1 Low apparent $\Delta^{12}\text{CH}_2\text{D}_2$ in microbialgenic methane
2 result from combinatorial isotope effects

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15 **Abstract**

Methane generated by microorganisms is most often depleted in the doubly substituted isotopologue $^{12}\text{CH}_2\text{D}_2$ relative to the stochastic reference distribution. To constrain the controls on depleted $\Delta^{12}\text{CH}_2\text{D}_2$ values, we experimentally isolated the root cause with microorganisms that produce methane from methylphosphonate via the C-P lyase pathway. This mechanism of methane production preserves the three hydrogens from methylphosphonate and adds one hydrogen from water. When maintaining the same methylphosphonate source, but varying the D/H composition of growth medium water, we observed significant shifts in methane $\Delta^{12}\text{CH}_2\text{D}_2$ values, but little to no change in $\Delta^{13}\text{CH}_3\text{D}$ values. We reproduced these observations with a model that considers only the combinatorial isotope effect. The variation in $\Delta^{12}\text{CH}_2\text{D}_2$ values of product methane resulted from the differences in D/H between reactants water and methylphosphonate. This work validates the hypothesis that combinatorial effects can strongly influence methane $\Delta^{12}\text{CH}_2\text{D}_2$ values, and must be considered for low temperature, abiotic or biotic systems where methane hydrogen is derived from multiple reservoirs.

16 **Keywords:** methane, clumped isotopes, methanogenesis, C-P lyase

1. Introduction

Multiply-substituted ("clumped") isotopologues are powerful tracers of molecular formation reactions (Eiler and Schauble, 2004; Stolper et al., 2014; Wang et al., 2015; Yeung et al., 2015; Young et al., 2017; Yeung et al., 2017; Popa et al., 2019). Exploring variations in the relative abundance of the two doubly-substituted mass-18 isotopologues of methane, $^{12}\text{CH}_2\text{D}_2$ and $^{13}\text{CH}_3\text{D}$, have provided promising constraints on provenance, even where bulk C and H isotopic ratios ($^{13}\text{C}/^{12}\text{C}$, D/H) yielded ambiguous information as to source and process (Young et al., 2017; Haghnegahdar et al., 2017; Young, 2019; Giunta et al., 2019; Ash et al., 2019). The relative abundance of the two mass-18 clumped isotopologues in a given population of methane molecules formed at high temperature (≥ 1000 K) in thermodynamic equilibrium, are approximated by a random distribution of all possible isotopologue combinations. This apportioning of isotopes is referred to as a "stochastic distribution," and provides the reference frame for reporting the relative abundances of clumped isotopologues (Eiler and Schauble, 2004). The abundance of a given multiply-substituted isotopologue relative to the most abundant isotopologue (e.g. $^{13}\text{CH}_3\text{D}/^{12}\text{CH}_4$) is reported as a fractional difference from the stochastic relative abundances, Δ_i , where subscript i refers to the specific clumped species, and Δ_i are reported in permil (‰). This stochastic reference frame highlights whether a process has favored or discriminated against the formation of molecules with bonds containing multiple heavy isotopes (e.g., $^{13}\text{CH}_3\text{D}$), rather than only differences in bulk isotope ratios. Under equilibrium conditions at low temperatures (< 300 K), the stability associated with bonds between heavy isotopes in a molecule increases, promoting the formation of the isotopically clumped molecular species. As temperature approaches infinity, this effect diminishes and the relative abundance of multiply-substituted molecules decreases, approaching $\Delta_i = 0$. Under non-equilibrium conditions at low temperatures, departures from the stochastic distribution may provide insights into the kinetics and reaction(s) of formation for a given methane population's natural history.

Methane produced by microorganisms is characterized by significant depletion or "anti-clumping" in the $\Delta^{12}\text{CH}_2\text{D}_2$ values by more than 20 to 50‰, relative to thermodynamic equilibrium (Young et al., 2017). These $\Delta^{12}\text{CH}_2\text{D}_2$ deficits are accompanied by negligible depletion in $\Delta^{13}\text{CH}_3\text{D}$ values (Figure 1). Several hypotheses have been put forward to explain these observed departures from thermodynamic equilibrium. The two hypotheses in the literature thus

