Clumped isotopes link older carbon substrates with slower rates of methanogenesis in northern lakes

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Supplementary Materials

Supplementary Text and Figures:

ST1- Details on culture experiment sterile media:

The sterile media used in the pure and enrichment culture experiments contained (g/L):

NaCl (23.4), $MgSO_4$•7 H_2O (9.44), $NaHCO_3$ (5.0), KCl (0.8), NH_4Cl (1.0), Na_2HPO_4 (0.6), $CaCl_2$•2 H_2O (0.14), cysteine-HCl (0.25), with the addition of 10 mL DSM 141 Trace Element solution, 10 mL of DSM 141 Vitamin solution, and 2.5 mL of a 50 mM H_2S^- solution.

ST2- Details on $\Delta^{13}CH_3D$ and $\Delta^{12}CH_2D_2$ Measurements

CH_4 samples from the Batch 3 culture experiments were measured for δD, $\delta^{13}C$, $\Delta^{13}CH_3D$, and for three samples additionally $\Delta^{12}CH_2D_2$. For these measurements the relevant equations are:

$$\Delta^{13}CH_3D = ({}^{13CH3D}R/{}^{13CH3D}R^* - 1) \quad (6)$$

$$\Delta^{12}CH_2D_2 = ({}^{12CH2D2}R/{}^{12CH2D2}R^* - 1) \quad (7)$$

Where the measured and expected random isotope ratios are defined as:

$${}^{13CH3D}R = ([{}^{13}CH_3D] / [{}^{12}CH_4] \quad (8)$$

$${}^{12CH2D2}R = ([{}^{12}CH_2D_2] / [{}^{12}CH_4] \quad (9)$$

$${}^{13CH3D}R^* = \left(4 \times {}^{2}R \times {}^{13}R\right) \quad (10)$$

and

$${}^{12CH2D2}R^* = \left(6 \times \left[{}^{2}R\right]^2\right) \quad (11)$$

A complete description of the analytical methods used for this measurement is found in *Eldridge et al.* [2019]. Samples were measured against a working reference gas with calibrated $\Delta^{13}CH_3D$ and $\Delta^{12}CH_2D_2$ values of 2.59 ± 0.14 ‰ and 5.86 ± 0.60 ‰, respectively *Eldridge et al.* [2019]. External reproducibility of δD, $\delta^{13}C$, $\Delta^{13}CH_3D$, and $\Delta^{12}CH_2D_2$ measurements, as determined from repeated measurements of laboratory standards, was 0.17, 0.02, 0.38 and 1.52 ‰ (1σ standard deviations; n=16), respectively.

ST3- Rationale for assumptions in model of kinetic isotope effects and comparison to data

We make three assumptions to model the relationship between CH_4 production rate and Δ_{18} or $\Delta^{13}CH_3D$ values, and to compare these model results to empirical data from culture experiments. First, we assume a constant rate of reverse methanogenesis (r_{rev}). This implies that reverse methanogenesis is a zero-order reaction. There are few constraints on the rate of reverse methanogenesis and its variability. However, anaerobic oxidation of methane (AOM) is thought to proceed using a reverse methanogenesis pathway [*Timmers et al.*, 2017], and previous studies have inferred zero-order kinetics for

AOM at high CH_4 concentrations [*Vavilin*, 2013; *Vavilin and Rytov*, 2013], which would be applicable to our culture experiments.

Second, to compare empirical results from culture experiments and model predictions, we express r_{rev} and r_{net} normalized to culture media volume. This comparison requires an assumption of uniform cell density between the culture experiments. This assumption is an oversimplification and could be responsible for some of the scatter in our data-model comparison (See ST-5). Accounting for cell density would be valuable in future studies relating Δ_{18} and CH_4 production rates.

