

## Supplementary Material

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

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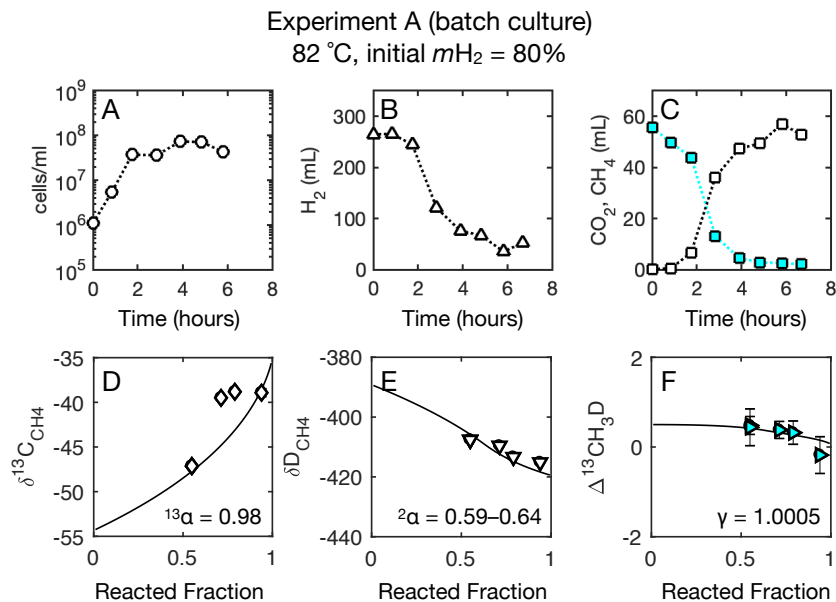


Figure S1. Temporal changes in cell density, headspace gases, and estimates of isotope fractionation factors during a batch culture experiment (Experiment B.82). *Methanocaldococcus bathoardescens* was grown at 82 °C and 80%  $H_2$ . Notations for symbols: open circle (cell density; cells/mL), open up-pointing triangle ( $H_2$ ; mL), filled squares ( $CO_2$ ; mL), open squares ( $CH_4$ ; mL), open diamonds ( $\delta^{13}C_{CH_4}$ ; ‰), open down-pointing triangles ( $\delta D_{CH_4}$ ; ‰), and filled right-pointing triangles ( $\Delta^{13}CH_3D$ ; ‰). The  $\delta^{13}C_{CO_2}$  of the source  $CO_2$  was  $-35\text{‰}$ , and the  $\delta D_{H_2O}$  of the source water was  $-45\text{‰}$ .

