The Stable Carbon Isotope Fractionation of Methanogenesis Products at Complete Carbon Consumption

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Supplementary Information

The Supplementary Information includes:

- Methods
- Calculations
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- Supplementary Information References

Methods

Media and Culture Conditions

Methanococcus maripaludis S2 cultures were grown anaerobically and hydrogenotrophically in a modified DSMZ141 media (Deutsche Sammlung von Mikroorganismen und Zellkulturen; Braunschweig, Germany) with the following concentrations in mM: MOPS buffer, 50; $NaHCO_3$, 27; NaCl, 308; MgCl x $6H_2O$, 19.7; KH_2PO_4, 1.0287; KCl, 4.561; NH_4Cl, 4.674; $CaCl_2$ X $2H_2O$, 0.952; $Fe(NH_4)_2(SO_4)_2$, 0.005; Resazurin, 0.005; Na_2S x $9H_2O$, 2. In addition, 10 mL of DSMZ141 trace elements solution and 10 mL of DSMZ141 vitamins solution were added per litre of media. All chemicals except $NaHCO_3$, Na_2S x $9H_2O$, and DSMZ141 vitamins solution were added to the media before autoclaving. Pre-autoclave, media was stirred and boiled for 5 minutes, followed with N_2 sparging until cool to promote degassing. Then, 40 mL of media was aliquoted to N_2 sparged 160 mL serum vials containing stir bars, which were then sealed with butyl rubber stoppers and crimped shut. Post-autoclave, serum vials were sparged with H_2 for three minutes and then sterile, anoxic solutions of $NaHCO_3$, Na_2S x $9H_2O$, DSMZ141 vitamins solution were added via syringe and needle. At inoculation, 1 % culture was transferred in from a living culture with the same conditions as the experiment, and serum vial headspace was pressurized to 20 psi with H_2. In total, seven cultures were grown for this experiment, not including two negative controls.

The initial pH of the media was ~ 6.7 and cultures were kept at a temperature of 37 °C in an incubator. Serum vials were placed in Modular Internet Controllable Real-time Optical-density Loggers (microLogger) which

stirred and measured the optical density (OD) of cultures as described in Kopf and Younkin (2025). Cultures were stirred continuously at a speed of 625 rpm. OD measurements were recorded automatically every 10 min using a custom 3D-printed bottle adapter fitted with a narrow beam angle 630 nm LED (Marktech Optoelectronics, MTE7063NK2-UR) as light source and adjustable gain optical sensor (Texas Instruments, OPT101) as detector controlled by an ARM Cortex M3 powered microcontroller (Particle Industries, PHOTONH).

Of the seven cultures grown, four were terminated at various points of the growth curve and three were allowed to reach early stationary phase. Of the terminated cultures, one culture was stopped at early exponential phase, one at early-mid exponential phase, one at mid-late exponential phase, and one at late exponential phase. At termination, cultures were acidified with 0.5 mL 4 M phosphoric acid to stop growth and release all remaining DIC into the headspace. 6 mL of media from the three stationary samples was transferred to sealed, N_2 sparged 60 mL serum vials and acidified with 0.1 mL 4 M phosphoric acid to release any remaining DIC in the media into the headspace. The remaining early stationary cultures were opened and sampled immediately.

Headspace Measurements

Headspace gas from exponential cultures was sampled directly from acidified cultures and analysed immediately. Headspace gas from early stationary samples was extracted from culture vials and then transferred and stored in butyl stoppered and crimped 30 mL serum vials filled with 30 % NaCl saline solution until analysis. One negative control was acidified with 0.5 mL 4 M phosphoric to release all DIC into the headspace and analysed for initial DIC concentration measurements.

Headspace gas was run on a gas chromatograph with flame ionization and thermal conductivity detectors (SRI GC-FID/TCD 8610C Multi-Gas #5 Configuration) with argon as a carrier gas (12 psi) for quantification of CO_2, CH_4, and H_2. Pure methane gas (Airgas) and a 1 % standard gas mix of CO, CO_2, CH_4, and H_2 (Scott) were used as quantification standards. Standards were injected in multiple volumes to create calibration curves for CO_2, CH_4, and H_2. Either 25 µL or 100 µL of sample headspace was injected into the GC-FID/TCD using a N_2 sparged gas-tight syringe and needle. Samples were equilibrated with atmospheric pressure before injection. Standards and samples were run for 4 minutes at 15 °C. Each standard and sample was measured at least 3x and the final quantification value was an average of the replicate measurements.