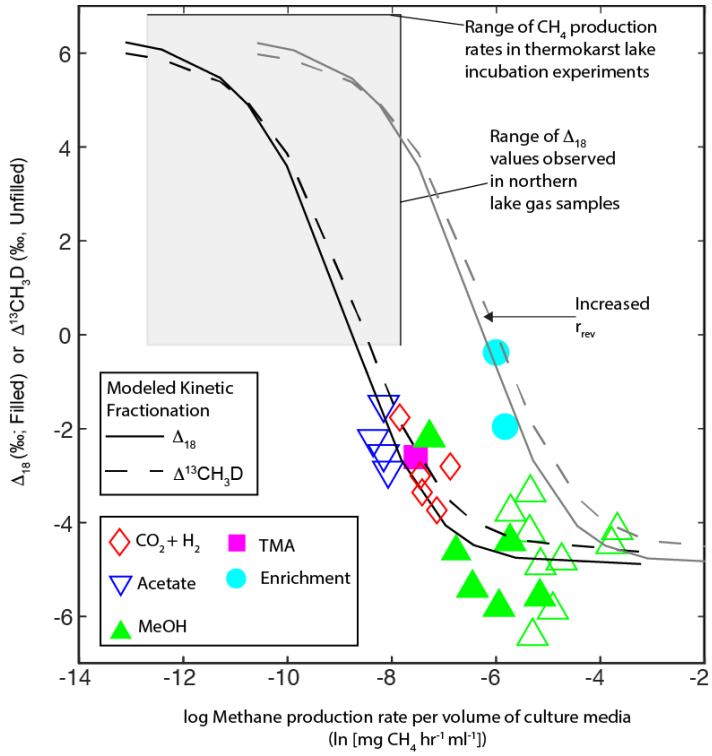
Third, the model was developed for hydrogenotrophic methanogenesis [*Stolper et al.*, 2015], but we are comparing it to CH_4 produced using four different carbon substrates (Figure 1). However, the model specifically describes kinetic isotope effects associated with the addition of a hydrogen atom to methyl-coenzyme-M. This step is common to all of the CH_4 production pathways studied here, and therefore kinetic isotope effects for clumped isotopologues can be reasonably assumed to be similar for these different methanogenesis pathways, as discussed by *Gruen et al.* [2018].

To compare the range of CH_4 production rates from thermokarst lake incubation experiments [*Heslop et al.*, 2015] with the modeled relationship between Δ_{18} and r_{net}, we took the full range of reported CH_4 production potentials (0.02 to 8.08 μg C-CH_4 g dry sediment^{-1} day^{-1}) and converted this to mg CH_4 ml pore water^{-1} hour^{-1} using mean values of the gravimetric water content for the two sample types (organic rich mud and thawed permafrost) with the highest and lowest CH_4 production rates.

ST-4 Details on enrichment culture experiment results

The enrichment culture experiments are clearly differentiated from the pure culture experiments in Figure 1. With this limited dataset we cannot clearly identify the cause of this difference. One possibility is a higher rate of the reverse reactions of methanogenesis. If r_{rev} is increased by a factor of 10 (to $5x10^{-3}$ mg CH_4 hr^{-1} ml^{-1}) the kinetic isotope effect model provides a relatively good fit to these data (Supplementary Figure 1). This is plausible given that with the diverse consortium of microbes potentially present in these experiments there would have been the possibility of sulfate reduction or other bacterial metabolisms acting as an electron acceptor for AOM, which could have increased the rates of reverse methanogenesis and increased Δ_{18} values [*Ash et al.*, 2019]. We note that sulfate was present in the culture media, and there was anecdotal evidence (i.e. odor) of the presence of hydrogen sulfide during gas sampling of these cultures.

Alternately, if the methanogen cell density in these experiments was substantially higher than in the pure culture experiments, it is possible that the relatively high net CH_4 production rate is not an accurate reflection of the cell-specific CH_4 production rate relative to the pure cultures. However, it is unlikely that the enrichment culture methanogen community would have reached the large turbid densities of the pure culture experiments.