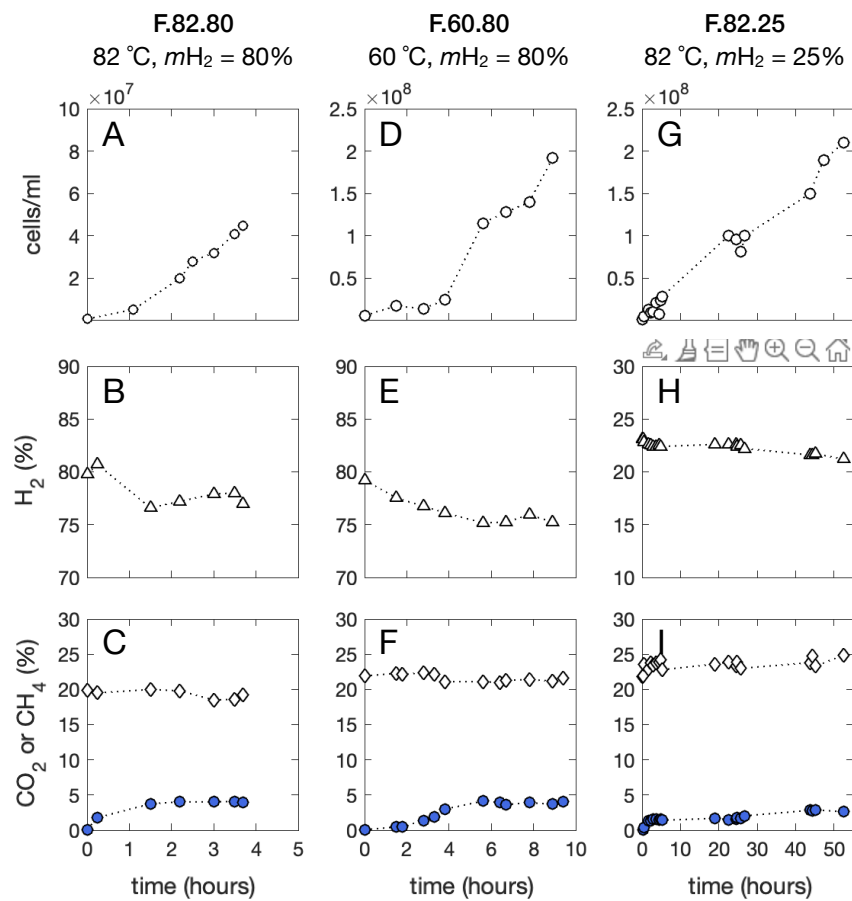


Figure S2. Temporal changes in cell density and headspace mixing ratios during fed-batch incubations of *Methanocaldococcus bathoardescens*. Experiment F.82.80 (panels A–C) was conducted at 82 °C and 80%  $H_2$ ; Experiment F.60.80 (panels D–F) at 60 °C and 80%  $H_2$ ; and Experiment F.82.25 (panels G–I) at 82 °C and 25%  $H_2$ . Notations for symbols: open circle (cell density; cells/ml), open up-pointing triangle ( $H_2$ ; %), open diamonds ( $CO_2$ ; %) and filled circles ( $CH_4$ ; %). Note that timescales are different among experiments.

