i.e., low $\Delta^{13}CH_3D$ values) observed during F.65.20 and F.65.5 are comparable to those observed during batch experiments with mesophilic methanogens, which tend to produce lower $\Delta^{13}CH_3D$ values compared to thermophilic methanogens (open *vs*. filled circles; Figure 4C). The changes in bulk isotope values ($\delta^{13}C_{CO2}$, $\delta^{13}C_{DIC}$, $\delta^{13}C_{CH4}$, δD_{H2O} and δD_{CH4}) during F.65.20 and F.65.5 are reported in Table 5 and Supplementary Material, Figure S5.

3.4 Dissolved H_2 in the liquid medium

The results of $[H_2]$ calculations are shown in Figure 3. Overall, the results show undersaturation in $[H_2]$ with respect to headspace for high density and fast-growing cultures (Figure 3A1–4E1).

Media become more undersaturated over time (i.e., at higher cell density; Figure 3A2–3E2) due to the increase in total H_2 consumption rate, which corresponds with the increase in *MPR* (Figure 3A3–3E3). The difference between the maximum $[H_2]^{eq}$ and minimum $[H_2]$ values (with lower k_La) was the largest, between 2-fold and >10-fold, at the highest temperature (82 °C; F.82.80 and F.82.25), whereas the difference was <2-fold at lower temperatures of 65°C and 60°C. The lower range of $[H_2]$ was calculated using the minimum k_La value required to avoid a negative $[H_2]$ in all experiments (350 h^{-1}), and the higher range of $[H_2]$ was calculated with a k_La value of 350 h^{-1}, twice as much as the minimum value (Figure 3A3–3E3).

3.5 Isotopologue flow network model

The equilibrium end-member scenario shows uniform values across the $[H_2]$ range that correspond to values expected at equilibrium (–51‰ for $\delta^{13}C$, –151‰ for δD and 6‰ for $\Delta^{13}CH_3D$ at 82 °C, yellow dotted line; Figure 5). The isotope values change as a function of $[H_2]$ in the uniform reversibility and differential reversibility scenarios (red dashed line and blue solid line, respectively; Figure 5). The $\delta^{13}C$ and $\Delta^{13}CH_3D$ profiles show significant difference between uniform and differential reversibility scenarios; in the differential reversibility scenario, $\delta^{13}C$ and $\Delta^{13}CH_3D$ values decrease to local minima at a $[H_2]$ range between the two K_m values assigned for reversibility terms (i.e., 5×10^{-5} M for φ_{1-3}; 10^{-8} M for φ_4), and values increase toward the equilibrium values when all four steps become reversible ($[H_2] \lesssim 10^{-7}$ M) (Figure 5A and 5C). The low $\delta^{13}C$ values between the two K_m values are less than the value expected for equilibrium. In the Discussion section below, we provide further interpretations for the patterns of isotope fractionation factors ($^{13}\alpha$, $^{2}\alpha$) and clumped isotopologue abundance ($\Delta^{13}CH_3D$) observed during fed-batch experiments in this study in light of $[H_2]$ and isotopologue flow network model results.

4 Discussion

Our results—from combined analyses of $\delta^{13}C_{CO2}$, $\delta^{13}C_{CH4}$, δD_{H2O}, δD_{CH4} and $\Delta^{13}CH_3D$—confirm previous observations that carbon isotope fractionation increases with decreasing pH_2 (Penning et al., 2005; Londry et al., 2008; Okumura et al., 2016; Topçuoğlu et al., 2019; Nguyen et al., 2020) and shed some new light on the behavior of hydrogen isotope and clumped isotopologue systems at different growth phases and [H_2]. The observed values of consistently low δD_{CH4} and $\Delta^{13}CH_3D$ (relative to equilibrium) suggest primarily kinetic fractionations for hydrogen and clumped isotope systems under our experimental conditions. However, the apparent decrease in $\Delta^{13}CH_3D$ values (i.e., further departure from equilibrium) for later growth phase and low [H_2] was unexpected and contrasts previous model predictions based on the differential reversibility hypothesis (e.g., Stolper et al., 2015; Wang et al., 2015). Using the modeled values of [H_2] in the fed-batch system and results of isotopologue flow network model, we discuss the observed patterns of $^{13}\alpha$ (section 4.1); $^{2}\alpha$ and $\Delta^{13}CH_3D$ (section 4.2); limitations of the model and broader implications for interpreting the isotopic signatures of natural methane samples (section 4.3).

4.1 High cell density during stationary growth phase leads to low [H_2] and higher than equilibrium carbon isotope fractionations

The magnitude of carbon isotope fractionation is higher (lower $^{13}\alpha$ values) for later growth phase within a single experiment and at lower xH_2 across experiments (Figure 3A4–3E4; Figure 4A). The decrease in $^{13}\alpha$ coincides with the transition from exponential phase to stationary phase for *M. thermautotrophicus* (Figure 3D2 and 3E2) or later linear growth for *M. bathoardescens* (Figure 3A2–3C2). Our observation is consistent with previous culture studies that reported increasing carbon isotope fractionation as a function of growth phase (Botz et al., 1996; Valentine et al., 2004)

435 and experiments with lower pH_2 (Valentine et al., 2004; Londry et al., 2008; Okumura et al., 2016;
436 Topçuoğlu et al., 2019; Nguyen et al., 2020). Some studies have also reported larger than
437 equilibrium isotope fractionation (e.g., Botz et al., 1996; Valentine et al., 2004; Penning et al.,
438 2005; Okumura et al., 2016; Topçuoğlu et al., 2019), similar to the observations in this study (e.g.,
439 F.82.80, F.82.25 and F.65.5 in Figure 3A4–3E4 and Figure 4A).

440 In addition to causing physiological changes, later growth phase with high cell density
441 leads to low [H_2] due to the increase in total H_2 consumption rate. The decrease in [H_2] at high cell
442 density is important to consider for the hydrogenotrophic methanogenesis reaction because of its
443 4:1 H_2:CH_4 stoichiometry (Eqn. 7) and poor solubility of H_2. While the dissolved concentrations
444 of highly soluble gases (e.g., CO_2) can be close to equilibrium with the headspace, the dissolved
445 concentrations of poorly soluble gases (e.g., H_2 and CH_4) can be far away from equilibrium with
446 the gas phase. Higher partial pressure of water vapor at saturation (pH_2O_{sat}) in the reactor
447 headspace during (hyper)thermophilic incubations should also be considered (Eqn. 11), as it would
448 further lower the [H_2] in liquid media. The observed linear growth for *M. bathoardescens* (Figure
449 3A2–3C2) suggests that growth and methane production rates were limited by the supply
450 (=dissolution) rate of H_2.

451 Accurate measurement of [H_2] for methanogenic media can be challenging. Previous
452 studies measured [H_2] for fed-batch reactors or chemostats by sampling liquid media into serum
453 vials and measuring pH_2 in the headspace (e.g., Ver Eecke et al., 2012; Stewart et al., 2016;
454 Topçuoğlu et al., 2018, 2019). For example, Topçuoğlu et al. (2019) reported [H_2] values (prior to
455 inoculation) of 82 ± 2 and 21 ± 6 μM for high and low H_2 experiments, respectively. These values
456 are lower and higher than the saturation concentrations (516 and 10 μM) based on the dry H_2
457 mixing ratios of 86.6 and 1.6% for high and low H_2 experiments, respectively. Here, high pH_2O_{sat}

458 at higher temperatures (0.51 bars at 82°C) can explain lower [H_2] compared to calculations for dry
459 headspace. Higher than saturation [H_2] values may indicate entrainment of H_2 microbubbles in
460 addition to dissolved H_2 (e.g., McGinnis et al., 2015). After inoculation, microbial consumption
461 of H_2 would affect the steady state dissolved concentrations of gases in the liquid due to relatively
462 slow rate of H_2 dissolution (Eqn. 12). For example, in anaerobic fermentors where H_2 is produced
463 by microbial processes, the liquid-to-gas mass transport limit resulted in as much as 80 times
464 oversaturation of H_2 compared to the headspace gas (Pauss et al., 1990). On the other hand, in
465 chemostat cultures where methanogens consume H_2 (i.e., gas-to-liquid transport), the dissolved H_2
466 concentration was found to be 10 times lower than the saturation with respect to gas phase (Jud et
467 al., 1997). Because methanogens use dissolved form of H_2, we estimated [H_2] under each
468 experimental condition to assess the effect of the dissolved H_2 concentration on isotope
469 fractionation.

470 As shown in Figure 6A, the modeled [H_2] range under our experimental conditions (*ca*. 7
471 to 410 μM) is lower than the [H_2] range expected for typical batch cultures (e.g., 0.6 to 1.2 mM
472 for 1 to 2 bars of 80% H_2 headspace) and partially overlaps with the [H_2] range found in cow rumen
473 (0.1 to 50 μM; Smolenski and Robinson, 1988; Janssen, 2010, and references therein; Wang et al.,
474 2015); but it is higher than the [H_2] range found in typical freshwater (5 to 75 nM; Robinson and
475 Tiedje, 1982; Conrad et al., 1985; Conrad et al., 1987; Kuivila et al., 1989) and marine sediments
476 (2 to 60 nM; Lin et al., 2012). In the differential reversibility scenario for the isotopologue flow
477 network model, $\delta^{13}C$ values decrease with a decrease in [H_2] for [H_2] <1 mM (between 10^{-6} and
478 10^{-3} M; Figure 5A). This is consistent with the decreasing $^{13}\alpha$ values observed during fed-batch
479 experiments with a decrease in [H_2] (due to increase in cell density) or xH_2 (mixing ratios for
480 different experiments) for the modeled [H_2] range between 7 and 410 μM (between 7×10^{-6} and

4×10^{-4} M; Figure 6B). In addition, the $^{13}\alpha$ values during the fed-batch experiment conducted at 82 °C and 80% H_2 (F.82.80; $^{13}\alpha = 0.95 \pm 0.01$; Table 4, Figure 4A) were lower compared to the $^{13}\alpha$ value for batch experiment conducted at the same temperature and initial $x$$H_2$ (B.82.80; $^{13}\alpha$ = 0.98; Supporting Information, Figure S1D). Considering that [H_2] in batch cultures at high headspace pressure (1 to 2 bars) and $x$$H_2$ (80%) can reach millimolar levels (0.6 to 1.2 mM; Figure 6A), the difference in $^{13}\alpha$ values observed between fed-batch and batch experiments is also consistent with the overall correlation between low $^{13}\alpha$ and low [H_2].