Headspace gas was run on a Picarro Ring-Down Spectrometer G2201-I for $\delta^{13}C$ of CH_4 and CO_2. Samples were diluted with N_2 to be within the CO_2 range of 380-2000 ppm and the CH_4 range of 10-1000 ppm. Samples were diluted within a 500 mL gas-tight syringe, which was attached to the sample port so that sample could be gradually pulled into the Picarro. IAEA-C2 (- 8.24 ‰), USGS44 (- 42.21 ‰), and IAEA-603 (2.46 ‰) were used as $\delta^{13}C$ CO_2 calibration standards and three pure CH_4 gasses (- 23.6 ‰, - 38.3 ‰, and - 68.6 ‰) were used as $\delta^{13}C$ CH_4 calibration standards. Isotopic measurements of CO_2 and CH_4 on the Picarro Cavity Ringdown Spectrometer G2201-i have been factory calibrated in the presence of ambient air or ultra zero grade air, both of which have approximately 80 % N_2 and 20 % O_2. Changes in the proportion or composition of the gas matrix can rapidly change the absorbance across the spectrum. At the high sample dilution used in these measurements, the impact of H_2 and the lack of O_2 were not found to significantly impact the isotopic measurements. Each $\delta^{13}C$ value represents one measurement on the Picarro.

The hydrogen isotopic compositions (δ^2H, expressed in the VSMOW framework) of CH_4 was analysed at the Centre for Isotope Geochemistry at the Lawrence Berkeley National Laboratory using a gas-chromatograph isotope ratio mass spectrometer (GC-IRMS) system (Thermo Scientific GC TraceGas Ultra system connected

to a Thermo Scientific Delta V Plus). The methodology employed here follows that described in Turner *et al.* (2021). Briefly, headspace gas was sampled using a gas-tight syringe and injected into a 25 µL stainless-steel loop attached to a 6-port valve (VICI-Valco). The CH_4 was separated chromatographically on an HP-mole sieve fused silica capillary column (30 m ± 0.32 mm) using helium as carrier gas. Following chromatographic separation, CH_4 was passed through a ceramic tube at 1420 °C, pyrolyzed, and converted to H_2 for hydrogen isotopic measurements. Ceramic tubes were pre-conditioned by injecting 250 µL of pure CH_4 three times the day before the measurement session. Measured δ^2H values were corrected relative to external natural gas standards acquired from the United States Geological Service HCG-1 (δ^2H-CH_4 of – 64.0 ‰), HCG-2 (δ^2H-CH_4 of – 183.2 ‰), and HCG-3 (δ^2H-CH_4 of – 224.3 ‰) (Dias *et al.*, 2022).

Biomass Harvesting and Lipid Extraction

Biomass was harvested from early stationary samples through centrifugation. ~ 40 mL media was transferred to a 50 mL falcon tube and spun down at 5000 RPM for 10 minutes at 20 C, 9 ACC, and 8 DEC. After the first round of centrifugation, most of the media was discarded except 1000 µL which was used to resuspend cells. Resuspended cells were then transferred to 2 mL microcentrifuge tubes, and subsequently spun down 13,300 RPM for 10 minutes at room temperature. After the second round of centrifugation, the supernatant was discarded, and biomass pellets were frozen overnight at – 71 °C. The next day, pellets were freeze-dried for ~ 24 hours. Biomass pellets were stored at – 71 °C after being freeze-dried.

Lipids were extracted from freeze-dried biomass pellets through acidic hydrolysis-methanolysis. 1 µg of squalane was added as an internal quantification standard to biomass pellets. Pellets were physically destroyed with furnaced glass disruptor beads in a 1:1 V:V ratio with methanol for 10 minutes at 3000 RPM using a Disruptor Genie (Scientific 126 Industries, SI-DD38). Excess methanol was evaporated off before beginning acid hydrolysis. 3 M HCl with 33 % H_2O content was added to samples, which were then heated for 90 minutes at 65 °C. Methyl tert-butyl ether (MTBE) and hexane was then added to samples, followed by 5 minutes of sonification (Qsonica Q500, Newton, CT, USA). Samples were then centrifuged for 5 minutes at 15000 g at room temperature. The organic phase (hexane/MTBE) was pipetted into combusted 2 mL glass vials using a glass pipette and bulb. Hexane was added to samples two more times with centrifugation and pipetting of the organic phase. The organic phase contained total lipid extracts (TLE), which were then dried down under N_2 for ether cleavage.