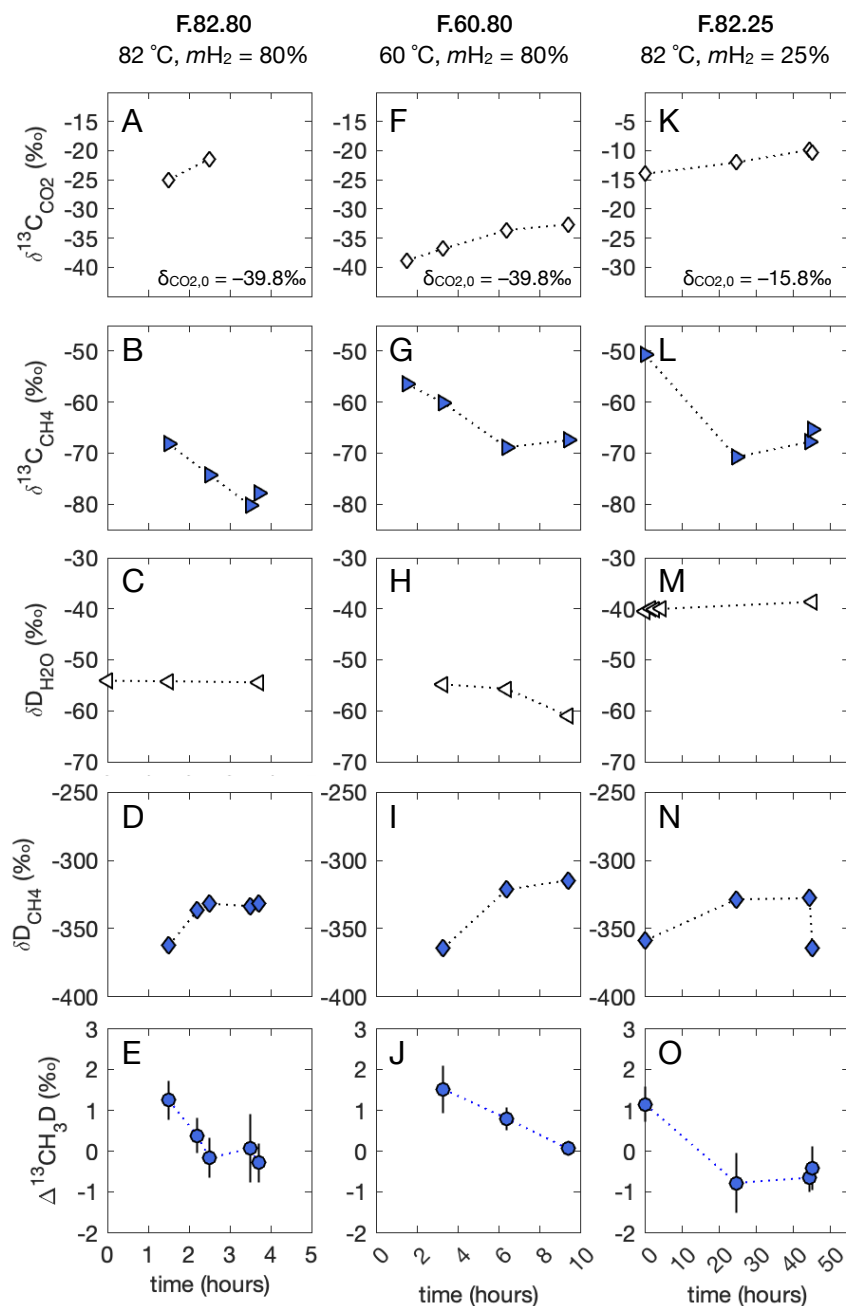


Figure S3. Temporal changes in bulk and clumped isotopologue ratios during fed-batch incubations of *Methanocaldococcus bathoardescens*. Experiment F.82.80 (panels A–E) was conducted at 82 °C and 80% H<sub>2</sub>; Experiment F.60.80 (panels F–J) at 60 °C and 80% H<sub>2</sub>; and Experiment F.82.25 (panels K–O) at 82 °C and 25% H<sub>2</sub>. Notations for symbols: open diamonds ( $\delta^{13}\text{C}_{\text{CO}_2}$ ; ‰), filled right-pointing triangles ( $\delta^{13}\text{C}_{\text{CH}_4}$ ; ‰), open left-pointing triangles ( $\delta\text{D}_{\text{H}_2\text{O}}$ ; ‰), filled diamonds ( $\delta\text{D}_{\text{CH}_4}$ ; ‰) and filled circles ( $\Delta^{13}\text{CH}_3\text{D}$ ; ‰).  $\delta^{13}\text{C}_{\text{CO}_2,0}$  values in panels A, F and K are the carbon isotopic compositions of CO<sub>2</sub> measured upstream. Note that timescales are different among experiments.