Supplementary Figure 1: Plot of log CH_4 production rate vs. Δ_{18} and $\Delta^{13}CH_3D$ in a subset of pure and enrichment culture experiments. Solid and dashed lines indicate predicted values based on a model of methanogenesis kinetic isotope effects[*Stolper et al.*, 2015], assuming a constant value of r_{rev} of $4x10^{-4}$ mg CH_4 hr^{-1} ml^{-1}. Gray solid and dashed lines indicate predicted values with an r_{rev} value of $5x10^{-3}$ mg CH_4 hr^{-1} ml^{-1}. Gray box as in Figure 1. Analytical errors for Δ_{18} or $\Delta^{13}CH_3D$ measurements (1σ) are smaller than the symbols. MeOH, methanol; TMA, Trimethylamine.

ST-5 Clumped isotope variability in pure culture experiment results and model sensitivity to fractionation factors

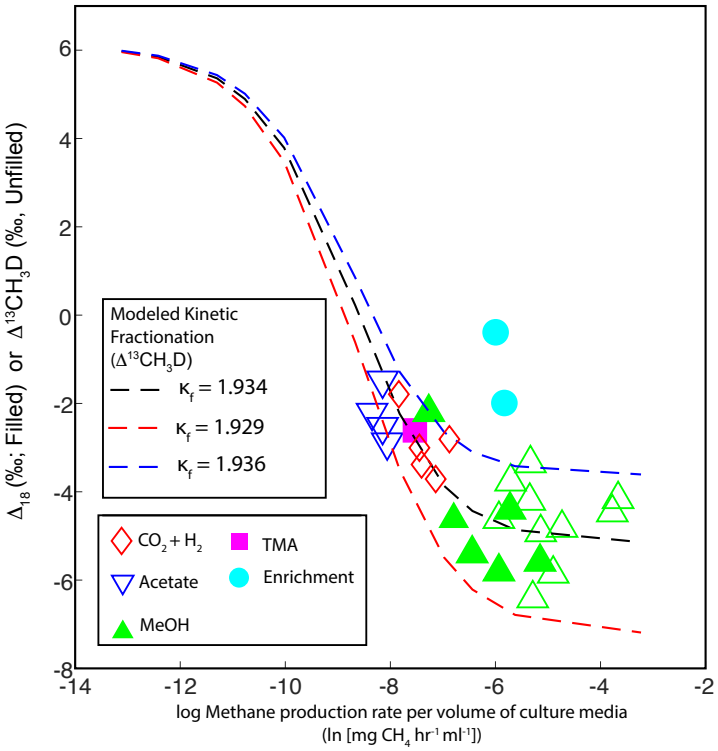
The model of kinetic isotope effects predicts the general trend of the pure culture results shown in Figure 1, but there is clearly variability in these results that is not accounted for by the model. It is beyond the scope of this study to thoroughly assess the causes of this variability. However, we observe that varying the value of $\kappa_{f-}{}^{13}CH_3D$ (as defined in the Methods) from 1.929 to 1.936 generates model curves that encompass the variability in the pure culture data (Supplementary Figure 2). It is important to note that differences in

120 $\kappa_{f\text{-}}{}^{13}CH_3D$ would have a much smaller effect at the lower rates of methanogenesis that are
121 generally found in natural environments.