54 far are: i) contributions from distinct pools of hydrogen with disparate D/H
 55 ratios and/or differentially fractionated H sites during formation, and ii)
 56 quantum tunneling of hydrogen during methane formation (Young et al.,
 57 2017; Yeung, 2016; Röckmann et al., 2016; Young, 2019; Cao et al., 2019).
 58 The former refers to the so-called “combinatorial effect.” The importance of
 59 the combinatorial effect as a source of anti-clumping, wherein the measured
 60 clumped isotopologue abundance is less than predicted by theory, was first
 61 hypothesized during the study of molecular oxygen clumped isotopologues
 62 (Yeung et al., 2015; Yeung, 2016; Röckmann et al., 2016) and was suggested
 63 as relevant to microbially produced (microbialgenic) methane (Young et al.,
 64 2017). However, the combinatorial effect has not been directly tested or
 65 demonstrated in any experimental study. While this effect can arise from
 66 drawing on two or more reservoirs, it is not simply mixing in the general sense.
 67 To interpret the significance of depleted (anti-clumped) $\Delta^{12}\text{CH}_2\text{D}_2$ values re-
 68 quires a thorough understanding of if, when and how the combinatorial effect
 69 is expressed. This understanding is essential to the application of clumped
 70 isotope signatures as tracers of methane provenance, and extends to other
 71 gasses that could hypothetically experience combinatorics in altering the rela-
 72 tive abundance of multiply substituted isotopologue ratios, such as molecular
 73 oxygen ($\Delta^{18}\text{O}^{18}\text{O}$) or dinitrogen ($\Delta^{15}\text{N}^{15}\text{N}$).

74 To experimentally test for the combinatorial effect and quantify its in-
 75 fluence on $\Delta^{12}\text{CH}_2\text{D}_2$ values, we examined the bulk and clumped methane
 76 isotopologue abundances produced by bacteria operating the C-P lyase path-
 77 way. This microbialgenic methane production pathway is related to phospho-
 78 rous acquisition and not core energy metabolism (White and Metcalf, 2004;
 79 Sosa et al., 2019), the canonical anaerobic microbial methanogens (Valen-
 80 tine, 2011). This metabolism provides a unique opportunity to study the
 81 combinatorial effect because this pathway incorporates hydrogen from two
 82 distinct reservoirs during methane formation: three from the methyl group
 83 of methylphosphonate and one from water (Kamat et al., 2011, 2013). More-
 84 over, this methane is generated by rapidly growing bacteria where the bulk
 85 isotopic fractionations are independent of energy metabolism and growth
 86 rate (Taenzer et al., 2020). By varying the D/H composition of the water in
 87 which the bacteria grew and generated methane, we observed dramatic shifts
 88 in $\Delta^{12}\text{CH}_2\text{D}_2$ with minimal effect on $\Delta^{13}\text{CH}_3\text{D}$. Our experimental and model
 89 results together indicate that shifts in methane $\Delta^{12}\text{CH}_2\text{D}_2$ produced by the
 90 C-P lyase pathway were primarily due to the relative differences in D/H
 91 between water and methylphosphonate methyl hydrogens. The consistency

Figure 1: The distribution of laboratory axenic cultures (circles with crosses) and naturally-produced methane (open circles) by microbial methanogenesis in $\Delta^{12}\text{CH}_2\text{D}_2$ vs. $\Delta^{13}\text{CH}_3\text{D}$ space. The curve representing thermodynamic equilibrium is shown for reference, annotated with temperatures. Calculations and values are from Young (2019) and references therein.

between model predicted and experimentally generated isotopologue abundances demonstrates that the combinatorial effect can yield markedly negative $\Delta^{12}\text{CH}_2\text{D}_2$ values (below $< -50\%$) with little influence on $\Delta^{13}\text{CH}_3\text{D}$ values ($< 1\%$). Implications for the interpretation of experimental and natural methane clumped isotopologue studies are discussed.

2. Principles of the Combinatorial Effect

The stochastic distribution is the reference frame for reporting multiply substituted (clumped) isotopologue abundances. It is defined by the most probable abundance of the multiply-substituted isotopologue of interest assuming that the isotopes comprising the molecules were randomly distributed among all possible isotopologues. The stochastic reference is calculated by equating fractional isotopic abundances with probabilities. To illustrate the essence of the combinatorial effect and for simplicity we examine cases for the formation of two diatomic molecules, carbon monoxide (CO) and molecular oxygen (O_2).