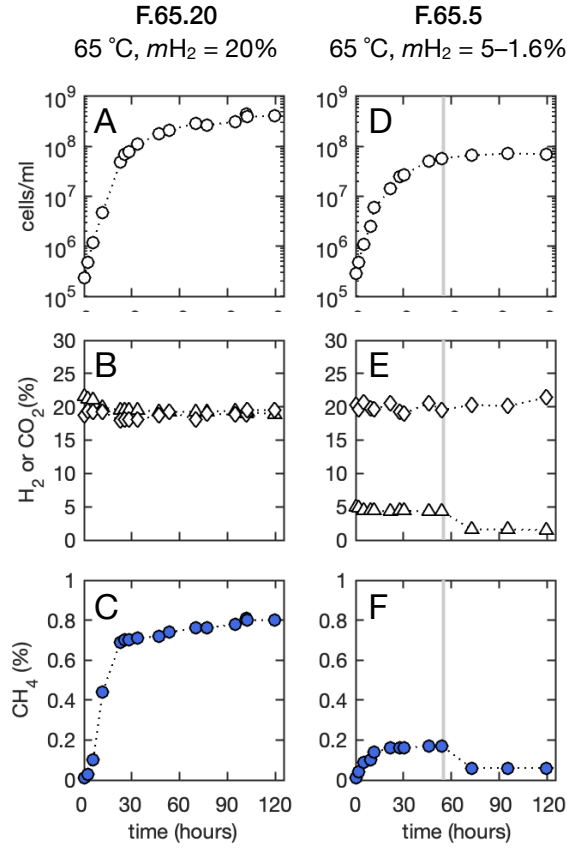


Figure S4. Temporal changes in cell density and headspace mixing ratio during fed-batch incubations of *Methanothermobacter thermaulithotrophicus*. Experiment F.65.20 (panels A–C) was conducted at 65 °C and 20% H<sub>2</sub> and Experiment F.65.5 (panels D–F) at 65 °C and 5% to 1.6% H<sub>2</sub>. After 55 hours (grey vertical line, panels D–F),  $x_{H_2}$  was switched from 5% to 1.6%. Notations for symbols are as follow: open circles (cell density; cells/ml), open triangles (H<sub>2</sub>; %), open diamonds (CO<sub>2</sub>; %) and filled circles (CH<sub>4</sub>; %).

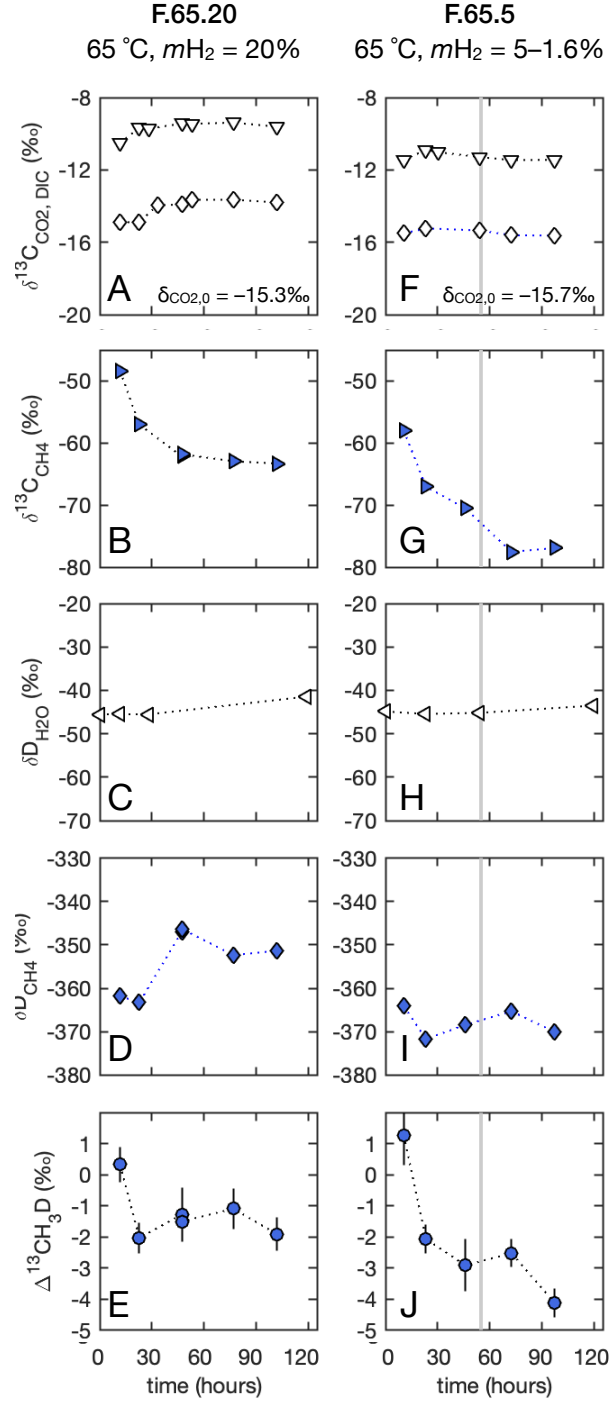


Figure S5. Temporal changes in bulk and clumped isotopologue ratios during fed-batch incubations of *Methanothermobacter thermaulithotrophicus*. Experiment F.65.20 (panels A–E) was conducted at 65 °C and 20%  $H_2$ . Experiment F.65.5 (panels F–J) at 65 °C and 5% to 1.6%  $H_2$ . After 55 hours (grey vertical line, panels D–F),  $xH_2$  was switched from 5% to 1.6%. Notations for symbols: open down-pointing triangles ( $\delta^{13}C_{DIC}$ ; ‰), open diamonds ( $\delta^{13}C_{CO_2}$ ; ‰), filled right-pointing triangles ( $\delta^{13}C_{CH_4}$ ; ‰), open left-pointing triangles ( $\delta D_{H_2O}$ ; ‰), filled diamonds ( $\delta^{13}C_{CH_4}$ ; ‰) and filled circles ( $\Delta^{13}CH_3D$ ; ‰).  $\delta^{13}C_{CO_2,0}$  values in panels A, F and K are the carbon isotopic compositions of  $CO_2$  measured upstream.