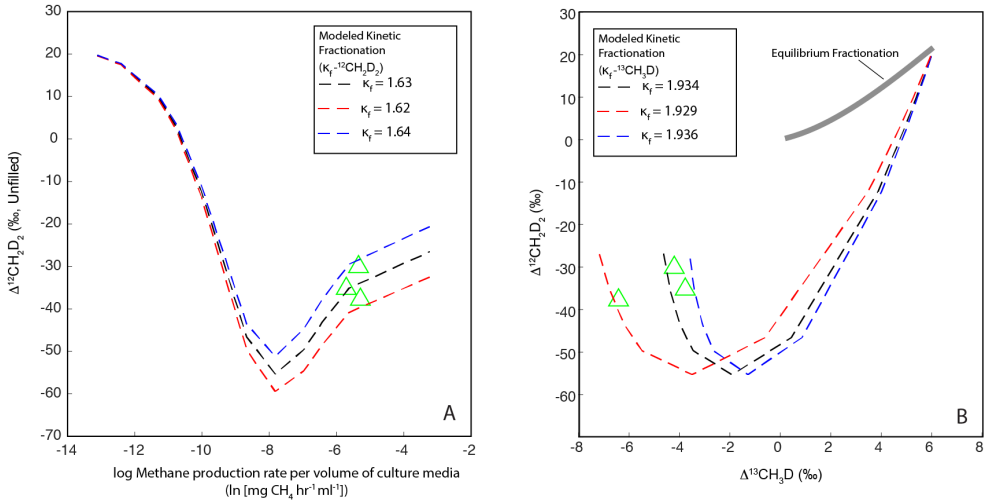
122 The largest differences from the model predicted values occur in the Batch 3
123 experiments (Table 2). These experiments were all conducted using methanol as a
124 substrate, suggesting that differences between methanogenesis pathways are not
125 producing the observed variability. Additionally, these experiments were conducted in
126 smaller batch experiments with less overall substrate provided, and the observed
127 variability may be partly caused by closed-system Rayleigh fractionation effects that
128 would potentially be more pronounced in smaller batch experiments. We observe
129 substantial enrichment of the $\delta^{13}C$ and δD of CH_4 in our experiments (Supplementary
130 Table 2) that would be consistent with substrate depletion during closed-system Rayleigh
131 fractionation [*Whiticar*, 1999; *Hayes*, 2001].

132 Three measurements of the Batch 3 samples included both $\Delta^{13}CH_3D$ and
133 $\Delta^{12}CH_2D_2$ values, and therefore afford an opportunity to examine whether the model
134 predictions are consistent with measurements of two distinct clumped isotopologues.
135 These measurements are consistent with the modeled relationship between r_{net} and
136 $\Delta^{12}CH_2D_2$ across a relatively narrow range of $\kappa_{f\text{-}}{}^{12}CH_2D_2$ values (1.62 to 1.64)
137 (Supplementary Figure 3A). Likewise, the measurements are consistent with the model
138 predicted relationship between $\Delta^{13}CH_3D$ and $\Delta^{12}CH_2D_2$, applying the same range of $\kappa_{f\text{-}}$
139 $^{13}CH_3D$ as applied in Supplementary Figure 2 (Supplementary Figure 3B). Currently
140 there are few available measurements of $\Delta^{12}CH_2D_2$ in the literature, but as this
141 measurement becomes more widespread it will provide additional constraints that can be

used to test hypothesized relationships between clumped isotope values and rates of methanogenesis.



Supplementary Figure 2: Plot of log CH_4 production rate vs. Δ_{18} and $\Delta^{13}CH_3D$ in a subset of pure and enrichment culture experiments. Dashed lines indicate predicted values based on a model of methanogenesis kinetic isotope effects [*Stolper et al.*, 2015], with varying values of κ_f-$^{13}CH_3D$ (see Methods), assuming a constant value of r_{rev} of $4x10^{-4}$ mg CH_4 hr^{-1} ml^{-1}. Analytical errors for Δ_{18} or $\Delta^{13}CH_3D$ measurements (1σ) are smaller than the symbols. MeOH, methanol; TMA, Trimethylamine.



Supplementary Figure 3: $\Delta^{12}CH_2D_2$ results from the Batch 3 pure culture experiments plotted against (A) the natural log of CH_4 production rate and (B) $\Delta^{13}CH_3D$. Dashed lines indicate predicted values based on a model of methanogenesis kinetic isotope effects [*Stolper et al.*, 2015], with varying values of $\kappa_{f\text{-}}{}^{12}CH_2D_2$ (A) and $\kappa_{f\text{-}}{}^{13}CH_3D$ (B), assuming a constant value of r_{rev} of $4x10^{-4}$ mg CH_4 hr^{-1} ml^{-1}. Analytical errors for $\Delta^{12}CH_2D_2$ and $\Delta^{13}CH_3D$ measurements (1σ) are smaller than the symbols. Gray line in (B) indicates the predicted values for equilibrium fractionation [Young et al., 2017].