Besides the general correlation between low [H_2] and larger carbon isotope fractionation, our isotopologue flow network model reproduced the large carbon isotope fractionation (larger than equilibrium) observed during later growth phases in the differential reversibility scenario (Figure 5A). When [H_2] is between 5×10^{-5} and 10^{-8} M (i.e., two K_m values assigned for φ_1 to φ_3 and φ_4, respectively), reactions up to CH_3-CoM are largely reversible (φ_1 to $\varphi_3 \simeq 1$). As a result, $\delta^{13}C$ of the methyl group of CH_3-CoM approaches to equilibrium value (–52‰ at 82°C; Gropp et al., 2021). The reversibility of the last step is relatively low above 10^{-8} M H_2 in our model, and this step can result in kinetic isotope fractionation of up to –40‰ ($^{13}\alpha = 0.96 \pm 0.01$, assayed at 60 °C; Scheller et al., 2013). Accordingly, the maximum overall fractionation of ~–92‰ (–52 – 40‰) can be achieved when the first three steps are fully reversible (φ_1 to $\varphi_3 \simeq 1$) and the last step is fully kinetic ($\varphi_4 \simeq 0$). This is consistent with the minimum $^{13}\alpha$ value (0.908, or fractionation of –92‰) in the differential reversibility scenario simulated at 60 °C in our study (yellow solid line; Figure 6B). At much lower [H_2] (i.e., $<10^{-8}$ M), the last step of methanogenesis becomes reversible and equilibrium fractionation is expected (–57‰ at 60 °C, –51‰ at 82 °C; Figure 6B).

4.2 Differential reversibility can explain the observed hydrogen and clumped isotopologue systematics

Previous culture studies have so far exclusively produced non-equilibrium hydrogen isotope fractionation (Valentine et al., 2004; Yoshioka et al., 2008; Hattori et al., 2012; Kawagucci et al., 2014; Stolper et al., 2015; Okumura et al., 2016; Gruen et al., 2018). In this study, hydrogen isotope system similarly indicated significant departure from equilibrium ($^2\alpha = 0.69 \pm 0.02$, Figure 3A5–3E5, Figure 4B *vs*. $^2\alpha^{eq} = 0.81$ at 60 °C and 0.82 at 82 °C). In the differential reversibility scenario (blue solid line; Figure 5B) δD values slightly increase for $[H_2] \geq 10^{-5}$ M, stay relatively constant for the $[H_2]$ range between 10^{-5} M and 10^{-8} M, and significantly increase toward equilibrium value for $[H_2] \leq 10^{-8}$ M (note that the two inflection points in the δD profile occur around the two K_m values assigned for φ_1 to φ_3 and φ_4, respectively). This is consistent with our isotope data for fed-batch experiments for the modeled $[H_2]$ range between 7 and 410 μM (between 7×10^{-6} and 4×10^{-4} M), where relatively constant $^2\alpha$ values were observed (Figure 6B).

The relatively constant $^2\alpha$ for modeled $[H_2]$ between 10^{-5} M and 10^{-8} M in the differential reversibility scenario (Figure 5B) and observed in our experiments (Figure 6C) can be explained with a large KIE associated with the last step of methanogenesis (CH_3-CoM to CH_4 reduction; reaction 4, Figure 2A). As described above in section 4.1, reactions up to CH_3-CoM are reversible (φ_1 to $\varphi_3 \simeq 1$) in the differential reversibility scenario for the $[H_2]$ range between 5×10^{-5} and 10^{-8} M. Consequently, the three H atoms in the methyl group of CH_3-CoM are isotopically equilibrated with the surrounding water ($\delta D_{CH3-CoM} = -122$‰ at 82 °C; Gropp et al., 2021). The reversibility of the last step is relatively low at $[H_2] \geq 10^{-8}$ M in our model, and this step can result in large kinetic isotope fractionation. For reference, Scheller et al., (2013) reported experimentally determined values of primary KIE (k_H/k_D) of 2.44 (i.e., $^{2,P}\alpha$ of 0.41) and secondary KIE of 1.17

525 (i.e., $^{2,S}\alpha$ of 0.85) at 60 °C for the last step in reverse direction. The addition of the last H atom
526 from water (e.g., δD_{H2O} = –50‰) to the equilibrated CH_3-CoM with the $^{2,P}\alpha$ and $^{2,S}\alpha$ values above
527 would result in the final δD_{CH4} of –343‰ (= [¾ · (–122/1000+1) · (0.85) + ¼ · (–50/1000+1) ·
528 (0.41)] – 1), which is comparable to the range observed in our experiments (–339 ± 34‰; Table 4;
529 Table 5).

530 These experimental and model results together suggest that the persistent non-equilibrium
531 signatures observed for hydrogen isotope system in this study and previous studies are results of
532 differential reversibility with a less reversibility (larger KIE) at the last H-addition step. It follows
533 that, in our model setup, hydrogen isotope fractionation approaches to equilibrium values at sub-
534 μM ranges of [H_2] (solid line, Figure 6C). Future experiments with direct measurements and
535 precise control of [H_2] (e.g., continuous cultures) at sub-μM levels are needed to validate this
536 hypothesis.

537 Similar to $^2\alpha$ values, $\Delta^{13}CH_3D$ values were depleted and also indicated significant departure
538 from equilibrium; unlike in hydrogen isotope system, however, a distinct pattern of decreasing
539 $\Delta^{13}CH_3D$ values was observed for clumped isotope system (Figure 3A6–3E6; Figure 6D). This is
540 in sharp contrast with previous isotope model results that suggest a positive correlation between
541 the overall metabolic reversibility and $\Delta^{13}CH_3D$ (e.g., Stolper et al., 2015; Wang et al., 2015). In
542 this case, more reversibility would result in the increase of $\Delta^{13}CH_3D$ toward the value expected at
543 internal isotopologue equilibrium (*ca*. 4‰ at 82 °C). This unexpected pattern $\Delta^{13}CH_3D$ values was
544 reproduced in the differential reversibility scenario, with a distinct decrease in $\Delta^{13}CH_3D$ values for
545 the [H_2] range between *ca*. 5×10^{-4} and 10^{-7} M (blue solid line, Figure 5C). Note that the two
546 inflection points in the $\Delta^{13}CH_3D$ profile occur around the two assigned K_m values of 5×10^{-5} and
547 10^{-8} M for φ_1 to φ_3 and φ_4, respectively. The effect of assigned K_m values on the $\Delta^{13}CH_3D$ profile

548 was evident from sensitivity test results (Supplemental Material, Figure S6). The K_m value of
549 5×10^{-5} M for φ_1 to φ_3 resulted in the best fit to the $\Delta^{13}CH_3D$ values measured from the fed-batch
550 experiments in this study (e.g., panel C *vs*. panels A, B, D or E; Supplemental Material, Figure S6).
551 This is likely not a coincidence, given that the experimentally determined K_m value for
552 hyperthermophilic methanogens is close to the assigned value (6.6×10^{-5} M for three
553 *Methanocaldococcus* species; Ver Eecke *et al.*, 2012). Because our experiments were done at the
554 modeled $[H_2]$ range between 7 and 410 μM, we cannot empirically assess the fit of the lower K_m
555 value assigned for φ_4 in the differential reversibility scenario. For example, varying the $[H_2]$ value
556 for φ_4 between 10^{-10} and 10^{-6} M has negligible effect on the $\Delta^{13}CH_3D$ profile for $[H_2] \geq 10^{-6}$ M
557 (panels F–J; Supplemental Material, Figure S6). Future experiments at sub-μM levels of $[H_2]$ are
558 needed to properly determine the threshold K_m value for the last step of methanogenesis (which
559 may depend on the species) that would equilibrate $\Delta^{13}CH_3D$ signatures. However, the K_m value of
560 10^{-8} M for φ_4 used in this study is reasonable, considering that equilibrium $\Delta^{13}CH_3D$ signatures in
561 microbial methane have been found in marine sediments where the typical $[H_2]$ range is between
562 2 and 60 nM (Lin et al., 2012; Figure 6A).

563 Another characteristic pattern of the clumped isotope system observed in our experiments
564 was significant depletion in $^{13}CH_3D$ with anti-clumped (negative) $\Delta^{13}CH_3D$ values at low $[H_2]$ or
565 xH_2 (Figure 3A6–3E6; Figure 6D). The differential reversibility scenario shown in Figure 5C and
566 Figure 6D successfully reproduces not only the decreasing trend but also anti-clumped $\Delta^{13}CH_3D$
567 values that fits the range of $\Delta^{13}CH_3D$ values observed in this study (minimum of –4.1‰).
568 Sensitivity test results indicated that secondary clumped isotope fractionation of ≥6‰ (i.e., $^{S}\gamma \geq$
569 0.994) is required to produce a distinct decreasing pattern with negative $\Delta^{13}CH_3D$ values at $[H_2] \geq$
570 10^{-5} M (i.e., lower end of our experimental range) (panels A–F; Supplemental Material, Figure

S7). The $^{S}\gamma$ value of 0.990 (i.e., 10‰ secondary clumped isotope fractionation) resulted in the profile that best fits both the decreasing trend and magnitude of depletion in measured $\Delta^{13}CH_3D$ values (Figure 5C, Figure 6D). Varying the magnitude of primary clumped isotope fractionation ($^{P}\gamma$) without any secondary isotope fractionation ($^{S}\gamma$=1) did not reproduce the observed patterns in $\Delta^{13}CH_3D$ (panels G–L; Supplemental Material, Figure S7).

4.3 Limitations of the isotopologue flow network model and implications for interpreting the isotope signatures of natural methane samples

While we explored three different cases—including the differential reversibility scenario—for the isotopologue flow network model, there is much complexity associated with the biochemical inner workings of methanogenesis that is not captured in the model. The model uses a constant KIE value per H-addition step, assuming that each step is catalyzed by the same enzyme across the range of $[H_2]$ examined in this study (10^{-9} to 10^{-2} M). This may not be true for H-addition steps that can be catalyzed by more than one enzyme (i.e., isoenzymes). There are at least two sets of isoenzymes known to catalyze the second and fourth H-addition steps during hydrogenotrophic methanogenesis. The fourth step of methanogenesis (the reduction of methenyl-H_4MPT^+ to methylene-H_4MPT) involves either the oxidation of H_2 or the oxidation of the reduced form of F_{420} ($F_{420}H_2$), where the former is catalyzed by H_2-forming N^5,N^{10}-methylene-H_4MPT dehydrogenase (Hmd) and the latter is catalyzed by F_{420}-dependent N^5,N^{10}-methylene-H_4MPT dehydrogenase (Fmd) (von Bünau et al., 1991; Reeve et al., 1997). Previous studies have shown that Mtd increases in expression, relative to Hmd, under H_2 limitation (Reeve et al., 1997; Hendrickson et al., 2007; Topçuoğlu et al., 2019). The isoenzyme switching from Hmd to Mtd under H_2 limitation has been suggested to allow a greater expression of carbon isotope fractionation (Valentine et al., 2004) and may apply for hydrogen and clumped isotope systems as well.

Another set of isoenzymes, MCR I and MCR II (sometimes referred to as MR I and MR II), catalyzes the fourth and last H-addition steps of hydrogenotrophic methanogenesis (Pihl et al., 1994; Reeve et al., 1997). The relative abundances of MCR I and MCR II have been shown to be determined largely by growth phase, where MCR II is preferentially expressed during exponential phase and MCR I during linear or stationary phase (Rospert et al., 1990; Bonacker et al., 1992; Pihl et al., 1994). Whether distinct KIEs are associated with these isoenzymes remains unclear; however, considering that MCR I and MCR II have different substrate affinities (i.e., K_m values for CH_3-S-CoM and H-S-HTP) and catalytic rates (Bonacker et al., 1993), it is possible that these isoenzymes for the rate-limiting step of last H-addition impart distinct KIEs. Future studies combining a proteomic or transcriptomic approach and isotope analyses are needed to evaluate the effect of isoenzyme switching on isotope fractionation during hydrogenotrophic methanogenesis.