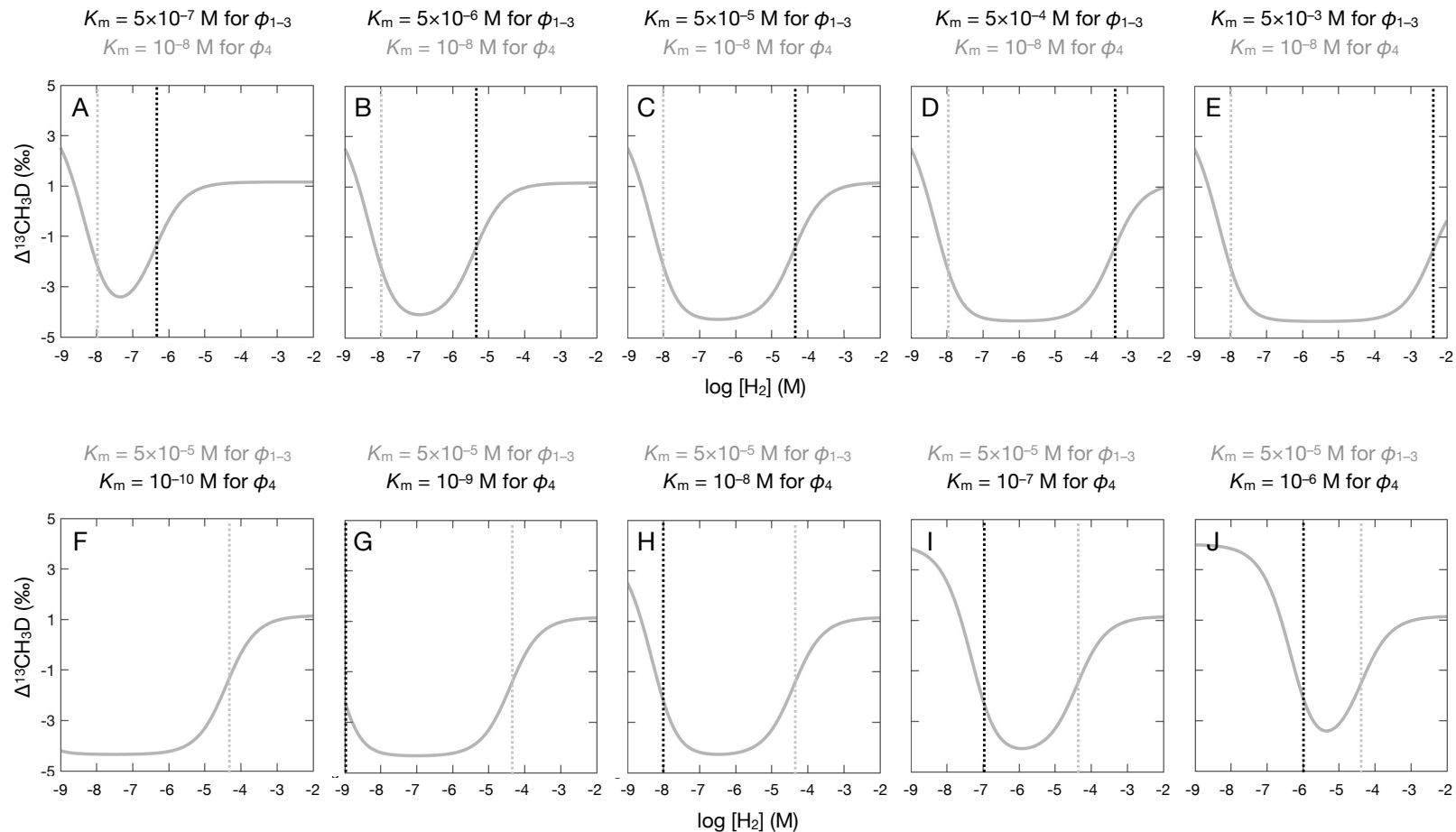


Figure S6. Modeled clumped isotopologue compositions ( $\Delta^{13}\text{CH}_3\text{D}$ ) of methane produced via hydrogenotrophic methanogenesis with different effective half-saturation constants ( $K_m$ ) for the differential reversibility scenario in **Error! Reference source not found.C**. The top row (panels A–E) shows  $\Delta^{13}\text{CH}_3\text{D}$  profiles with the same  $K_m$  value for the fourth H-addition step ( $K_m = 10^{-8}$  M for  $\phi_4$ , where  $\phi$  represents metabolic reversibility) and different  $K_m$  values for the first three H-addition steps ( $K_m = 5 \times 10^{-7}$  to  $5 \times 10^{-3}$  M for  $\phi_{1-3}$ ). The bottom row (panels F–J) shows  $\Delta^{13}\text{CH}_3\text{D}$  profiles with the same  $K_m$  value for the first three H-addition steps ( $K_m = 5 \times 10^{-5}$  M for  $\phi_{1-3}$ ) and different  $K_m$  values for the first three H-addition steps ( $K_m = 10^{-10}$  to  $10^{-6}$  M for  $\phi_4$ ). Note that panels C and H are the same  $\Delta^{13}\text{CH}_3\text{D}$  profile for the differential reversibility scenario in **Error! Reference source not found.C**. See section **Error! Reference source not found.** for more details about the model