For ether cleavage, hydroiodic acid was added to the dried TLE extract and heated at 120 °C for 4 hours. After heating, nanopure water and hexane were added to samples. Samples were vortexed for 1 minute and then the organic phase (hexane) was pipetted into a new set of 2 mL glass vials. The hexane addition, vortexing, and pipetting were done an additional two times. The samples were then dried down under N_2. A magnetic stir bar, PtO2, and hexane were added to each vial. Vials were bubbled with H_2 for 90 minutes. Samples were then filtered through ashed glass Pasteur pipette filter columns with glass wool and collected in 2 mL glass vials. 5 ug of quantification standard palmitic acid isobutyl ester (PAIBE) was then added to samples. Samples, which now contained the target analyte phytane, were dried under N_2 and stored at – 20 °C until isotope analysis.

Biomass and Lipid Analyses

The $\delta^{13}C$ of bulk biomass was measured on a Thermo Delta V continuous-flow stable isotope ratio mass spectrometer attached to a Thermo Flash2000 Elemental Analyzer. Oven-dried samples (~ 50 µg) and a suite of isotope standards, pugel (- 13.74 ‰), L_glut (- 26.51 ‰), EDTA2 (- 39.59 ‰), and act1 (- 29.49 ‰), were

weighed into tin capsules before being put onto the instrument. Stable isotope ratios are reported in delta (δ) notation relative to the Vienna Pee Dee Belemnite (VPDB) standard for carbon, where $\delta = ([(R_{Sample}/R_{Standard}) - 1])$, R is the ratio of the heavier mass isotope to the lighter mass isotope, as per mil (‰). In-house R scripts using R Statistical Software (v4.2.0; R Core Team 2022) and the RStudio 2022.07.0 interface (RStudio Team, 2022) with Tidyverse and IsoVerse packages (Kopf *et al.*, 2021; Wickham *et al.*, 2023) were used for data correction. Sample $\delta^{13}C$ values were corrected for either blank, offset, drift, linearity, and/or linearity+drift with a final scale correction.

Lipids were quantified on a GC-flame ionization detector (GC-FID; Thermo TRACE 1310 and identified on a GC-MS (Thermo ISQ LT with TRACE 1310). Compounds were identified on the GC-MS by comparing samples to an n-alkane mixture containing pristine and phytane. Phytane was quantified with peak size comparison between samples and quantification standards squalane and PAIBE.

The $\delta^{13}C$ of phytane was measured on a GC-pyrolysis-isotope ratio MS on a GC IsoLink 184 II IRMS System (Thermo Scientific). The GC-P-IRMS system contained a Trace 1310 GC fitted with a programmable temperature vaporization (PTV) injector, a 30 m ZB5HT column (i.d. = 0.25 mm, 0.25 µm, Phenomenex, Torrance, CA, USA), ConFlo IV interface, and MAT 253 Plus mass spectrometer (Thermo Scientific). The lipid $\delta^{13}C$ calibration was done in R, using the packages isoreader (Kopf *et al.*, 2021) and isoprocessor available at github.com/isoverse. The biomass $\delta^{13}C$ data correction and calculations were performed using in-house R scripts using R Statistical Software (v4.2.0; R Core Team 2022) and the RStudio 2022.07.0 interface (RStudio Team, 2022) with Tidyverse and IsoVerse packages (Kopf *et al.*, 2021, Wickham *et al.*, 2023).