The stochastic distribution for the relative abundance of the multiply-substituted isotopologue $^{13}\text{C}^{18}\text{O}$ in a sample of carbon monoxide gas is calculated as

$$x(^{13}\text{C}^{18}\text{O}) = x(^{13}\text{C})x(^{18}\text{O}) \quad (1)$$

where $x^{13}\text{C}$ is the fractional abundance of ^{13}C relative to all carbon isotopes by number (i.e., $^{13}\text{C}/(^{12}\text{C} + ^{13}\text{C})$), $x^{18}\text{O}$ is the fractional abundance of ^{18}O relative to all oxygen isotopes (i.e., $^{18}\text{O}/(^{16}\text{O} + ^{17}\text{O} + ^{18}\text{O})$), and $x(^{13}\text{C}^{18}\text{O})$ is the fractional abundance of the $^{13}\text{C}^{18}\text{O}$ isotopologue relative to all CO molecules. The ratio of this multiply-substituted isotopologue to the most abundant isotopic species is therefore $x(^{13}\text{C}^{18}\text{O})/x(^{12}\text{C}^{16}\text{O})$. It is common to express ratios of relative isotopologue fractions in terms of the isotope ratios of their constituent elements. In this case, $x(^{13}\text{C}^{18}\text{O})/x(^{12}\text{C}^{16}\text{O}) = (^{13}\text{C}/^{12}\text{C})(^{18}\text{O}/^{16}\text{O}) = ^{13}R^{18}R$. Similarly, the stochastic fractional abundance of the multiply-substituted isotopologue of molecular oxygen (O_2) composed of two ^{18}O atoms is

$$x(^{18}\text{O}^{18}\text{O}) = x(^{18}\text{O})x(^{18}\text{O}) \quad (2)$$

where the symbols are analogous to those in Equation 1. In this case $x(^{18}\text{O}^{18}\text{O})/x(^{16}\text{O}^{16}\text{O}) = (^{18}\text{O}/^{16}\text{O})(^{18}\text{O}/^{16}\text{O}) = ^{18}R^2$.

123 For both $^{18}\text{O}^{18}\text{O}$ and $^{13}\text{C}^{18}\text{O}$, the accuracy of the estimated stochastic
 124 tic distribution is dictated by the fidelity of the bulk isotope ratios (e.g.,
 125 $^{13}R = ^{13}\text{C}/^{12}\text{C}$ and $^{18}R = ^{18}\text{O}/^{16}\text{O}$) relative to these ratios at the time the
 126 molecules formed, and the degree to which the sample separation chemistry
 127 and mass-spectrometry can capture these ratios, free of subsequent fraction-
 128 ation effects. The cases of CO and O₂ represent contrasting examples of the
 129 ability to reconstruct the actual isotope ratios of the constituent atoms dur-
 130 ing assembly of the molecules. In the case of CO, the isotopic compositions
 131 of C and O unambiguously represent the isotope ratios of the constituent
 132 atoms since carbon and oxygen are distinguishable; the analysis of carbon
 133 monoxide yields $^{13}\text{C}/^{12}\text{C}$ and $^{18}\text{O}/^{16}\text{O}$ bulk ratios that faithfully record the
 134 actual isotopic compositions of the two constituent atoms (there is no confus-
 135 ing C and O, see Figure 2). In contrast, in the case of O₂, the isotopologue of
 136 interest is composed of two atoms of the same element and so the molecular
 137 positions can not be distinguished from one another. Therefore, despite the
 138 fact that the two atoms comprising the O₂ molecule may have originated
 139 from different reservoirs with distinct $^{18}\text{O}/^{16}\text{O}$ ratios (Yeung et al., 2015),
 140 the stochastic abundance of the $^{18}\text{O}^{18}\text{O}$ molecule is calculated on the basis
 141 of the average $^{18}\text{O}/^{16}\text{O}$ ratio. That is, the $^{18}\text{O}/^{16}\text{O}$ of the O₂ sample reflects
 142 an average of the two individual molecular sites and their source reservoirs.
 143 By necessity the $x(^{18}\text{O}^{18}\text{O})/x(^{16}\text{O}^{16}\text{O}) = ^{18}R^2$ value for the stochastic iso-
 144 topologue ratio calculation assumes that the two molecular positions were
 145 formed from identical oxygen isotope ratios of ^{18}R . In reality, the oxygen
 146 isotope ratio is the average of two potentially distinct ratios, $^{18}R_1$ and $^{18}R_2$,
 147 which individually contributed to the O₂ molecule, such that:

$$[x(^{18}\text{O}^{18}\text{O})/x(^{16}\text{O}^{16}\text{O})]_{\text{stochastic}} = \left(\frac{(^{18}R_1 + ^{18}R_2)}{2} \right)^2 \quad (3)$$