Despite the limitations mentioned above, insights gained from the empirical relationships among bulk ($^{13}\alpha$, $^{2}\alpha$) and clumped ($\Delta^{13}CH_3D$) isotope systems investigated in this study help us better interpret the isotope signatures in natural samples of methane. Our experimental and model results show that the nuances of isotope fractionation at a given time point warrant further consideration of physiological state and the amount of H_2 available for methanogens in the dissolved form. For example, rather than characterizing carbon isotope values with either an equilibrium or kinetic end-member signature, one would need to consider the large carbon isotope fractionation (larger than equilibrium) that can result from differential reversibility at an intermediate range of $[H_2]$ (between 10^{-5} M and 10^{-8} M under our experimental conditions; Figure 5A).

Clumped isotope results also suggest that low $\Delta^{13}CH_3D$ values are not necessarily associated with specific growth temperatures or metabolic pathways (e.g., hydrogenotrophic *vs*.

617 acetoclastic *vs*. methylotrophic). In studies where hydrogenotrophic species were grown on
618 H_2/CO_2 at different temperatures, mesophilic cultures in general were associated with larger
619 depletion in $\Delta^{13}CH_3D$ compared to thermophilic cultures (Figure 4C; Stolper et al., 2015; Wang et
620 al., 2015; Gruen et al., 2018). In studies where methanogens were grown on different substrates
621 using different biochemical pathways, methylotrophic and acetoclastic cultures in general carried
622 more depleted $\Delta^{13}CH_3D$ (and $\Delta^{12}CH_2D_2$) signatures (Young et al., 2017; Gruen et al., 2018). The
623 wide range of $\Delta^{13}CH_3D$ values (–4.1 to 2.1‰) observed in this study, where all cultures were
624 grown under hyperthermophilic and hydrogenotrophic conditions, suggests that the magnitude of
625 depletion in clumped isotopologues is not always associated with growth temperatures or
626 methanogenic pathways.

627 **5 Conclusion**

628 We cultured two different species of methanogens, *Methanocaldococcus bathoardescens* and
629 *Methanothermobacter thermautotrophicus* (ΔH), in batch and fed-batch systems and measured the
630 $\delta^{13}C$ and δD of the substrates (CO_2 and H_2O) as well as the $\delta^{13}C$, δD and $\Delta^{13}CH_3D$ of the product
631 (CH_4). The results of the fed-batch experiments confirm previous observations, where carbon
632 isotope fractionation ($^{13}\alpha = 0.96 \pm 0.02$) approaches and often exceed the magnitude expected at
633 CH_4-CO_2 equilibrium (e.g., Botz et al., 1996; Valentine et al., 2004; Penning et al., 2005; Okumura
634 et al., 2016; Topçuoğlu et al., 2019), while hydrogen isotope fractionation remains significantly
635 larger ($^{2}\alpha = 0.67 \pm 0.01$) than that expected at CH_4-H_2O equilibrium (Figure 4). The observed low
636 $\Delta^{13}CH_3D$ values indicate kinetic isotope effects, with an apparent decrease in $\Delta^{13}CH_3D$ values with
637 decreasing pH_2. The isotopologue flow network model presented showed our observations—large
638 carbon isotope fractionation, depleted hydrogen isotope signatures, and distinct decreasing
639 $\Delta^{13}CH_3D$ values with decreasing pH_2—can be explained by differential reversibility, in which the

last step of methanogenesis is less reversible compared to the preceding three H-addition reactions. In addition, including secondary clumped KIE in the model reproduced the magnitude of depletion in $\Delta^{13}CH_3D$ values observed in our experiments. Future studies focusing on controlled H_2 limitation experiments at sub-μM concentrations and conducting proteomic or transcriptomic analyses in parallel may improve our interpretations of the bulk and clumped isotope signatures used for methane source identification.

Acknowledgment

We thank J. F. Holden (University of Massachusetts, Amherst) for providing the *M. bathoardescens* culture used in this study; T. Bosak for providing lab facilities to support our culturing work; J. Sun for laboratory assistance for *M. thermautotrophicus* experiments; and D. Boutt (University of Massachusetts, Amherst) for analyzing δD_{H2O}. This work was supported by the NASA Astrobiology Institute "Rock-Powered Life" project under cooperative agreement NNA15BB02A. J.H.R. was also supported by the Grayce B. Kerr Fellowship, the Robert R. Shrock Fellowship and the Callahan Dee Fellowship at MIT.

Appendix A. Supplementary Material

Research Data that supports this research publication can be found in the Supplementary Material at [Link].

References

Balabane M., Galimov E., Hermann M. and Létolle R. (1987) Hydrogen and carbon isotope fractionation during experimental production of bacterial methane. *Org. Geochem.* **11**, 115–119.

Baldassare F. J., McCaffrey M. A. and Harper J. A. (2014) A geochemical context for stray gas investigations in the northern Appalachian Basin: Implications of analyses of natural gases from Neogene-through Devonian-age strata. *Am. Assoc. Pet. Geol. Bull.* **98**, 341–372.

Belyaev S. S., Wolkin R., Kenealy W. R., DeNiro M. J., Epstein S. and Zeikus J. G. (1983) Methanogenic Bacteria from the Bondyuzhskoe Oil Field: General Characterization and Analysis of Stable-Carbon Isotopic Fractionation. *Appl. Environ. Microbiol.* **45**, 691–697.

Bonacker L. G., Baudner S., Mörschel E., Böcher R. and Thauer R. K. (1993) Properties of the two isoenzymes of methyl-coenzyme M reductase in Methanobacterium thermoautotrophicum. *Eur. J. Biochem.* **217**, 587–595.

Bonacker L. G., Baudner S. and Thauer R. K. (1992) Erratum: Differential expression of the two methyl-coenzyme M reductases in Methanobacterium thermoautotrophicum as described immunochemically via isoenzyme-specific antisera (European Journal of Biochemistry, Vol. 206, No. 1). *Eur. J. Biochem.* **207**, 1129.

Botz R., Pokojski H.-D., Schmitt M. and Thomm M. (1996) Carbon isotope fractionation during bacterial methanogenesis reduction. **25**, 255–262.

von Bünau R., Zirngibl C., Thauer R. K. and Klein A. (1991) Hydrogen-forming and coenzyme-F420-reducing methylene tetrahydromethanopterin dehydrogenase are genetically distinct enzymes in Methanobacterium thermoautotrophicum (Marburg). *Eur. J. Biochem.* **202**, 1205–1208.

683 Cao X., Bao H. and Peng Y. (2019) A kinetic model for isotopologue signatures of methane
684 generated by biotic and abiotic CO2 methanation. *Geochim. Cosmochim. Acta* **249**, 59–75.
685 Chabab S., Théveneau P., Coquelet C., Corvisier J. and Paricaud P. (2020) Measurements and
686 predictive models of high-pressure H2 solubility in brine (H2O+NaCl) for underground
687 hydrogen storage application. *Int. J. Hydrogen Energy* **45**, 32206–32220.
688 Conrad R., Goodwin S. and Zeikus J. G. (1987) Hydrogen metabolism in a mildly acidic lake
689 sediment (Knaack Lake). *FEMS Microbiol. Lett.* **45**, 243–249.
690 Conrad R., Phelps T. J. and Zeikus J. G. (1985) Gas metabolism evidence in support of the
691 juxtaposition of hydrogen-producing and methanogenic bacteria in sewage sludge and lake
692 sediments. *Appl. Environ. Microbiol.* **50**, 595–601.
693 Coplen T. B. (2011) Guidelines and recommended terms for expression of stable-isotope-ratio
694 and gas-ratio measurement results. *Rapid Commun. Mass Spectrom.* **25**, 2538–2560.
695 Douglas P. M. J., Stolper D. A., Eiler J. M., Sessions A. L., Lawson M., Shuai Y., Bishop A.,
696 Podlaha O. G., Ferreira A. A., Santos Neto E. V., Niemann M., Steen A. S., Huang L.,
697 Chimiak L., Valentine D. L., Fiebig J., Luhmann A. J., Seyfried W. E., Etiope G., Schoell
698 M., Inskeep W. P., Moran J. J. and Kitchen N. (2017) Methane clumped isotopes: Progress
699 and potential for a new isotopic tracer. *Org. Geochem.* **113**, 262–282. Available at:
700 https://doi.org/10.1016/j.orggeochem.2017.07.016.
701 Ver Eecke H. C., Butterfield D. A., Huber J. A., Lilley M. D., Olson E. J., Roe K. K., Evans L.
702 J., Merkel A. Y., Cantin H. V. and Holden J. F. (2012) Hydrogen-limited growth of
703 hyperthermophilic methanogens at deep-sea hydrothermal vents. *Proc. Natl. Acad. Sci. U.*
704 *S. A.* **109**, 13674–13679.
705 Etiope G. and Sherwood Lollar B. (2013) Abiotic methane on earth. *Rev. Geophys.* **51**, 276–299.

706 Flores R. M., Rice C. A., Stricker G. D., Warden A. and Ellis M. S. (2008) Methanogenic
707 pathways of coal-bed gas in the Powder River Basin, United States: The geologic factor.
708 *Int. J. Coal Geol.* **76**, 52–75.
709 Fuchs G., Moll J., Scherer P. and Thauer R. (1979a) Activity, acceptor specificity and function
710 of hydrogenase in Methanobacterium thermoautotrophicum. *Hydrog. Their Catal. Act.,*
711 *Struct. Funct., Work. Meet.*, 83–92.
712 Fuchs G., Thauer R., Ziegler H. and Stichler W. (1979b) Carbon Isotope Fractionation by
713 Methanobacterium thermoautotrophicum. **9**, 135–139.
714 Games L. M. and Hayes J. M. (1978) Methane-producing bacteria : natural frac ~ onatio ~ s of
715 tbe stable carbon isotopes. **42**, 1295–1297.
716 Gonzalez Y., Nelson D. D., Shorter J. H., McManus J. B., Dyroff C., Formolo M., Wang D. T.,
717 Western C. M. and Ono S. (2019) Precise Measurements of 12CH2D2 by Tunable Infrared
718 Laser Direct Absorption Spectroscopy. *Anal. Chem.* **91**, 14967–14974.
719 Gropp J., Iron M. A. and Halevy I. (2021) Theoretical estimates of equilibrium carbon and
720 hydrogen isotope effects in microbial methane production and anaerobic oxidation of
721 methane. *Geochim. Cosmochim. Acta* **295**, 237–264. Available at:
722 https://doi.org/10.1016/j.gca.2020.10.018.
723 Gruen D. S., Wang D. T., Ko M., Stewart L. C., Goldhammer T., Holden J. F., Hinrichs K. and
724 Ono S. (2018) ScienceDirect Experimental investigation on the controls of clumped
725 isotopologue and hydrogen isotope ratios in microbial methane. **237**, 339–356.
726 Hattori S., Nashimoto H., Kimura H., Koba K., Yamada K., Shimizu M., Watanabe H., Yoh M.
727 and Yoshida N. (2012) Hydrogen and carbon isotope fractionation by thermophilic
728 hydrogenotrophic methanogens from a deep aquifer under coculture with fermenters.