ST-6: *Comparison of CH_4 concentration and stable carbon and hydrogen isotopic composition with Fm values*

In both lake types we do not observe a significant correlation ($p < 0.05$) between CH_4 concentration or $\delta^{13}C$ values and CH_4 Fm values (Supplementary Figure 4). For the Alaskan dataset there are also measurements of CO_2 $\delta^{13}C$, and we do not observe a significant correlation between $^{13}\alpha_{CO2\text{-}CH4}$ and Fm values. We do observe a significant negative correlation between CH_4 δD and Fm in the Stordalen dataset, although this correlation is weaker than that between Δ_{18} and Fm. We do not observe a similar correlation in the Alaskan dataset.

In general, a greater proportion of CH_4 derived from acetoclastic methanogenesis is associated with higher $\delta^{13}C$ values and lower δD values [*Whiticar*,

173 1999]. In the Alaskan dataset we do not observe a clear correlation between Fm and these
174 variables. In the Stordalen dataset we observe correlations that are consistent with higher
175 Fm with more acetoclastic methanogenesis. However, culture experiments do not show a
176 significant difference between these pathways for Δ_{18} if CH_4 is produced at the same rate
177 (Figure 1). Furthermore, neither δD nor $\delta^{13}C$ is as strong a predictor of Fm as is Δ_{18}.
178 Given the inconsistent and relatively weak correlations between δD or $\delta^{13}C$ and Fm, we
179 conclude that rates of methanogenesis, as opposed to methanogenic pathway, is a more
180 important variable for understanding variability in Fm in these lakes.

181 Some studies have inferred that variable degrees of anaerobic or aerobic oxidation
182 can influence Δ_{18} values [*Wang et al.*, 2016; *Young et al.*, 2017; *Giunta et al*., 2019],
183 although the direction of this effect is different for aerobic or anaerobic oxidation, with
184 predicted lower values for aerobic oxidation and higher values for anaerobic oxidation.
185 Greater CH_4 oxidation will generally lead to higher δD and $\delta^{13}C$ values in CH_4, and
186 smaller values of $^{13}\alpha_{CO2\text{-}CH4}$ [*Whiticar*, 1999]. We do not observe a pattern that is
187 consistent with oxidation being linked to Fm in either lake type. As discussed above, at
188 Stordalen higher Fm is correlated with higher $\delta^{13}C$ but with lower δD values, which is
189 not consistent with an oxidation effect. In Alaska there is no apparent correlation between
190 Fm and $\delta^{13}C$ or $^{13}\alpha_{CO2\text{-}CH4}$.

191 Substrate depletion in a closed system is expected to lead to enrichment of $\delta^{13}C$ in
192 CH_4 [*Whiticar* et al., 1999]. Currently the effects of substrate depletion on Δ_{18} have not
193 been explicitly studied. We suggest that the absence of a significant correlation between
194 CH_4 $\delta^{13}C$ and Fm in either lake system means that this process is not strongly influencing
195 the correlation between Δ_{18} and Fm. As discussed above (ST-5), closed-system substrate