148 where subscripts 1 and 2 refer to two distinct oxygen reservoirs. The dif-
 149 ference between using the actual constituent ratios $^{18}R_1^{18}R_2$ and using the
 150 average ratio $(^{18}R_1 + ^{18}R_2)/2$ (by necessity) to calculate the stochastic ratio
 151 for the calculation of $\Delta^{18}\text{O}^{18}\text{O}$ is a manifestation of the mathematical truism
 152 that

$$(^{18}R_1)(^{18}R_2) \leq \left(\frac{(^{18}R_1) + (^{18}R_2)}{2} \right)^2. \quad (4)$$

153 In particular, we have for the true value for the fractional departure in

Figure 2: Schematic illustrations showing the pairing of oxygen-oxygen or oxygen-carbon isotopes when molecules of O_2 or CO are formed, respectively. The potential for two separate reactant reservoirs to contribute atoms from pools of distinct isotopic composition is illustrated for each case. For CO , the oxygen and carbon reservoirs are always distinguishable by virtue of their different elemental identities. The composition of the C and O sources are reflected in the isotopic ratios of the product CO , offset by any fractionation, which can be individually traced. On the contrary, in the case of two oxygen reservoirs contributing to O_2 , only a single average isotopic composition can be determined for the product O_2 which obscures contributions from two separate, and potentially distinct oxygen sources and any fractionation between product and either of the sources.

154 $x(^{18}\text{O}^{18}\text{O})/x(^{16}\text{O}^{16}\text{O})$ from stochastic

$$\Delta^{18}\text{O}^{18}\text{O} = \frac{[x(^{18}\text{O}^{18}\text{O})/x(^{16}\text{O}^{16}\text{O})]_{\text{sample}}}{(^{18}R_1)(^{18}R_2)} - 1 \quad (5)$$

155 where "sample" refers to the measured isotopologue ratio. The $\Delta^{18}\text{O}^{18}\text{O}$
 156 value from Equation 5 cannot be reconstructed *a priori*. Rather, the estimate
 157 obtained from the measured, average bulk isotopic composition of the O_2 gas
 158 itself is actually

$$\Delta^{18}\text{O}^{18}\text{O} = \frac{4[x(^{18}\text{O}^{18}\text{O})/x(^{16}\text{O}^{16}\text{O})]_{\text{sample}}}{\left((^{18}R_1) + (^{18}R_2)\right)^2} - 1. \quad (6)$$

159 Only when $^{18}R_1 = ^{18}R_2$ will the two estimates be equal. Since the left-hand
 160 side of Equation 4 is the square of the geometric mean and the right-hand side
 161 is the square of the arithmetic mean, the inequality in this simple example is
 162 an expression of the well-known inequality of the geometric and arithmetic
 163 means, and is a general result (Yeung, 2016). Comparing Equations 4, 5,
 164 and 6 illustrates the general principle that when molecular positions are in-
 165 distinguishable, and the average isotope ratios rather than the site-specific
 166 isotope ratios are all that is available for calculating the stochastic distri-
 167 bution of a multiply-substituted isotopologue, the stochastic abundance of
 168 the isotopologue i is overestimated, resulting in a spuriously low Δ_i value.
 169 The spuriously low and even negative Δ_i values are the result of the refer-
 170 ence frame (Figure 3). Yeung and colleagues showed that for the case of
 171 multiply-substituted oxygen isotopologue in molecular oxygen that the mea-
 172 sured $\Delta^{18}\text{O}^{18}\text{O}$ diverged drastically from the stochastic prediction (Yeung
 173 et al., 2015), and predicted that this is potentially due to two contributing
 174 sources (reservoirs) of isotopically distinct oxygen – that is, the combinatorial
 175 effect (Yeung, 2016). To date, however, no study has tested this experimen-
 176 tally for any other multiply substituted isotope system, be it oxygen, methane
 177 or another.

178 Given these examples above, we predict the combinatorial effect is possi-
 179 ble whenever a molecule composed of multiple isotopes of the same element
 180 is constructed from more than one isotopic pool. The source of the different
 181 pools may result from different isotope effects for each step involved in con-
 182 structing the molecule, or from drawing on isotopically distinct reservoirs.
 183 Candidates include, but are not limited to, multiply substituted isotopo-
 184 logues of N_2 , O_2 , H_2 , CH_4 , and N_2O . Despite the established theoretical

Figure 3: An illustration of the combinatorial effect seen when plotting $\Delta^{18}\text{O}^{18}\text{O}$ as a function of the ratio of the isotopic compositions, R_1 and R_2 , of the distinct contributing oxygen reservoirs.