729 *Geochem. J.* **46**, 193–200.
730 Hayes J. M. (2001) Fractionation of carbon and hydrogen isotopes in biosynthetic processes.
731 *Rev. Mineral. Geochemistry* **43**, 225–277.
732 Haynes W. M., Lide D. R. and Bruno T. J. (2016) *CRC handbook of chemistry and physics: a*
733 *ready-reference book of chemical and physical data*. 97th Editi., CRC Press, Boca Raton,
734 Florida.
735 Hendrickson E. L., Haydock A. K., Moore B. C., Whitman W. B. and Leigh J. A. (2007)
736 Functionally distinct genes regulated by hydrogen limitation and growth rate in
737 methanogenic Archaea. **2007**.
738 Horibe Y. and Craig H. (1995) D/H fractionation in the system methane-hydrogen-water.
739 *Geochim. Cosmochim. Acta* **59**, 5209–5217.
740 Horita J. (2001) Carbon isotope exchange in the system CO2-CH4 at elevated temperatures.
741 *Geochim. Cosmochim. Acta* **65**, 1907–1919.
742 Horita J. and Wesolowski D. J. (1994) Horita and Wesolowski 1994. *Geochim. Cosmochim. Acta*
743 **58**, 1–13.
744 Janssen P. H. (2010) Influence of hydrogen on rumen methane formation and fermentation
745 balances through microbial growth kinetics and fermentation thermodynamics. *Anim. Feed*
746 *Sci. Technol.* **160**, 1–22.
747 Jud G., Schneider K. and Bachofen R. (1997) The role of hydrogen mass transfer for the growth
748 kinetics of Methanobacterium thermoautotrophicum in batch and chemostat cultures. *J. Ind.*
749 *Microbiol. Biotechnol.* **19**, 246–251.
750 Kawagucci S., Kobayashi M., Hattori S., Yamada K., Ueno Y., Takai K. and Yoshida N. (2014)
751 Hydrogen isotope systematics among H2–H2O–CH4 during the growth of the

752 hydrogenotrophic methanogen Methanothermobacter thermautotrophicus strain ΔH.
753 *Geochim. Cosmochim. Acta* **142**, 601–614.
754 Krzycki J. A., Kenealy W. R., DeNiro M. J. and Zeikus J. G. (1987) Stable Carbon Isotope
755 Fractionation by Methanosarcina barkeri during Methanogenesis from Acetate, Methanol,
756 or Carbon Dioxide-Hydrogen. *Appl. Environ. Microbiol.* **53**, 2597–2599.
757 Kuivila K. M., Murray J. W., Devol A. H. and Novelli P. C. (1989) Methane production, sulfate
758 reduction and competition for substrates in the sediments of Lake Washington. *Geochim.*
759 *Cosmochim. Acta* **53**, 409–416.
760 Lin Y. S., Heuer V. B., Goldhammer T., Kellermann M. Y., Zabel M. and Hinrichs K. U. (2012)
761 Towards constraining H2 concentration in subseafloor sediment: A proposal for combined
762 analysis by two distinct approaches. *Geochim. Cosmochim. Acta* **77**, 186–201.
763 Londry K. L., Dawson K. G., Grover H. D., Summons R. E. and Bradley A. S. (2008) Stable
764 carbon isotope fractionation between substrates and products of Methanosarcina barkeri.
765 *Org. Geochem.* **39**, 608–621.
766 Lovley D. R. (1985) Minimum threshold for hydrogen metabolism in methanogenic bacteria.
767 *Appl. Environ. Microbiol.* **49**, 1530–1531.
768 Macpherson J. V and Unwin P. R. (1997) Determination of the Diffusion Coefficient of
769 Hydrogen in Aqueous Solution Using Single and Double Potential Step
770 Chronoamperometry at a Disk Ultramicroelectrode. *Anal. Chem.* **69**, 2063–2069.
771 Mariotti A., Germon J. C., Hubert P., Kaiser P., Letolle R., Tardieux A. and Tardieux P. (1981)
772 Experimental determination of nitrogen kinetic isotope fractionation: Some principles;
773 illustration for the denitrification and nitrification processes. *Plant Soil* **62**, 413–430.
774 McGinnis D. F., Kirillin G., Tang K. W., Flury S., Bodmer P., Engelhardt C., Casper P. and

Grossart H. P. (2015) Enhancing surface methane fluxes from an oligotrophic lake: Exploring the microbubble hypothesis. *Environ. Sci. Technol.* **49**, 873–880.

Nguyen T. B., Topçuoğlu B. D., Holden J. F., LaRowe D. E. and Lang S. Q. (2020) Lower hydrogen flux leads to larger carbon isotopic fractionation of methane and biomarkers during hydrogenotrophic methanogenesis. *Geochim. Cosmochim. Acta* **271**, 212–226.

Okumura T., Kawagucci S., Saito Y., Matsui Y., Takai K. and Imachi H. (2016) Hydrogen and carbon isotope systematics in hydrogenotrophic methanogenesis under H2-limited and H2-enriched conditions: implications for the origin of methane and its isotopic diagnosis. *Prog. Earth Planet. Sci.* **3**, 1–19.

Ono S., Rhim J. H., Gruen D. S., Taubner H., Kölling M. and Wegener G. (2021) Clumped isotopologue fractionation by microbial cultures performing the anaerobic oxidation of methane. *Geochim. Cosmochim. Acta* **293**, 70–85.

Ono S., Wang D. T., Gruen D. S., Sherwood Lollar B., Zahniser M. S., McManus B. J. and Nelson D. D. (2014) Measurement of a doubly substituted methane isotopologue, 13CH3D, by tunable infrared laser direct absorption spectroscopy. *Anal. Chem.* **86**, 6487–6494.

Park S. H., Park C., Lee J. Y. and Lee B. (2017) A Simple Parameterization for the Rising Velocity of Bubbles in a Liquid Pool. *Nucl. Eng. Technol.* **49**, 692–699.

Pauss A., Andre G., Perrier M. and Guiot S. R. (1990) Liquid-to-Gas mass transfer in anaerobic processes: Inevitable transfer limitations of methane and hydrogen in the biomethanation process. *Appl. Environ. Microbiol.* **56**, 1636–1644.

Penning H., Plugge C. M., Galand P. E. and Conrad R. (2005) Variation of carbon isotope fractionation in hydrogenotrophic methanogenic microbial cultures and environmental samples at different energy status. *Glob. Chang. Biol.* **11**, 2103–2113.

798 Pihl T. D., Sharma S. and Reeve J. N. (1994) Growth phase-dependent transcription of the genes
799 that encode the two methyl coenzyme M reductase isoenzymes and N5-
800 methyltetrahydromethanopterin:Coenzyme M methyltransferase in Methanobacterium
801 thermoautotrophicum ΔH. *J. Bacteriol.* **176**, 6384–6391.
802 Pohlman J. W., Kaneko M., Heuer V. B., Coffin R. B. and Whiticar M. (2009) Methane sources
803 and production in the northern Cascadia margin gas hydrate system. *Earth Planet. Sci. Lett.*
804 **287**, 504–512.
805 Rees C. E. (1973) A steady-state model for sulphur isotope fractionation in bacterial reduction
806 processes. *Geochim. Cosmochim. Acta* **37**, 1141–1162.
807 Reeve J. N., Nölling J., Morgan R. M. and Smith D. R. (1997) Methanogenesis: Genes,
808 Genomes, and Who's on First? *J. Bacteriol.* **179**, 5975–5986.
809 Robinson J. A. and Tiedje J. M. (1982) Kinetics of hydrogen consumption by rumen fluid,
810 anaerobic digestor sludge, and sediment. *Appl. Environ. Microbiol.* **44**, 1374–1384.
811 Rospert S., Linder D., Ellermann J. and Thauer R. K. (1990) Two genetically distinct methyl-
812 coenzyme M reductases in Methanobacterium thermoautotrophicum strain Marburg and
813 ΔH. *Eur. J. Biochem.* **194**, 871–877.
814 Scheller S., Goenrich M., Thauer R. K. and Jaun B. (2013) Methyl-Coenzyme M Reductase from
815 Methanogenic Archaea: Isotope E ff ects on the Formation and Anaerobic Oxidation of
816 Methane.
817 Schoell M. (1988) Multiple origins of methane in the Earth. *Chem. Geol.* **71**, 1–10.
818 Sherwood Lollar B., Lacrampe-Couloume G., Voglesonger K., Onstott T. C., Pratt L. M. and
819 Slater G. F. (2008) Isotopic signatures of CH4 and higher hydrocarbon gases from
820 Precambrian Shield sites: A model for abiogenic polymerization of hydrocarbons. *Geochim.*

Cosmochim. Acta **72**, 4778–4795.

Sherwood Lollar B., Westgate T. D., Ward J. A., Slater G. F. and Lacrampe-Couloume G. (2002) Abiogenic formation of alkanes in the earth's crust as a minor source for global hydrocarbon reservoirs. *Nature* **416**, 522–524.

Smolenski W. J. and Robinson J. A. (1988) In situ rumen hydrogen concentrations in steers fed eight times daily, measured using a mercury reduction detector. *FEMS Microbiol. Lett.* **53**, 95–100.

Stewart L. C., Llewellyn J. G., Butterfield D. A., Lilley M. D. and Holden J. F. (2016) Hydrogen and thiosulfate limits for growth of a thermophilic, autotrophic Desulfurobacterium species from a deep-sea hydrothermal vent. *Environ. Microbiol. Rep.* **8**, 196–200.

Stolper D. A., Martini A. M., Clog M., Douglas P. M., Shusta S. S., Valentine D. L., Sessions A. L. and Eiler J. M. (2015) Distinguishing and understanding thermogenic and biogenic sources of methane using multiply substituted isotopologues. *Geochim. Cosmochim. Acta* **161**, 219–247.

Stolper D. A., Sessions A. L., Ferreira A. A., Santos Neto E. V., Schimmelmann A., Shusta S. S., Valentine D. L. and Eiler J. M. (2014) Combined 13C-D and D-D clumping in methane: Methods and preliminary results. *Geochim. Cosmochim. Acta* **126**, 169–191. Available at: http://dx.doi.org/10.1016/j.gca.2013.10.045.

Teramoto M., Fujita S., Kataoka M. and Hashimoto K. (1970) Effect of Bubble Size on the Selectivity of Consecutive Gas-Liquid Reactions. In *Meeting of the Society of the Chemical Engineers (Japan)* pp. 79–82.