Calculations

All calculations and data are available at https://github.com/KopfLab/2025_batther_et_al_C_limitation

Simple Rayleigh Distillation

Catabolism only

$$
\begin{aligned}
\text{Rxn: } CO_2 &\rightarrow CH_4 \\
\alpha &= \alpha_{CO2\rightarrow CH4} \\
\frac{R_{CO2}}{R_{CO2_0}} &= f^{\alpha_{CO2\rightarrow CH4}-1} \\
\text{MB: } R_{CO2_0} &= f \cdot R_{CO2} + (1-f) \cdot R_{CH4} \\
\rightarrow \frac{R_{CH4}}{R_{CO2_0}} &= \frac{1 - f \cdot R_{CO2}/R_{CO2_0}}{1-f} = \frac{1 - f^{\alpha_{CO2\rightarrow CH4}}}{1-f}
\end{aligned}
$$

Catabolism + anabolism

$$
\begin{aligned}
\text{Rxn: } CO_2 &\rightarrow (1-Y) \cdot CH_4 + Y \cdot B \\
\alpha &= (1-Y) \cdot \alpha_{CO2\rightarrow CH4} + Y \cdot \alpha_{CO2\rightarrow B} \\
&= (1-Y) \cdot \alpha_{CO2\rightarrow CH4} + Y \cdot \alpha_{B/CH4} \cdot \alpha_{CO2\rightarrow CH4} \\
&= \alpha_{CO2\rightarrow CH4} \cdot \left(1 + Y \cdot \left(\alpha_{B/CH4} - 1\right)\right) \\
&= \alpha_{CO2\rightarrow CH4} \cdot k_1 \quad | \text{ with } k_1 = 1 + Y \cdot \left(\alpha_{B/CH4} - 1\right) \\
\frac{R_{CO2}}{R_{CO2_0}} &= f^{\alpha_{CO2\rightarrow CH4} \cdot k_1 - 1} \\
R_B &= \alpha_{B/CH4} \cdot R_{CH4} \\
\text{MB: } R_{CO2_0} &= f \cdot R_{CO2} + (1-f) \cdot (1-Y) \cdot R_{CH4} + (1-f) \cdot Y \cdot R_B \\
\rightarrow \frac{R_{CH4}}{R_{CO2_0}} &= \frac{1 - f \cdot R_{CO2}/R_{CO2_0}}{(1-f) \cdot \left(1 + Y \cdot \left(\alpha_{B/CH4} - 1\right)\right)} \\
&= \frac{1 - f \cdot R_{CO2}/R_{CO2_0}}{(1-f) \cdot k_1} \\
&= \frac{1 - f^{\alpha_{CO2\rightarrow CH4} \cdot k_1}}{(1-f) \cdot k_1}
\end{aligned}
$$

Distillation with change in fractionation factors

Same as the one previous model (with anabolism and catabolism) except for the fractionation factor change at some amount of CO2 remaining f_{s1}:

$$\begin{aligned}
\text{as before: } k_1 &= 1 + Y \cdot \left(\alpha_{B/CH4} - 1\right) \\
R_B &= \alpha_{B/CH4} \cdot R_{CH4} \\
\text{new: } \frac{R_{CO2}}{R_{CO2_0}} &= \begin{cases} f^{\alpha_1 \cdot k_1 - 1} \text{ for } f \in [1, f_{a_1}] \\ f_{s1}^{\alpha_1 \cdot k_1 - 1} \cdot \left(\frac{f}{f_{s1}}\right)^{\alpha_2 \cdot k_1 - 1} \text{ for } f \in]f_{a_1}, 0] \end{cases} \\
\text{MB: } R_{CO2_0} &= f \cdot R_{CO2} + (1-f) \cdot (1-Y) \cdot R_{CH4} + (1-f) \cdot Y \cdot R_B \\
\rightarrow \frac{R_{CH4}}{R_{CO2_0}} &= \frac{1 - f \cdot R_{CO2}/R_{CO2_0}}{(1-f) \cdot k_1}
\end{aligned}$$