185 impact of combinatorics in influencing clumped isotopologue Δ_i values, it
186 has yet to be demonstrated unambiguously in the laboratory. Substantiat-
187 ing the existence of the combinatorial effect and quantifying its magnitude
188 are prerequisites to interpreting existing and future clumped isotopologue
189 signatures in molecules composed of multiple atoms of the same element.

190 3. Methods

191 3.1. Microbial cultivation and gas production

192 The model microorganism *Pseudomonas stutzeri* strain HI00D01 (here-
193 after, strain HI00D01) was cultivated following the protocols in our recent
194 study (Taenzer et al., 2020), with modifications to generate large enough
195 quantities of methane for clumped isotope analyses. For methane generation
196 experiments all sources of P were removed and the medium was amended
197 with methylphosphonic acid (aka MPn; Sigma-Aldrich, CAS Number 993-
198 13-5) to a concentration of 2mM, with glucose (electron donor and carbon
199 source) to a concentration of 3g/L, and sodium nitrate (NaNO_3) used as
200 the terminal electron acceptor to a final concentration of 40mM. The growth
201 medium was filter-sterilized then transferred to 160mL, 1 or 5L borosilicate
202 bottles and vigorously degassed with ultra-high purity helium (UHP-He) for
203 one hour per liter. Cell pellets from aerobic cultures were re-suspended and
204 incubated on amended MPn and NaNO_3 media in 160mL butyl stoppered
205 serum bottles. After reaching exponential phase on the MPn-amended media
206 and positive head-space methane detection (via GC-FID), 1 or 5L cultures
207 were inoculated to an optical density (OD600) of 0.001 and incubated at
208 30°C in the dark. Medium for D-enriched water experiments was prepared
209 following the approach of Zhang and colleagues (Zhang et al., 2009), where
210 it was enriched by adding a calculated quantity of D_2O (Sigma-Aldrich, CAS
211 Number 7789-20-0) to the stock media prior to UHP-Helium flushing and
212 sterilization.

213 The gaseous methane, dinitrogen, and carbon dioxide produced by strain
214 HI00D01 were captured into gas-impermeable 0.25, 1, or 4L Tedlar bags,
215 along with trace water vapor. The gas bags were built with straight thru
216 1/4 inch stainless steel tubing (GSB-P-1 Calibrated Instruments) to which
217 we fitted with a 1/4 inch stainless steel needle valve by compression fitting
218 to seal the gas bags before and after sample collection. To allow sample
219 into the bag, we attached to the needle valve another 1/4" stub of stainless
220 tubing, followed by a tube-to-pipe adapter 1/4" compression to 1/8" NPT,

221 followed by an 1/8" NPT to 1/4-28 adapter to then a luer lock and finally
 222 a threaded 18-G stainless steel dispensing needle (all plumbing parts from
 223 McMaster-Carr or Swagelok). The needle was inserted through black butyl
 224 stopper (Glasger&tebau Ochs GmbH) into the top of the bags through
 225 plastic funnels that were added for stability. At the end of the incubations,
 226 UHP-He sparged water was used to displace all remaining headspace into
 227 the Tedlar bags, allowed to equilibrate, then sealed by the needle valves
 228 and removed from experiment bottles, and the valves plugged with metal
 229 compression septa until preparation on the vacuum line.

230 3.2. Bulk isotope ratio measurements

231 Bulk carbon ($\delta^{13}\text{C}$) and hydrogen (δD) isotope compositions of methylphos-
 232 phonic acid (Sigma-Aldrich, CAS Number 993-13-5) were determined at the
 233 Northwestern University Stable Isotope Laboratory. Briefly, the $\delta^{13}\text{C}$ value
 234 of MPn was determined via EA-IRMS on a Costech 4010 Elemental An-
 235 alyzer, coupled to a Delta V Plus isotope ratio mass spectrometer via a
 236 Conflo IV interface. Analyses were done in triplicate, and placed on the
 237 VPDB-LSVEC scale via analysis of organic standards supplied by Indiana
 238 University (acetanilide #1, urea #2a, and D-glucose) (Schimmelmann, 1991;
 239 Qi and Coplen, 2011). The bulk hydrogen (δD) isotope composition of MPn
 240 was determined via TC/EA-IRMS, operated at 1420°C, and coupled to a
 241 Delta V Plus isotope ratio mass spectrometer via a Conflo IV. Analyses
 242 were done in triplicate, and cross-referenced to the Ag-tube water isotope
 243 standards supplied by the USGS (GISP2, VSMOW, and UC03). The aver-
 244 age composition of the non-exchangeable hydrogens on MPn (i.e., R-CH₃)
 245 was determined by equilibration with D-enriched waters (2mL) in a custom-
 246 designed vacuum chamber modified from a Costech zero-blank auto-sampler.
 247 Incubations were done at room temperature (25°C) for 6 days. At the end
 248 of the equilibration, water vapor was pumped to <50 mTorr for 2 hours,
 249 followed by subsequent TC/EA analyses with Ag-tube USGS standards as
 250 above, following published methods (Qi and Coplen, 2011).