196 depletion may have influenced our pure culture results, but any substrate depletion effects
197 appear to be secondary to the effect of net CH_4 production rate (Supplementary Figure 2).
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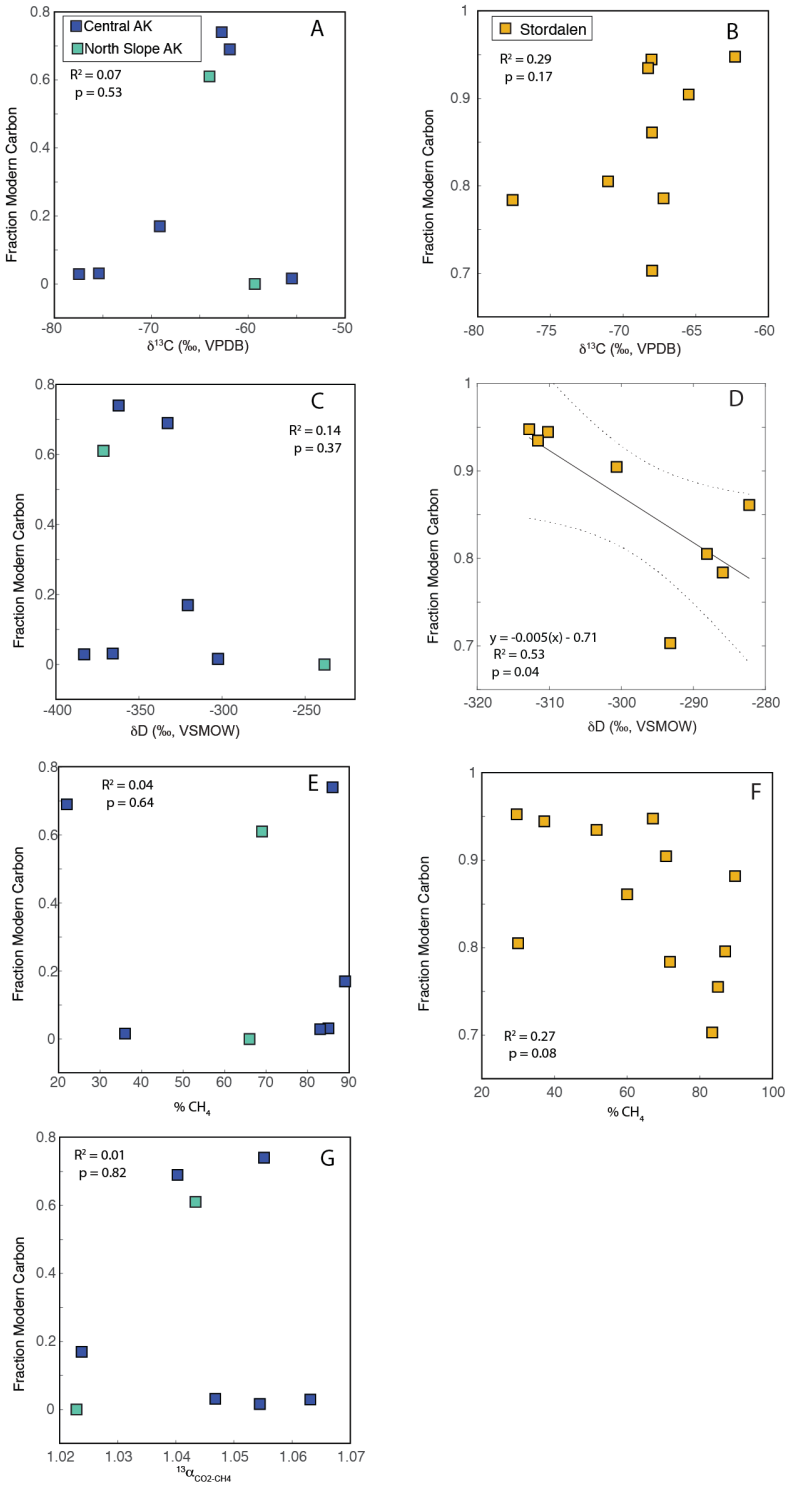

Supplementary Figure 4: Scatter plots of CH_4 ^{14}C age vs. different isotopic or chemical parameters for the Alaskan and Stordalen datasets. (A) and (B): $\delta^{13}C$ of CH_4; (C) and (D) δD of CH_4; (E) and (F) CH_4 concentration (% by volume); (G) $^{13}\alpha_{CO2\text{-}CH4}$ (Alaskan dataset only). R^2 and p values are shown for each correlation. In (D) the solid line shows the best-fit regression curve, and the dotted lines show its 95% confidence interval.