Thauer R. K. (2011) Anaerobic oxidation of methane with sulfate: On the reversibility of the reactions that are catalyzed by enzymes also involved in methanogenesis from CO2. *Curr.*

844 *Opin. Microbiol.* **14**, 292–299.
845 Thauer R. K., Kaster A. K., Seedorf H., Buckel W. and Hedderich R. (2008) Methanogenic
846 archaea: Ecologically relevant differences in energy conservation. *Nat. Rev. Microbiol.* **6**,
847 579–591.
848 Topçuoğlu B. D., Meydan C., Orellana R. and Holden J. F. (2018) Formate hydrogenlyase and
849 formate secretion ameliorate H 2 inhibition in the hyperthermophilic archaeon
850 Thermococcus paralvinellae. *Environ. Microbiol.* **20**, 949–957.
851 Topçuoğlu B., Meydan C., Nguyen T., Lang S. and Holden J. F. (2019) Growth Kinetics, Carbon
852 Isotope Fractionation, and Gene Expression in the Hyperthermophile Methanocaldococcus
853 jannaschii during Hydrogen-Limited Growth and Interspecies Hydrogen Transfer Begüm.
854 **85**, 1–14.
855 Valentine D. L., Chidthaisong A., Rice A., Reeburgh W. S. and Tyler S. C. (2004) Carbon and
856 hydrogen isotope fractionation by moderately thermophilic methanogens. *Geochim.*
857 *Cosmochim. Acta* **68**, 1571–1590.
858 Wang D. T., Gruen D. S., Lollar B. S., Hinrichs K.-U., Stewart L. C., Holden J. F., Hristov A.
859 N., Pohlman J. W., Morrill P. L., Könneke M., Delwiche K. B., Reeves E. P., Sutcliffe C.
860 N., Ritter D. J., Seewald J. S., McIntosh J. C., Hemond H. F., Kubo M. D., Cardace D.,
861 Hoehler T. M. and Ono S. (2015) Nonequilibrium clumped isotope signals in microbial
862 methane. *Science (80-.).* **348**, 428–431.
863 Welhan J. A. and Lupton J. E. (1987) Light hydrocarbon gases in Guaymas Basin hydrothermal
864 fluids: thermogenic versus abiogenic origin. *Am. Assoc. Pet. Geol. Bull.* **71**, 215–223.
865 Whiticar M. J. (1990) A geochemial perspective of natural gas and atmospheric methane. *Org.*
866 *Geochem.* **16**, 531–547.

867 Whiticar M. J. (1999) Carbon and hydrogen isotope systematics of bacterial formation and
868 oxidation of methane. *Chem. Geol.* **161**, 291–314.
869 Wolfe R. S. (2011) *Techniques for cultivating methanogens*. 1st ed., Elsevier Inc.
870 Yoshioka H., Sakata S. and Kamagata Y. (2008) Hydrogen isotope fractionation by
871 Methanothermobacter thermoautotrophicus in coculture and pure culture conditions.
872 *Geochim. Cosmochim. Acta* **72**, 2687–2694.
873 Young E. D., Kohl I. E., Lollar B. S., Etiope G., Rumble D., Li (李姝宁) S., Haghnegahdar M.
874 A., Schauble E. A., McCain K. A., Foustoukos D. I., Sutclife C., Warr O., Ballentine C. J.,
875 Onstott T. C., Hosgormez H., Neubeck A., Marques J. M., Pérez-Rodríguez I., Rowe A. R.,
876 LaRowe D. E., Magnabosco C., Yeung L. Y., Ash J. L. and Bryndzia L. T. (2017) The
877 relative abundances of resolved l2CH2D2 and 13CH3D and mechanisms controlling
878 isotopic bond ordering in abiotic and biotic methane gases. *Geochim. Cosmochim. Acta* **203**,
879 235–264.
880 Young E. D., Rumble D., Freedman P. and Mills M. (2016) A large-radius high-mass-resolution
881 multiple-collector isotope ratio mass spectrometer for analysis of rare isotopologues of O2,
882 N2, CH4 and other gases. *Int. J. Mass Spectrom.* **401**, 1–10.
883
884

Tables

Table 1. Summary of experiments.

Experiment	Organism	Temp. (°C)	xH_2 (%)	System
B.82	*Methanocaldococcus bathoardescens*	82	80	Batch
F.82.80	*Methanocaldococcus bathoardescens*	82	80	Fed-batch
F.82.25	*Methanocaldococcus bathoardescens*	82	25	Fed-batch
F.60.80	*Methanocaldococcus bathoardescens*	60	80	Fed-batch
F65.20	*Methanothermobacter thermautotrophicus* (ΔH)	65	20	Fed-batch
F.65.5	*Methanothermobacter thermautotrophicus* (ΔH)	65	5→1.6	Fed-batch

Table 2. Parameters used for model calculation described in 2.3.7.

Parameter	Values used (range)	Sources/Notes
r_b	0.05 cm (0.025 to 0.1 cm)	Teramoto *et al.* (1970)
D	5×10^{-5} cm^2/sec	Macpherson and Unwin (1997)
A_b	200 cm^2	Calculated for a gas flow rate of 200 mL/min, medium height of 20 cm, and bubble radius of 0.05 cm; Park et al. (2017)
A_h	177 cm^2	Calculated for a known reactor I.D. = 15 cm

Table 3. Results for batch culture experiment (Experiment B.82). *Methanocaldococcus bathoardescens* was grown at 82 °C on 80% H_2. The headspace pressure decreased, as 5 moles of gas were consumed to produce 1 mole of gas (Eqn. 7). Therefore, volumes (in mL) of H_2, CO_2, and CH_4 were calculated assuming 4:1:1 reaction stoichiometries and mixing ratios for H_2: CO_2:CH_4 measure by GC (Table 3; Supplementary Material, Figure S1).

Time (h)	cells/mL	H_2 (mL)†	CO_2 (mL)†	CH_4 (mL)†	$\delta^{13}C_{CO2}$ (‰)	$\delta^{13}C_{CH4}$ (‰)	δD_{H2O} (‰)	δD_{CH4} (‰)	$\Delta^{13}CH_3D$ (‰)	CI
0.0	-	288.24	47.35	0.13	-34.9	-	-45.4	-	-	-
1.0	1.1E+6	281.61	50.49	0.53	-	-	-46.9	-	-	-
1.9	5.5E+6	258.65	44.57	6.83	-31.4	-	-	-	-	-
3.0	3.7E+7	125.88	13.33	36.98	-14.3	-47.1	-46.5	-407.6	0.48	0.20
4.0	3.6E+7	78.29	4.55	48.45	-25.1	-39.5	-	-409.6	0.38	0.19
5.0	7.4E+7	68.78	2.84	50.48	-	-	-	-	-	-
6.0	7.1E+7	35.52	2.60	58.35	-22.0	-	-	-	-	-
6.9	4.3E+7	53.28	2.11	53.90	-	-38.8	-46.0	-413.3	0.32	0.26

† volume of gas calculated based on mixing ratios and expected headspace pressure at the time of measurement; – values not determined; CI is the 95% confidence interval in permil (‰).

Table 4. Results for fed-batch experiments. *Methanocaldococcus bathoardescens* was grown at 82 °C and 80% H_2 (F.82.80); at 60 °C and 80% H_2 (F.60.80); and at 82 °C and 25% H_2 (F.82.25).

Experiment	Temp (˚C)	Time (h)	cells/mL	H_2 (%)	CO_2 (%)	CH_4 (%)	MPR (mol/h)	csMPR (pmol/cell/h)	$\delta^{13}C_{CO2}$ (‰)	$\delta^{13}C_{CH4}$ (‰)	$\alpha_{CH4\ CO2}$	δD_{H2O} (‰)	δD_{CH4} (‰)	$\alpha_{CH4\ H2O}$	$\Delta^{13}CH_3D$ (‰)	CI
F.82.80	82	0.0	6.8E+5	79.8	19.9	0.08	4.1E-04	0.33	-39.9	-	*	-53.7	-	*	-	-
F.82.80	82	0.0	-	80.7	19.5	1.78	8.0E-03	-	-	-	*	-	-	*	-	-
F.82.80	82	1.1	5.2E+6	-	-	-	-	-	-	-	*	-	-	*	-	-
F.82.80	82	1.5	-	76.6	20.0	3.75	1.6E-02	-	-25.8	-68.2	0.957	-53.8	-362.6	0.674	1.25	0.48
F.82.80	82	2.2	2.0E+7	77.2	19.8	4.03	1.7E-02	0.47	-21.3	-76.6	0.944	-	-336.9	*	0.38	0.43
F.82.80	82	2.5	2.8E+7	77.9	18.5	4.02	1.7E-02	0.34	-	-74.3	*	-	-331.8	*	-0.17	0.49
F.82.80	82	3.0	3.2E+7	78.0	18.6	4.09	1.7E-02	0.29	-	-80.2	*	-	-333.6	*	0.07	0.83
F.82.80	82	3.5	4.1E+7	-	-	-	-	-	-	-	*	-	-	*	-	-
F.82.80	82	3.7	4.5E+7	77.0	19.2	3.92	1.7E-02	0.21	-	-77.9	*	-	-331.8	*	-0.29	0.47
F.60.80	60	0.0	5.8E+6	75.5	21.9	0.06	3.1E-04	0.03	-	-	*	-	-	*	-	-
F.60.80	60	1.5	1.7E+7	73.9	22.3	0.47	2.4E-03	0.08	-38.8	-56.4	0.982	-	-305.0	*	2.06	-
F.60.80	60	1.8		75.3	22.1	0.50	2.6E-03	-	-	-	*	-	-	*	-	-
F.60.80	60	2.8	1.4E+7	73.1	22.4	1.38	6.9E-03	0.28	-	-	*	-	-	*	-	-
F.60.80	60	3.3		75.1	22.2	1.90	9.0E-03	-	-36.8	-60.1	0.976	-	-364.5	*	1.52	0.58
F.60.80	60	3.8	2.4E+7	72.5	21.1	2.98	1.4E-02	0.32	-	-	*	-	-	*	-	-
F.60.80	60	5.6	1.3E+8	74.0	21.1	4.14	1.8E-02	0.08	-	-	*	-	-	*	-	-
F.60.80	60	6.4		74.5	21.0	3.94	1.7E-02	-	-33.6	-68.9	0.964	-	-321.4	*	0.80	0.28
F.60.80	60	6.7	1.1E+8	71.7	21.3	3.64	1.7E-02	0.08	-	-	*	-	-	*	-	-
F.60.80	60	7.8	1.4E+8	73.9	21.4	3.91	1.7E-02	0.07	-	-	*	-	-	*	-	-
F.60.80	60	8.9	1.9E+8	71.7	21.2	3.7	1.7E-02	0.05	-	-	*	-	-	*	-	-
F.60.80	60	9.4		73.7	21.6	4.07	1.8E-02	-	-32.6	-67.5	0.964	-	-314.6	*	0.06	0.17
F.82.25	82	0.0	1.0E+6	23.1	21.8	BDL	-	-	-	-	*	-	-	*	-	-
F.82.25	82	0.2		23.1	21.9	0.03	1.6E-04	-	-14.0	-50.8	0.963	-40.4	-358.8	0.668	1.15	0.42
F.82.25	82	0.5	4.4E+6	22.8	23.6	0.41	2.1E-03	0.27	-	-	*	-	-	*	-	-
F.82.25	82	1.7	1.3E+7	22.6	22.9	1.30	5.8E-03	0.25	-	-	*	-39.9	-	*	-	-
F.82.25	82	2.3	9.1E+6	22.5	23.8	1.39	6.1E-03	0.37	-	-	*	-	-	*	-	-
F.82.25	82	2.8	9.6E+6	22.4	23.5	1.53	6.6E-03	0.38	-	-	*	-40.1	-	*	-	-
F.82.25	82	3.6	2.1E+7	22.4	23.8	1.56	6.7E-03	0.18	-	-	*	-	-	*	-	-
F.82.25	82	4.4	7.4E+6	22.5	23.9	1.47	6.4E-03	0.48	-	-	*	-40.0	-	*	-	-
F.82.25	82	4.9	2.4E+7	22.4	24.2	1.57	6.7E-03	0.16	-	-	*	-	-	*	-	-
F.82.25	82	5.2	2.8E+7	16.7	22.8	1.41	7.8E-03	0.15	-	-	*	-	-	*	-	-
F.82.25	82	19.0	2.0E+8	22.6	23.6	1.69	7.1E-03	0.02	-	-	*	-	-	*	-	-
F.82.25	82	22.5	1.0E+8	22.6	23.9	1.51	6.5E-03	0.04	-	-	*	-	-	*	-	-
F.82.25	82	24.5	9.6E+7	22.6	23.3	1.57	6.7E-03	0.04	-	-	*	-	-	*	-	-
F.82.25	82	24.7		22.4	23.9	1.82	7.5E-03	-	-12.0	-70.8	0.941	-	-328.6	*	-0.78	0.73
F.82.25	82	25.8	8.1E+7	22.5	23.0	1.65	7.0E-03	0.05	-	-	*	-	-	*	-	-
F.82.25	82	26.8	1.0E+8	22.2	2.0	2.05	8.3E-03	0.05	-	-	*	-	-	*	-	-
F.82.25	82	43.7	1.5E+8	21.6	23.8	2.82	1.1E-02	0.04	-	-	*	-	-	*	-	-
F.82.25	82	44.4		21.6	24.8	2.71	1.0E-02	-	-9.9	-67.8	0.941	-	-327.7	*	-0.65	0.36
F.82.25	82	45.2		21.7	23.3	2.89	1.1E-02	-	-10.3	-65.4	0.944	-38.6	-364.5	0.661	-0.42	0.53
F.82.25	82	52.5	2.1E+8	21.2	24.9	2.62	1.0E-02	0.03	-	-	*	-	-	*	-	-