251 Isotopic analysis of the medium water was conducted at the Stable Isotope
 252 Laboratory at Dartmouth College following published procedures (Kopec
 253 et al., 2019). Briefly, water hydrogen isotopic ratios (δD) were measured us-
 254 ing the H-Device, in which water was reduced by hot chromium (850°C) and
 255 the resulting hydrogen gas was measured by an isotope ratio mass spectrom-
 256 eter (IRMS, Thermo Delta Plus XL). Isotopic ratios (D/H) are reported in

257 delta notation in permil (‰) deviation from the international standard VS-
 258 MOW on the VSMOW-SLAP (Vienna Standard Mean Ocean Water, Stan-
 259 dard Light Antarctic Precipitation) scale. The measurement 1σ uncertainty
 260 is 0.5‰ for δD , and the measured value was converted to the water isotope
 261 equivalent by calibration with known standards. When samples were pre-
 262 sumed to be outside the range of the standards (e.g. for the enrichment
 263 experiments), samples were first diluted gravimetrically with a water stan-
 264 dard of known composition. Uncertainty's due to dilution are accounted for
 265 and reported in Table 1 as the propagated 1se.

266 The stable isotopic compositions of methane samples generated by the C-
 267 P lyase in cultures of strain HI00D01 were measured on an ultra-high mass
 268 resolution isotope ratio mass spectrometer (Panorama, Nu Instruments) as
 269 part of the measurements to determine $\Delta^{12}CH_2D_2$ and $\Delta^{13}CH_3D$.

270 *3.3. Multiply-substituted isotopologue measurements*

271 The abundance of the two multiply-substituted mass-18 isotopologues
 272 of methane gas samples were measured on a Panorama (Nu Instruments)
 273 ultra-high-resolution gas-source isotope ratio mass spectrometer housed in
 274 the Department of Earth, Planetary, and Space Sciences at the University
 275 of California Los Angeles. Detailed descriptions of the mass spectrometry
 276 method were given by Young and colleagues (Young et al., 2016), and applied
 277 in recent studies (Young et al., 2017; Haghnegahdar et al., 2017; Giunta et al.,
 278 2019; Ash et al., 2019; Young, 2019).

279 Methane sample gases were purified on a vacuum line interfaced with a gas
 280 chromatograph (SRI GC-FID 8610) prior to isotopic analysis. This method
 281 was previously described in Young et. al (2016). Modification to the pub-
 282 lished procedures were as follows. The Tedlar gas bag holding the C-P lyase
 283 generated methane gas (mixed with UHP He, water vapor, and the other
 284 metabolic products N_2 and CO_2) was connected to the line with compression
 285 fittings and PTFE ferrules. Prior to gas introduction, the hydrocarbon-free
 286 vacuum line was evacuated to at least 4.0×10^{-7} mbar. Sample gas was in-
 287 troduced from the bag to the line in aliquots of 1000 mbar into a calibrated
 288 50cc volume. Water vapor was trapped in a U-trap by ethanol-liquid nitro-
 289 gen (LN_2) slurry at $-80^\circ C$. The rest of the sample gas was then trapped on
 290 silica gel in a U-trap at LN_2 temperature for 10min, after which the non-
 291 condensable He was slowly pumped away. The methane aliquot was then
 292 cryofocused to remove the N_2 and CO_2 via GC and re-trapping on silica

gel as previously described (Young et al., 2017). Due to the significant partial pressure of N₂ relative to methane, and because complete separation of methane from N₂ was not achieved on the first pass through the GC, multiple aliquots were first passed through the GC, cryofocused, and co-trapped onto the same silica gel in a single glass cold-finger (6 to 7 GC-passes per sample bag). Once the requisite sample size of methane was captured (> 40 micromoles), the combined sample was once again cryofocused and passed through the GC a final time to remove trace N₂. The purified methane was then transferred to the Panorama inlet system for the bulk and clumped isotope ratio analyses following published protocols (Young et al., 2017).