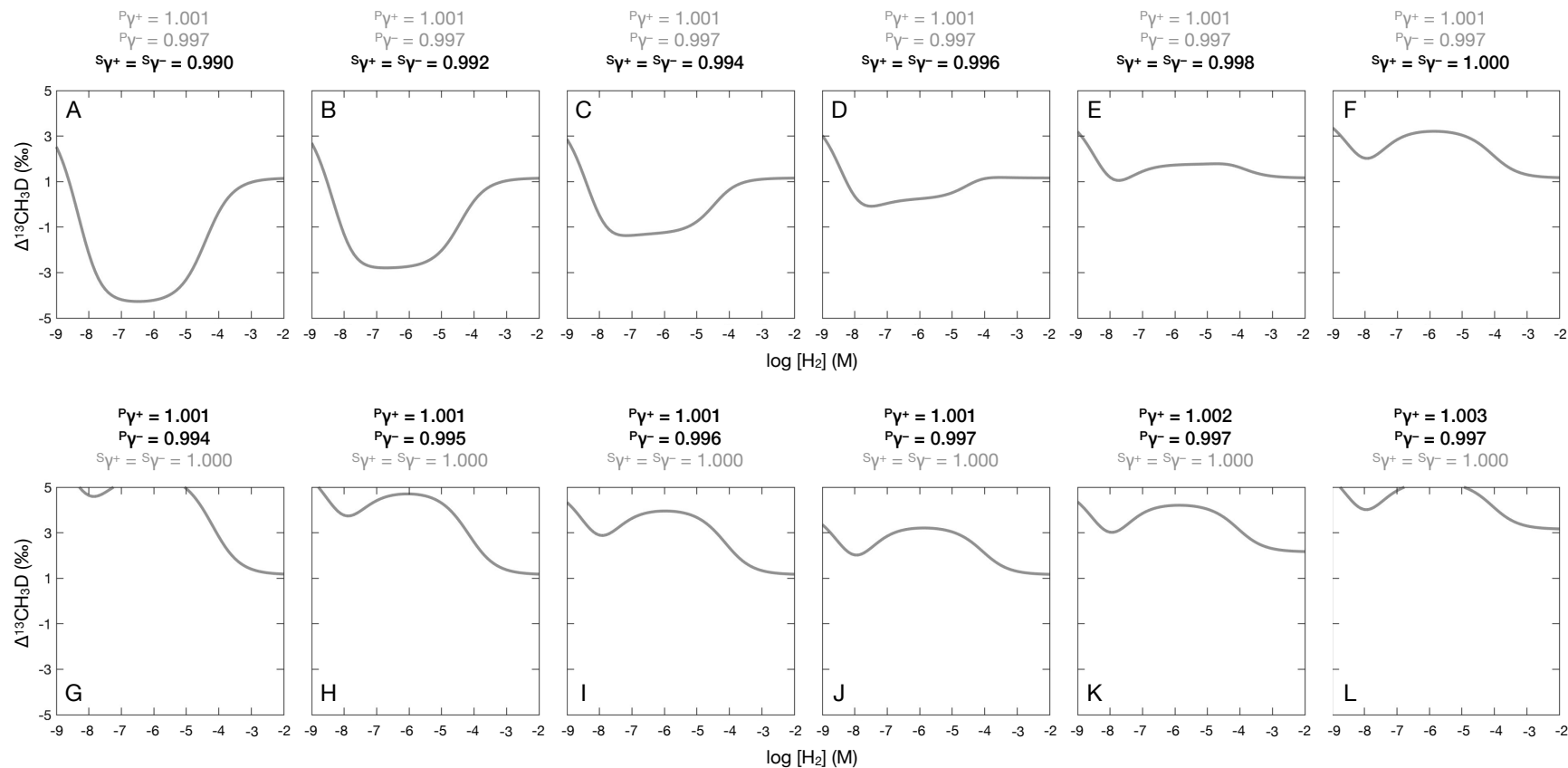


Figure S7. Modeled clumped isotopologue compositions ( $\Delta^{13}\text{CH}_3\text{D}$ ) of methane produced via hydrogenotrophic methanogenesis with different primary ( $P_\gamma$ ) and secondary ( $S_\gamma$ ) clumped isotope fractionation factors. The top row (panels A–F) shows  $\Delta^{13}\text{CH}_3\text{D}$  profiles with the same magnitude of equilibrium primary clumped isotope fractionation ( $P_\gamma = 4\%$  at  $82^\circ\text{C}$ ) and different magnitudes of secondary isotope fractionation ( $S_\gamma = 0$  to  $10\%$ ). The bottom row (panels G–L) shows  $\Delta^{13}\text{CH}_3\text{D}$  profiles with the same magnitude of equilibrium secondary clumped isotope fractionation ( $S_\gamma = 0\%$ ) and different magnitudes of primary isotope fractionation ( $P_\gamma = 4$  to  $7\%$ ). Note that panel A is the same  $\Delta^{13}\text{CH}_3\text{D}$  profile for the differential reversibility scenario in **Error! Reference source not found.C**. See section **Error! Reference source not found.** for more details about the model.