– values not determined; * not applicable; CI is the 95% confidence interval in permil (‰).

Table 5. Results for fed-batch experiments. *Methanothermobacter thermautotrophicus* was grown at 65 °C and 20% H_2 (Experiment F.65.20) and at 65 °C and 5–1.6% H_2 (Experiment F.65.5).

Experiment	Time (h)	cells/mL	H_2 (%)	CO_2 (%)	CH_4 (%)	MPR (mol/h)	csMPR (pmol/cell/h)	$\delta 13C_{CO2}$ (‰)	$\delta^{13}C_{DIC}$ (‰)	$\alpha_{CO2\text{-}DIC}$	$\delta^{13}C_{CH4}$ (‰)	$\alpha_{CH4\text{-}CO2}$	δD_{H2O} (‰)	δD_{CH4} (‰)	$\alpha_{CH4\text{-}H2O}$	$\Delta^{13}CH_3D$ (‰)	CI
F.65.20	0.0	2.3E+5	21.6	18.7	0.01	4.1E-05	0.098	-15.3	-11.7	0.996	-	*	-45.5	-	*	-	-
F.65.20	2.2	4.8E+5	21.2	19.4	0.03	1.4E-04	0.158	-	-	*	-	*	-	-	*	-	-
F.65.20	5.8	1.2E+6	21.1	19.3	0.10	4.5E-04	0.213	-	-	*	-	*	-	-	*	-	-
F.65.20	11.7	4.8E+6	19.9	19.3	0.44	2.0E-03	0.237	-14.9	-10.5	0.996	-48.4	0.966	-45.5	-361.8	0.669	0.33	0.57
F.65.20	22.8	4.8E+7	19.5	17.9	0.69	3.0E-03	0.035	-14.9	-9.7	0.995	-57.0	0.957	-	-363.3	0.667	-2.03	0.50
F.65.20	25.7	6.9E+7	19.5	18.1	0.70	3.1E-03	0.025	-	-	*	-	*	-	-	*	-	-
F.65.20	28.1	7.7E+7	19.5	18.1	0.70	3.1E-03	0.022	-	-9.7	*	-	*	-45.5	-	*	-	-
F.65.20	33.3	1.1E+8	19.5	18.1	0.71	3.1E-03	0.016	-14.0	-	*	-	*	-	-	*	-	-
F.65.20	47.2	1.8E+8	19.3	18.7	0.72	3.2E-03	0.010	-13.9	-9.4	0.995	-62.1	0.951	-	-347.0	0.684	-1.28	0.87
F.65.20	53.2	2.1E+8	19.2	19.3	0.74	3.3E-03	0.009	-13.6	-9.5	0.996	-	*	-	-	*	-	-
F.65.20	70.2	2.9E+8	19.3	18.1	0.76	3.3E-03	0.007	-	-	*	-	*	-	-	*	-	-
F.65.20	77.0	2.7E+8	19.3	19.0	0.76	3.3E-03	0.007	-13.6	-9.4	0.996	-62.9	0.950	-	-352.3	0.679	-1.09	0.65
F.65.20	94.9	3.1E+8	19.4	18.8	0.78	3.4E-03	0.006	-	-	*	-	*	-	-	*	-	-
F.65.20	101.9	4.5E+8	19.0	18.9	0.81	3.6E-03	0.004	-13.8	-9.6	0.996	-63.3	0.950	-	-351.4	0.677	-1.91	0.54
F.65.20	102.4	3.9E+8	19.2	19.5	0.80	3.5E-03	0.005	-	-	*	-	*	-	-	*	-	-
F.65.20	119.2	4.1E+8	18.9	19.5	0.80	3.5E-03	0.005	-	-	*	-	*	-41.4	-	*	-	-
F.65.5	0.0	2.8E+5	4.9	20.3	0.01	5.2E-05	0.103	-15.7	-11.4	0.996	-	-	-44.9	-	*	-	-
F.65.5	1.7	4.7E+5	4.8	19.6	0.04	1.8E-04	0.210	-	-	*	-	-	-	-	*	-	-
F.65.5	4.9	1.1E+6	4.5	20.7	0.09	4.7E-04	0.252	-	-	*	-	-	-	-	*	-	-
F.65.5	9.4	2.5E+6	4.4	19.7	0.1	7.0E-04	0.156	-	-	*	-58.0	0.957	-	-364.1	0.666	1.28	0.98
F.65.5	10.5		-	-	-	-	-	-15.5	-11.4	0.996	-	-	-	-	*	-	-
F.65.5	11.8	5.9E+6	4.4	19.6	0.14	7.1E-04	0.068	-	-	*	-	-	-	-	*	-	-
F.65.5	21.9	1.4E+7	4.3	20.5	0.16	7.9E-04	0.031	-	-	*	-67.0	0.947	-	-371.7	0.658	-2.06	0.47
F.65.5	23.3		-	-	-	-	-	-15.2	-10.9	0.996	-	-	-45.5	-	*	-	-
F.65.5	27.5	2.5E+7	4.4	19.3	0.16	7.7E-04	0.017	-	-	*	-	-	-	-	*	-	-
F.65.5	30.3	2.7E+7	4.4	19.0	0.16	7.7E-04	0.016	-	-11.0	*	-	-	-	-	*	-	-
F.65.5	46.0	5.1E+7	4.3	20.6	0.17	8.2E-04	0.009	-	-	*	-	-	-	-	*	-	-
F.65.5	54.0	5.8E+7	4.3	19.5	0.17	8.5E-04	0.008	-15.3	-11.3	0.996	-70.6	0.944	-45.2	-368.4	0.661	-2.92	0.84
F.65.5	72.5	6.6E+7	1.6	20.3	0.06	2.5E-04	0.002	-15.6	-11.5	0.996	-77.5	0.937	-	-365.3	0.665	-2.52	0.46
F.65.5	95.3	7.2E+7	1.6	20.1	0.06	2.5E-04	0.002	-	-	*	-76.9	0.938	-	-370.0	0.660	-4.13	0.46
F.65.5	97.3		-	-	-	-	-	-15.6	-11.4	0.996	-	-	-	-	*	-	-
F.65.5	119.4	7.0E+7	1.5	21.4	0.06	2.6E-04	0.002	-	-	*	-	-	-	-	*	-	-

– values not determined; * not applicable; CI is the 95% confidence interval in permil (‰)

Figure Captions

Figure 1. Schematic diagram of the fed-batch culturing system

Figure 2. Schematic overview of the biochemical pathway involved in hydrogenotrophic methanogenesis and isotopologue flow network model scenarios in this study. (A) Overview of the biochemical pathway and enzymes associated with each step. Grey bubbles represent pools of cellular carbon, grouped into those with the same redox state. The four H-addition steps are labeled with numbers. Fd_{red}, reduced ferredoxin; Fd_{ox}, oxidized ferredoxin; MFR, methanofuran; H_4MPT, tetrahydromethanopterin; F_{420}, coenzyme F_{420}; CoM-HS, coenzyme M; CoB-SH, coenzyme B; Ftr, formyl-MFR: H_4MPT formyltransferase; Mch, N^5,N^{10}-methenyl- H_4MPT cyclohydrolase; Mtd, F_{420}-dependent N^5,N^{10}-methylene-H_4MPT dehydrogenase; Hmd, H_2-forming N^5,N^{10}-methylene-H_4MPT dehydrogenase; Mer, F_{420}-dependent N^5,N^{10}-methylene-H_4MPT reductase; Mtr, N^5-methyl- H_4MPT:CoM methyltransferase; Mcr, methyl CoM reductase. Panels B, C and D show the three model scenarios tested in this study. K_m values are effective half-saturation constants used to assign reversibilities to H-addition steps. (B) Equilibrium end-member scenario has a K_m value of 10^4 M for all four H-addition steps. (C) Uniform reversibility scenario has a K_m value of 10^{-8} M for all four H-addition steps. (D) In differential reversibility scenario, the last step is less reversible compared to the preceding three H-addition steps ($K_m = 5 \times 10^{-5}$ M for the first three steps; $K_m = 10^{-8}$ M for the last step).

Figure 3. Summary of fed-batch experiment results of this study. Each column (columns A–E) shows the result for each fed-batch experiment. The experiment names and conditions can be found at the top of each column. Each row (rows 1–6) shows a type of data. Modeled values of dissolved hydrogen concentration or $[H_2]$ (μM; row 1), cell density (cells/mL; row 2), methane production rate or MPR (μmol/sec; row 3), carbon isotope fractionation factor or $^{13}\alpha$ (row 4), hydrogen isotope

928 fractionation factor or $^{2}\alpha$ (row 5), and methane clumped isotope abundance or $\Delta^{13}CH_3D$ (‰; row
929 6). For the [H_2] model results (row 1), open squares represent maximum [H_2] values expected at
930 equilibrium with the dry headspace mixing ratios ($x$$H_2$). Filled circles represent [H_2] values
931 expected at equilibrium that are corrected for saturation water vapor pressure ($p$$H_2O_{sat}$) at
932 respective temperatures (0.51 bars at 82 °C, 0.25 bars at 65 °C and 0.20 bars at 60 °C). Triangles
933 represent $p$$H_2O_{sat}$-corrected [$H_2$] values expected during methane production with two different
934 k_La values: 700 h^{-1} (down-pointing yellow triangles) and 350 h^{-1} (up-pointing blue triangles).
935 Refer to 2.3.7 for details. Grey horizontal lines in rows 4–6 represent the equilibrium $^{13}\alpha$, $^{2}\alpha$ and
936 $\Delta^{13}CH_3D$ values expected at respective experimental temperatures. Grey vertical lines in column
937 5 for F.65.5 indicate the time at which $x$$H_2$ was switched from 5% to 1.6%. Each panel shares the
938 y-axis with the panel to its left unless new axis tick marks are introduced. Note that the durations
939 of experiments vary across experiments.