3.4. Isotope notation

The isotopic compositions of carbon and hydrogen are reported as deviations from the carbon and hydrogen reference materials Vienna Pee Dee Belemnite and Vienna Standard Mean Ocean Water (VPDB and VSMOW, respectively). Standard delta notation is used to express the fractional differences:

$$\delta^{13}\text{C} = 10^3 \left(\frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{VPDB}}} - 1 \right) \quad (7)$$

and

$$\delta\text{D} = 10^3 \left(\frac{(\text{D}/\text{H})_{\text{sample}}}{(\text{D}/\text{H})_{\text{VSMOW}}} - 1 \right) \quad (8)$$

where all values are given in permil (‰), as shown. The relative abundances of the two multiply-substituted mass-18 isotopologues of methane are reported relative to the stochastic reference frame using the Δ_i notation, also in permil, where $\Delta^{13}\text{CH}_3\text{D}$ and $\Delta^{12}\text{CH}_2\text{D}_2$ are (Young et al., 2016):

$$\Delta^{13}\text{CH}_3\text{D} = 10^3 \left(\frac{x(^{13}\text{CH}_3\text{D})_{\text{sample}}}{x(^{13}\text{CH}_3\text{D})_{\text{stochastic}}} - 1 \right) \quad (9)$$

and

$$\Delta^{12}\text{CH}_2\text{D}_2 = 10^3 \left(\frac{x(^{12}\text{CH}_2\text{D}_2)_{\text{sample}}}{x(^{12}\text{CH}_2\text{D}_2)_{\text{stochastic}}} - 1 \right), \quad (10)$$

respectively (Young et al., 2016). The stochastic fractional abundances of the mass-18 isotopologues are obtained from the measured bulk isotope fractional abundances of atoms in the sample such that

$$x(^{13}\text{CH}_3\text{D})_{\text{stochastic}} = 4 \left(x(^{13}\text{C})x(\text{H})^3x(\text{D}) \right) \quad (11)$$

318 and

$$x(^{12}\text{CH}_2\text{D}_2)_{\text{stochastic}} = 6 \left(x(^{12}\text{C})x(\text{H})^2x(\text{D})^2 \right), \quad (12)$$

319 and where the coefficients of 4 and 6 account for the number of permutations
320 for the positions of the D and H isotopes (Young et al., 2016).

321 4. Results

322 The isotopic compositions of methane produced from the degradation of
323 MPn by strain HI00D01 in waters with different δD values in the range from
324 -376 to $+6800\text{‰}$ are given in Table 1. The δD of product methane correlates
325 closely with the δD of water (Figure 4). The methane $\delta^{13}\text{C}$ values vary little
326 between -97 to -101‰ (mean = $-99.9 \pm 1.2 \text{‰}$, 1σ), closely tracking the
327 $\delta^{13}\text{C}$ of the source MPn of near -100‰ relative to VPDB (Taenzer et al.,
328 2020). The three non-exchangeable H-sites on the MPn methyl-group have
329 a δD of $-138 \pm 5\text{‰}$ relative to VSMOW for all of the experiments except
330 those that utilized normal house water (-60‰ , both B1 and B2). In the
331 latter two experiments a different batch of MPn was used with the measured
332 MPn methyl-group δD being $-132 \pm 5\text{‰}$ relative to VSMOW. These results
333 show that the final hydrogen added to the methyl-group derives from medium
334 water during methane formation, and supports the recent finding that neg-
335 ligible carbon-isotope fractionation occurs during methane production from
336 MPn via the C-P lyase pathway (Taenzer et al., 2020).

337 The measured $\Delta^{13}\text{CH}_3\text{D}$ values of C-P lyase generated methane ranged
338 between -0.29 and 0.934‰ , with an average of $0.35 \pm 0.40 \text{‰}$ (Figure 5,
339 Table 1). The variation in $\Delta^{13}\text{CH}_3\text{D}$ values was only marginally greater
340 than measurement uncertainties, and did not vary systematically with water
341 δD , despite the significant contribution of water sourced from the medium
342 with a range of δD values from -376 to $+6800\text{‰}$. In sharp contrast, the
343 $\Delta^{12}\text{CH}_2\text{D}_2$ values varied from -69.2 to $+7.1\text{‰}$, a spread much greater than
344 measurement uncertainty of $< 1\text{‰}$ (Figure 5). The methane $\Delta^{12}\text{CH}_2\text{D}_2$
345 values correlated closely with the medium water δD in a non-linear, semi-
346 parabolic relationship (Figure 5).