## KIE values used in the isotopologue flow network model

We assign a KIE for the forward reaction ( $\alpha^+$ ) and derive the reverse KIE ( $\alpha^-$ ) that maintains internal consistency, and vice versa, using  $\alpha^{eq}$  values:

$$\alpha^{eq} = \frac{\alpha^-}{\alpha^+} \quad (\text{Eqn. S1})$$

For deuterated isotopologues, both primary ( $^P\alpha$ ) and secondary ( $^S\alpha$ ) KIEs are considered in the model (Table S1). Primary KIEs apply when a C–D bond is directly broken or formed during a reaction, whereas secondary KIEs apply when one or more C–D bond(s) is(are) transferred from a reactant to a product while a C–H bond is directly broken or formed instead. The following equations define the parameters reported in Table S1:

$$^{2,P}\alpha^{eq} = \frac{^{2,P}\alpha^-}{^{2,P}\alpha^+} \quad (\text{Eqn. S2})$$

$$^{2,S}\alpha^{eq} = \frac{^{2,S}\alpha^-}{^{2,S}\alpha^+} \quad (\text{Eqn. S3})$$

where the numeric superscript on the top left corner (e.g., 2) denotes the type of isotope system (e.g., 2 for hydrogen); the alphabetic superscripts, “P” and “S,” denote primary and secondary KIEs, respectively; and the “+” and “–” superscripts on the top right corner denote forward and backward reactions, respectively (Table S1).