Adding partial anabolism

$$\begin{aligned}
\text{Step 1: } f &\in [1, f_{s1}] \\
CO_2 &\rightarrow (1 - Y_{s1}) \cdot CH_4 + Y_{s1} \cdot B \\
Y_{s1} &= \frac{Y}{1 - f_{s1}} \\
\alpha_{s1} &= (1 - Y_{s1}) \cdot \alpha_{CO2 \rightarrow CH4} + Y_{s1} \cdot \alpha_{B/CH4} \cdot \alpha_{CO2 \rightarrow CH4} \\
&= \alpha_{CO2 \rightarrow CH4} \cdot k_2 \quad | \text{ with } k_2 = 1 + Y \cdot \frac{\alpha_{B/CH4} - 1}{1 - f_{s1}} \\
\text{MB: } R_{CO2_0} &= f \cdot R_{CO2} + (1-f) \cdot (1 - Y_{s1}) \cdot R_{CH4} + (1-f) \cdot Y_{s1} \cdot R_B \\
&= f \cdot R_{CO2} + \frac{1-f}{1-f_{s1}} \cdot (1 - f_{s1} - Y) \cdot R_{CH4} + \frac{1-f}{1-f_{s1}} \cdot Y \cdot R_B \\
\rightarrow \frac{R_{CH4}}{R_{CO2_0}} &= \frac{1 - f \cdot R_{CO2}/R_{CO2_0}}{(1-f) \cdot k_2} \\
\text{still: } R_B &= \alpha_{B/CH4} \cdot R_{CH4}
\end{aligned}$$

$$\begin{aligned}
\text{Step 2: } f &\in]f_{s1}, 0] \\
CO_2 &\rightarrow CH_4 \\
\alpha_{s2} &= \alpha_{CO2 \rightarrow CH4} \\
\text{MB: } R_{CO2_0} &= f \cdot R_{CO2} + \left(f_{s1} - f + (1 - f_{s1}) \cdot (1 - Y_{s1})\right) \cdot R_{CH4} + (1 - f_{s1}) \cdot Y_{s1} \cdot R_B \\
&= f \cdot R_{CO2} + (1 - f - Y) \cdot R_{CH4} + Y \cdot R_B \\
&= f \cdot R_{CO2} + (1 - f - Y) \cdot R_{CH4} + Y \cdot \alpha_{B/CH4} \cdot R_{CH4_{s1}} \\
\rightarrow \frac{R_{CH4}}{R_{CO2_0}} &= \frac{1 - f \cdot R_{CO2}/R_{CO2_0} - Y \cdot \alpha_{B/CH4} \cdot R_{CH4_{s1}}/R_{CO2_0}}{1 - f - Y} \\
\text{new: } R_B &= \alpha_{B/CH4} \cdot R_{CH4_{s1}}
\end{aligned}$$

The overall B/CH4 fractionation factor and f_{s1} are dependent on each other by the following equation whose solution can be found numerically by root finding:

$$\frac{R_{B_{final}}}{R_{CO2_0}} = \alpha_{B/CH4} \cdot \frac{R_{CH4_{s1}}}{R_{CO2_0}} = \alpha_{B/CH4} \cdot \frac{1 - f_{s1} \cdot R_{CO2_{s1}}/R_{CO2_0}}{(1 - f_{s1}) \cdot k_2}$$