940 **Figure 4.** Changes in carbon ($^{13}\alpha$) and hydrogen ($^{2}\alpha$) isotope fractionation factors and $\Delta^{13}CH_3D$
941 values during hydrogenotrophic methanogenesis. Panels A, B and C, respectively, show $^{13}\alpha$, $^{2}\alpha$
942 and $\Delta^{13}CH_3D$ values measured in this study and reported in the literature, as a function of H_2 partial
943 pressure ($p$$H_2$) in the supply gas. Color triangle and diamond symbols represent data from this
944 study. Grey circles in panels A and B represent the $^{13}\alpha$ and $^{2}\alpha$ values from the literature (Games
945 and Hayes, 1978; Fuchs *et al.*, 1979; Belyaev *et al.*, 1983; Balabane *et al.*, 1987; Krzycki *et al.*,
946 1987; Botz *et al.*, 1996; Valentine *et al.*, 2004; Yoshioka, Sakata and Kamagata, 2008; Hattori *et*
947 *al.*, 2012; Kawagucci *et al.*, 2014). Grey symbols in panel C represent the $\Delta^{13}CH_3D$ values obtained
948 from pure culture hydrogenotrophic methanogenesis experiments in closed systems (Gruen *et al.*,
949 2018 and references therein). Filled and open circles represent thermophilic and mesophilic
950 temperatures, respectively. Dashed lines represent the $^{13}\alpha$, $^{2}\alpha$ and $\Delta^{13}CH_3D$ values expected at

951 equilibrium at temperatures indicated in the legend.

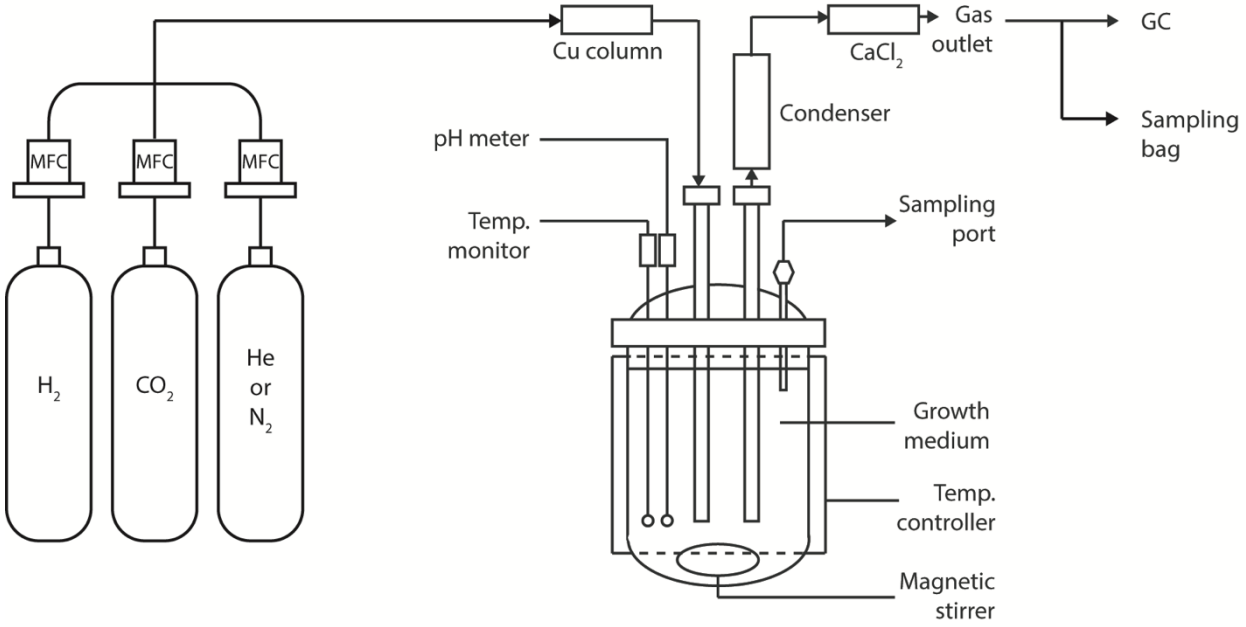
952 **Figure 5.** Modeled carbon ($\delta^{13}C$) and hydrogen (δD) isotope and clumped isotopologue
953 compositions ($\Delta^{13}CH_3D$) of methane produced via hydrogenotrophic methanogenesis. Panel A, B,
954 and C show the modeled $\delta^{13}C$, δD and $\Delta^{13}CH_3D$ values of methane, respectively. Dotted lines
955 (yellow) show the model results at 82 °C for the equilibrium end-member scenario ($K_m = 10^4$ M
956 for all φ values). Dashed lines (red) show the results for the uniform reversibility scenario (K_m =
957 10^{-8} M for all φ values). Solid lines (blue) show a differential reversibility scenario, where the last
958 H-addition step is less reversible compared to the preceding three H-addition steps ($K_m = 5\times10^{-5}$
959 M for φ_{1-3}, $K_m = 10^{-8}$ M for φ_4).

960 **Figure 6.** Carbon ($^{13}\alpha$) and hydrogen ($^{2}\alpha$) isotope fractionation factors and $\Delta^{13}CH_3D$ values as a
961 function of modeled dissolved H_2 concentration, $[H_2]$. Panel A shows the typical ranges of $[H_2]$
962 observed in natural environments and culture studies. The $[H_2]$ ranges for batch cultures studies
963 were calculated assuming 1–2 bars of 80% H_2 in the headspace at 25 °C. Note that the pH_2 values
964 for batch co-cultures (Okumura et al., 2016) are based on headspace mixing ratios and that $[H_2]$ in
965 the co-cultures are likely higher than $[H_2]^{eq}$ expected in equilibrium with pH_2. Panels B, C and D
966 compare the result of the isotopologue flow network model and experimental data from this study.
967 Color symbols and corresponding experiment names are shown in the legend. Horizontal dashed
968 lines represent the $^{13}\alpha$, $^{2}\alpha$ and $\Delta^{13}CH_3D$ values expected at equilibrium at corresponding
969 temperatures as shown in the legend. Solid lines are modeled trajectories of $^{13}\alpha$, $^{2}\alpha$ and $\Delta^{13}CH_3D$
970 for the differential reversibility scenario based on the isotopologue flow network model results
971 (see Figure 5).