KIEs for clumped isotopologues follow the rule of geometric mean (Bigeleisen, 1955). For example, the KIE for  $^{13}\text{CH}_3\text{D}$  is approximately the product of KIEs for  $^{13}\text{C}/^{12}\text{C}$  and D/H. The primary KIE for clumped isotopologues ( $^P\gamma$ ) is defined as the deviation from this product (Wang et al., 2015). Similar to  $\alpha$  values, the backward ( $^P\gamma^-$ ) clumped isotopologue KIE is derived by assigning a forward value ( $^P\gamma^+$ ) and using equilibrium values ( $^P\gamma^{eq}$ ; 1.004 at 82 °C):

$$P_{\gamma^{eq}} = \frac{P_{\gamma^{-}}}{P_{\gamma^{+}}} \quad (\text{Eqn. S4})$$

$$S_{\gamma^{eq}} = \frac{S_{\gamma^{-}}}{S_{\gamma^{+}}} \quad (\text{Eqn. S5})$$

Table S1.  $^{13}\text{C}/^{12}\text{C}$  and D/H isotope fractionation factors used as input parameters for the isotopologue flow network model in this study. See **Error! Reference source not found.** for model description. Values shown in italic are prescribed fractionation factors, and those in non-italic are derived values using Eqn. S2 and S3.

Reaction	$^{13}\alpha^{\text{eq}}$	$^{13}\alpha^{+}$	$^{13}\alpha^{-}$	$^{2,\text{P}}\alpha^{\text{eq}}$	$^{2,\text{P}}\alpha^{+}$	$^{2,\text{P}}\alpha^{-}$	$^{2,\text{S}}\alpha^{\text{eq}}$	$^{2,\text{S}}\alpha^{+}$	$^{2,\text{S}}\alpha^{-}$
1	0.9853 <sup>a</sup>	<i>0.9600<sup>b</sup></i>	0.9743	0.9077 <sup>a</sup>	<i>0.7<sup>b</sup></i>	0.7712	n.a.	n.a.	n.a.
2	0.9862 <sup>a</sup>	<i>0.9600<sup>b</sup></i>	0.9734	0.9609 <sup>a</sup>	<i>0.7<sup>b</sup></i>	0.7285	1.0587 <sup>a</sup>	<i>0.84<sup>b</sup></i>	0.7934
3	0.9758 <sup>a</sup>	<i>0.9600<sup>b</sup></i>	0.9838	0.8779 <sup>a</sup>	<i>0.7<sup>b</sup></i>	0.7974	0.9136 <sup>a</sup>	<i>0.84<sup>b</sup></i>	0.9194
4	1.0005 <sup>a</sup>	<i>0.9600<sup>b</sup></i>	0.9595	0.8494 <sup>a</sup>	<i>0.7<sup>b</sup></i>	0.8241	0.9675 <sup>a</sup>	<i>0.84<sup>b</sup></i>	0.8682

n.a., not applicable; <sup>a</sup> Gropp, Iron and Halevy (2020), 82 °C; <sup>b</sup> Scheller et al. (2013).

Table S2. Clumped isotope fractionation factors used as input parameters for the isotopologue flow network model in this study. See **Error! Reference source not found.** for model description. Values shown in italic are prescribed fractionation factors, and those in non-italic are derived values using Eqn. S4 and S5.

Reaction	$P_{\gamma}^{\text{eq}}$	$P_{\gamma}^{+}$	$P_{\gamma}^{-}$	$S_{\gamma}^{\text{eq}}$	$S_{\gamma}^{+}$	$S_{\gamma}^{-}$
1	1.004	<i>0.998</i>	0.994	n.a.	n.a.	n.a.
2	1.004	<i>0.998</i>	0.994	1.000	<i>0.991</i>	0.991
3	1.004	<i>0.998</i>	0.994	1.000	<i>0.991</i>	0.991
4	1.004	<i>0.998</i>	0.994	1.000	<i>0.991</i>	0.991

n.a., not applicable