Supplementary Figures

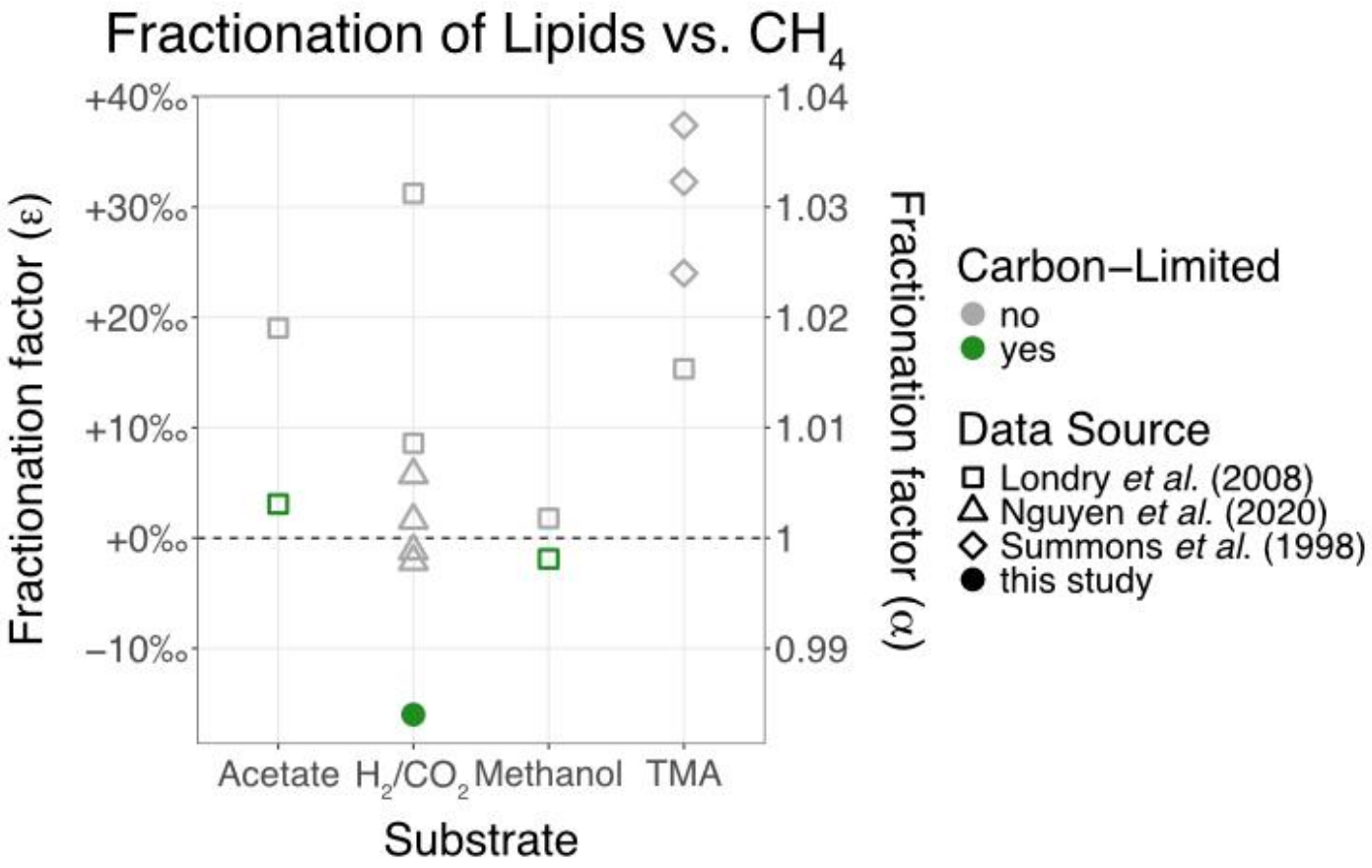

Figure S-1 Fractionation between methane and lipids from lab grown methanogen cultures from this experiment and literature data. TMA: trimethylamine.