972

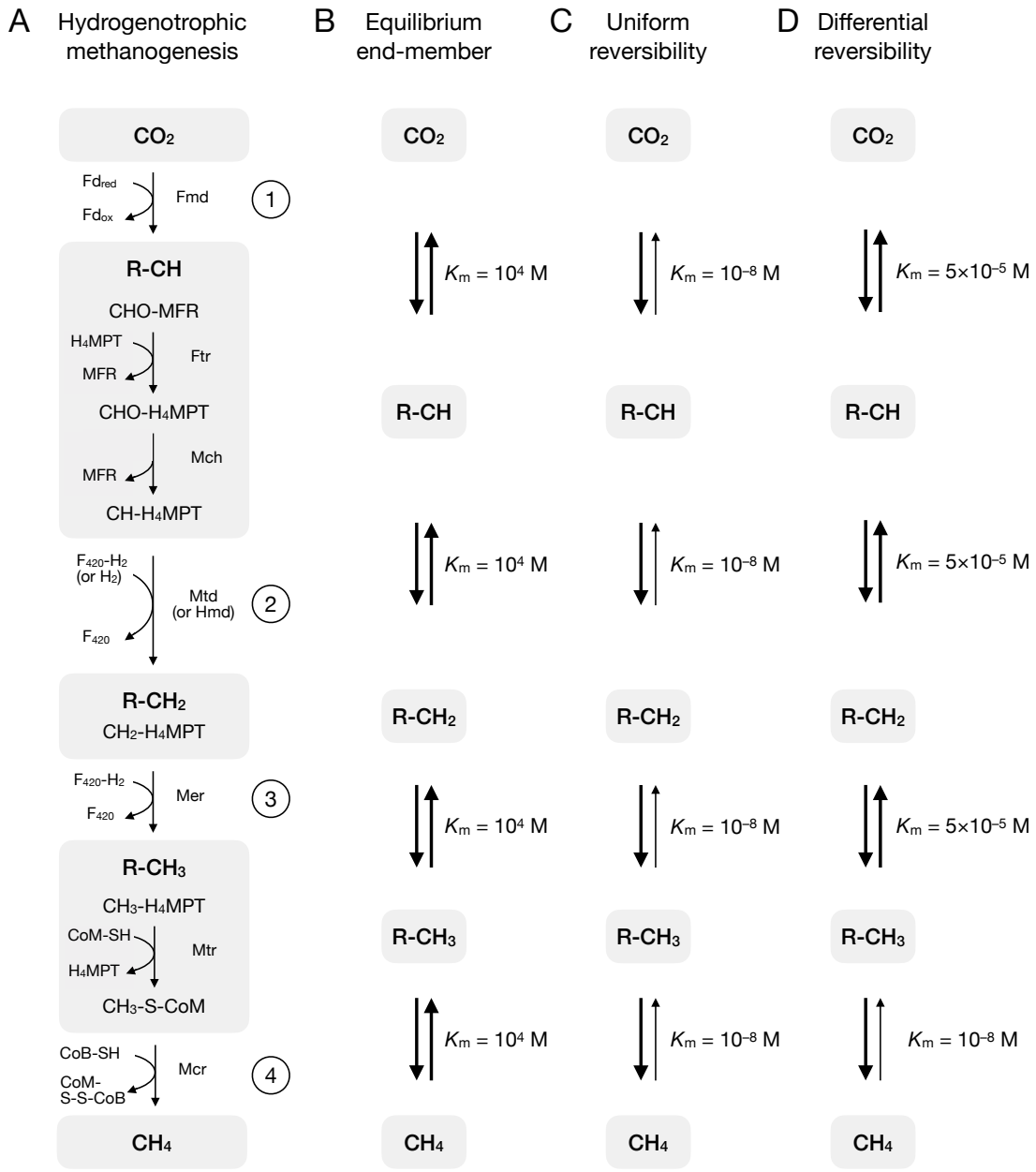
973 **Figures**

974



975

976 **Figure 1**



977
978 **Figure 2**

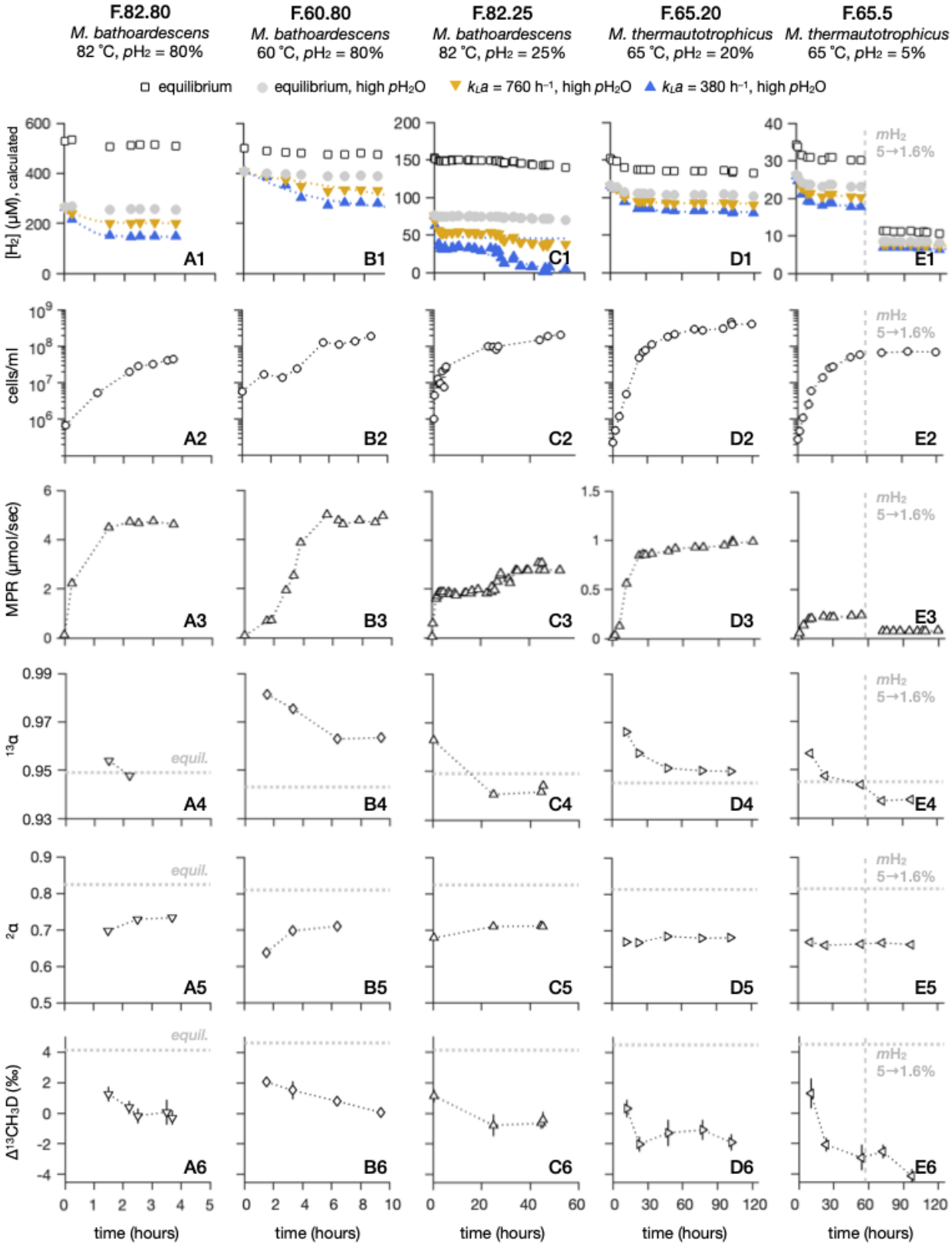

F.82.80
M. bathoardescens
82 °C, pH2 = 80%
F.60.80
M. bathoardescens
60 °C, pH2 = 80%
F.82.25
M. bathoardescens
82 °C, pH2 = 25%
F.65.20
M. thermautotrophicus
65 °C, pH2 = 20%
F.65.5
M. thermautotrophicus
65 °C, pH2 = 5%
equilibrium
equilibrium, high pH2O
kLa = 760 h-1, high pH2O
kLa = 380 h-1, high pH2O
[H2] (µM), calculated
cells/ml
MPR (µmol/sec)
13α
2α
Δ13CH3D (‰)
mH2 5→1.6%
equil.
A1
B1
C1
D1
E1
A2
B2
C2
D2
E2
A3
B3
C3
D3
E3
A4
B4
C4
D4
E4
A5
B5
C5
D5
E5
A6
B6
C6
D6
E6
time (hours)

Figure 3

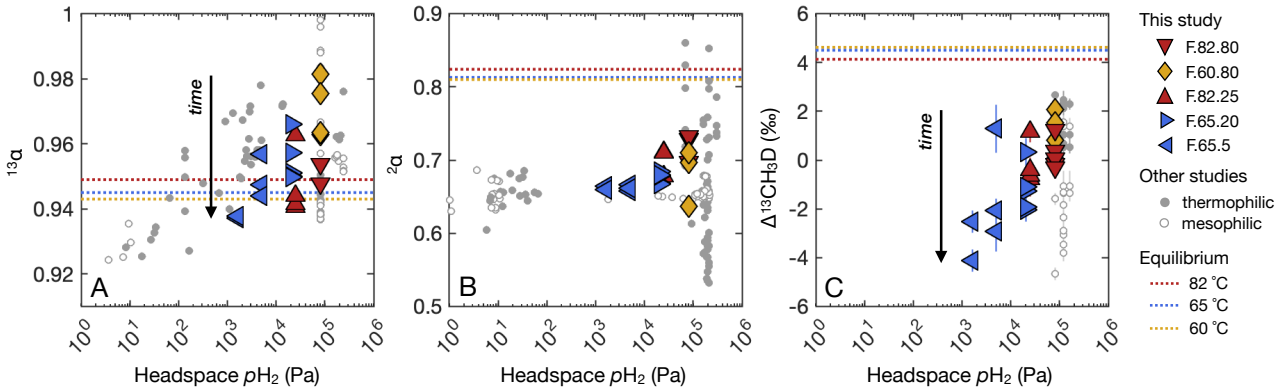


Figure 4

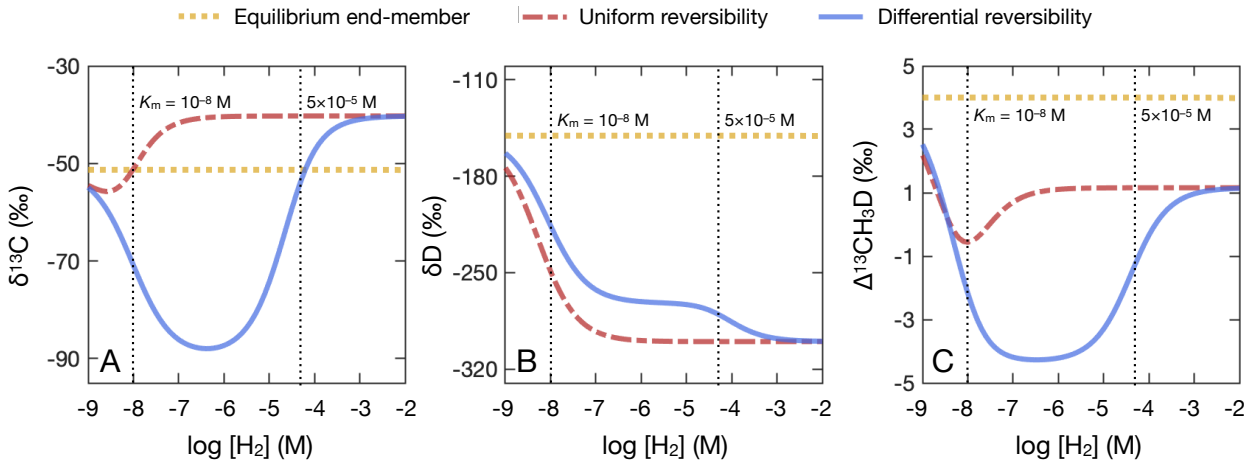
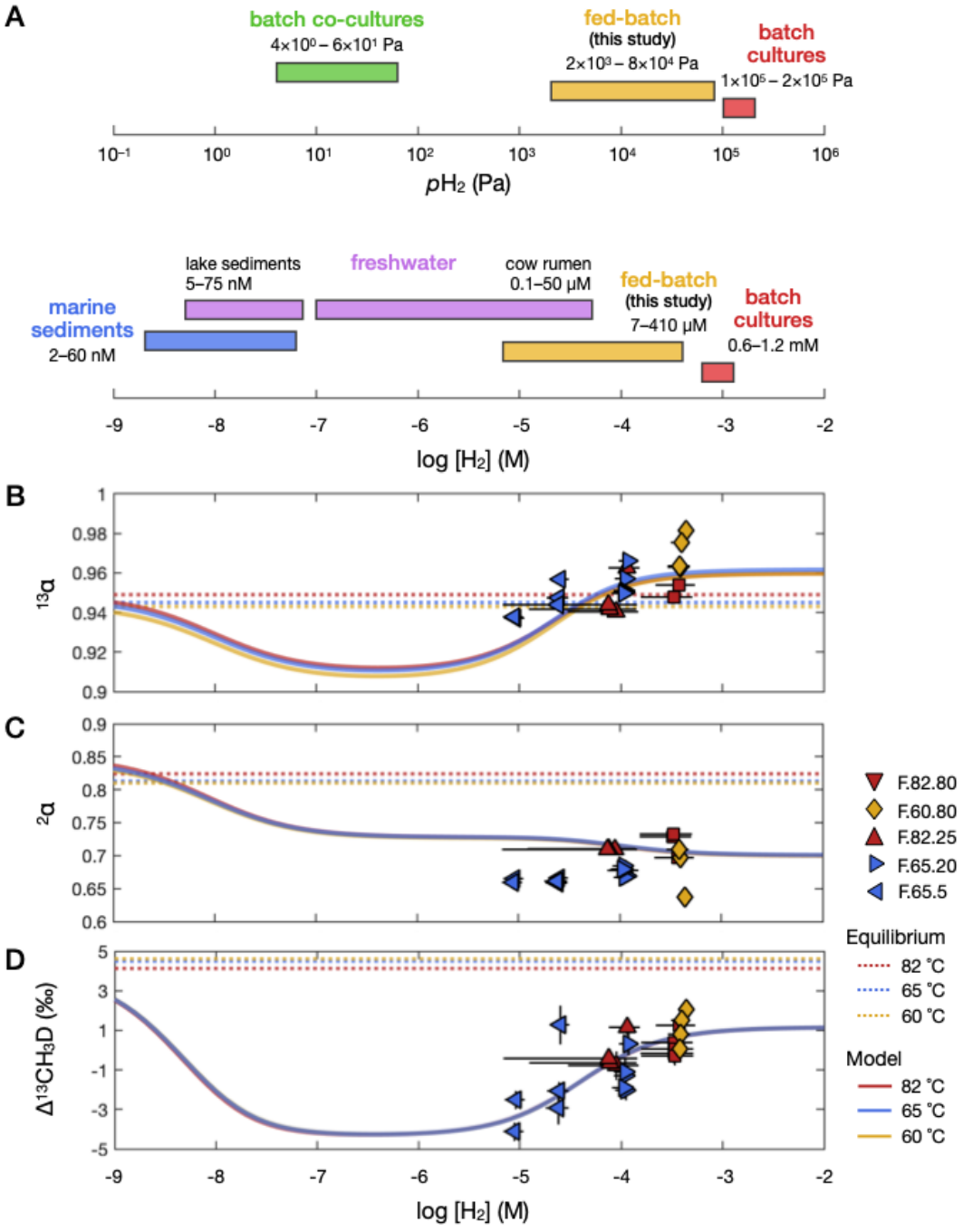


Figure 5



989

990 **Figure 6**