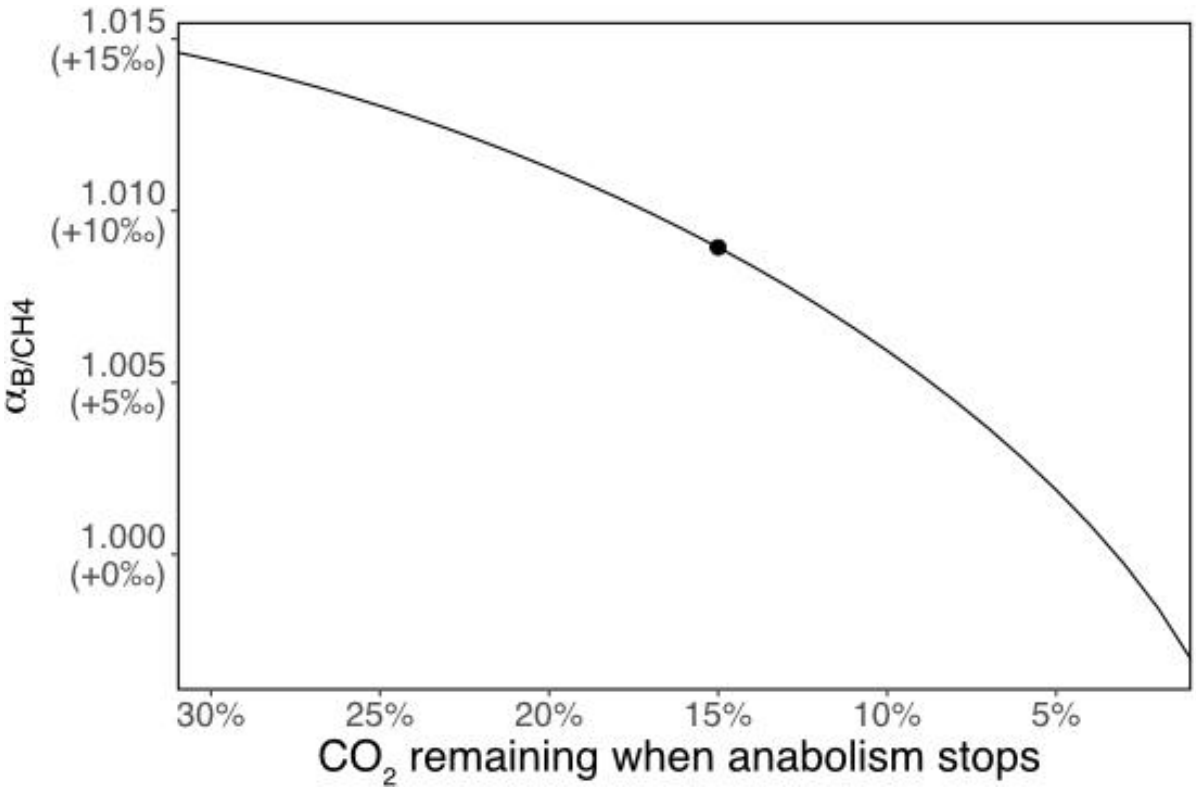

Figure S-2 Fractionation factors between biomass and methane (α) at various points of anabolism inhibition plotted against CO_2 remaining (%).

Supplementary Information References

Dias, R.F., Coleman, D.D., Ellis, G.S. (2022) Stable Isotopic Data (Delta13C and Delta2H) for Reference Materials HCG-1, HCG-2, HCG-3. *USGS Asset Identifier Service (AIS)* https://doi.org/10.5066/P9RBXUMD

Kopf, S., Younkin, A. (2025) µLogger: a Modular Internet Controllable Real-time Optical-density Logger. https://github.com/KopfLab/micrologger_device.

Kopf, S., Davidheiser-Kroll, B., Kocken, I. (2021) Isoreader: An R package to read stable isotope data files for reproducible research. *JOSS* 6, 2878. https://doi.org/10.21105/joss.02878

Londry, K.L., Dawson, K.G., Grover, H.D., Summons, R.E., Bradley, A.S. (2008) Stable Carbon Isotope Fractionation between Substrates and Products of Methanosarcina Barkeri. *Organic Geochemistry* 39, 608–621. https://doi.org/10.1016/j.orggeochem.2008.03.002

Nguyen, T.B., Topçuoğlu, B.D., Holden, J.F., LaRowe, D.E., Lang, S.Q. (2020) Lower hydrogen flux leads to larger carbon isotopic fractionation of methane and biomarkers during hydrogenotrophic methanogenesis. *Geochimica et Cosmochimica Acta* 271, 212–226. https://doi.org/10.1016/j.gca.2019.11.015

R Core Team (2022) R: A Language and Environment for Statistical Computing. *R Foundation for Statistical Computing, Vienna*. https://www.R-project.org

RStudio Team (2022) RStudio: Integrated Development for R. RStudio. *PBC*. http://www.rstudio.com/

Summons, R.E., Franzmann, P.D., Nichols, P.D. (1998) Carbon Isotopic Fractionation Associated with Methylotrophic Methanogenesis. *Organic Geochemistry* 28, 465–475. https://doi.org/10.1016/S0146-6380(98)00011-4

Turner, A.C., Korol, R., Eldridge, D.L., Bill, M., Conrad, M.E., Miller III, T.F., Stolper, D.A. (2021) Experimental and theoretical determinations of hydrogen isotopic equilibrium in the system CH_4-H_2-H_2O from 3 to 200 °C. *Geochimica et Cosmochimica Acta* 314, 223–269. https://doi.org/10.1016/j.gca.2021.04.026

Wickham, H. (2023) *Tidyverse: Easily Install and Load the Tidyverse*. https://CRAN.Rproject.org/package=tidyverse