Figure 4: The D/H ratios of product methane vs. the D/H of medium water expressed in per mil deviations from VSMOW. Linear regression yields $\delta D_{CH_4} = 0.05 \delta D_{H_2O} - 301.5$, with an R^2 of 0.999. The strong correlation between the isotopic ratios demonstrates addition of a consistent fraction of reactant water to product methane, and allows for the calculation of the fractionation factor between water D/H and the D/H of the single hydrogen added to the MPn CH_3 to form methane (see text).

Figure 5: The measured (left) $\Delta^{13}\text{CH}_3\text{D}$ and (right) $\Delta^{12}\text{CH}_2\text{D}_2$ of microbialgenic methane produced in experiments with water of varying δD water for methane produced in various D-spiked experiments. Errors are smaller than the symbols.

Table 1: The isotopic compositions of medium waters and product methane.

Water			Methane							
Sample ID	δD	1se	$\delta^{13}\text{C}$	1se	δD	1se	$\Delta^{13}\text{CH}_3\text{D}$	1se	$\Delta^{12}\text{CH}_2\text{D}_2$	1se
-400 B1	-376	0.5	-100.388	0.012	-319.26	0.04	0.93	0.35	-69.18	1.15
-400 B2	-376	0.5	-98.950	0.013	-319.22	0.04	0.85	0.44	-69.10	0.83
-60 B1	-63	0.5	-99.993	0.006	-299.49	0.04	0.02	0.13	-52.28	0.83
-60 B2	-63	0.5	-100.15	0.010	-300.22	0.03	0.50	0.24	-51.11	0.88
750 B1	1013	1.7	-97.047	0.006	-249.08	0.05	0.28	0.31	-14.90	1.32
750 B2	1022	7.3	-100.297	0.024	-250.12	0.05	0.77	0.42	-13.82	1.17
1500 B1	2007	5.1	-100.745	0.010	-194.72	0.04	0.26	0.18	-0.01	0.96
1500 B2	2188	5	-100.846	0.007	-190.06	0.04	0.54	0.27	0.79	1.11
3000 B1	3797	3.1	-100.213	0.009	-106.08	0.05	0.72	0.38	5.2	1.14
3000 B2	3868	2.3	-100.606	0.071	-104.59	0.06	-0.29	0.71	7.06	1.10
3900 B1+2	4871	7.9	-97.949	0.019	-46.43	0.03	0.24	0.36	0.12	0.72
5500 B1	6800	11.1	-100.480	0.006	42.39	0.04	-0.03	0.25	-25.46	0.85

5. Discussion

5.1. D/H fractionation between MPn and water

Understanding the sources of hydrogen used in methane formation by the C-P lyase enzyme is central to understanding the results of these experiments. The main substrate MPn is an organophosphonate synthesized by certain microbes in nature when faced with inorganic phosphate limitation (Metcalf et al., 2012; Yu et al., 2013). The methyl-group hydrogens on MPn

are ultimately derived from the water in which MPn is biosynthesized, offset by an unknown isotope effect. The hydrogen isotope fractionation between methyl-group hydrogens and water in more thoroughly studied processes, such as acetogenesis, show offsets from bulk water δD of hundreds of permil (Hattori et al., 2010). Here we utilized synthetic MPn with an average δD value distinct from all medium waters utilized (see Results). Phosphorus-starved microbes that encode the C-P lyase enzyme are able to grow on MPn as their sole source of phosphorus by breaking down MPn (Repeta et al., 2016; Sosa et al., 2019; Carini et al., 2014), and as a by-product, release methane (Kamat et al., 2011). The carbon-phosphorus bond in the methyl phosphonate intermediate, ribose-1-methylphosphonate 5-phosphate (PRPn), is attacked by the C-P lyase enzyme subunit PhnJ, producing ribose-1,2-cyclic-phosphate-5-phosphate (PRcP), and releasing the methyl group used to form methane. This reaction breaks the methyl group from the rest of the phosphonate compound. In vitro experiments with PhnJ showed that the hydrogen added to the methyl group originates from the bulk solvent (water) via a solvent exchangeable site on an amino acid in PhnJ (Kamat et al., 2011). The medium-derived hydrogen is delivered to form methane either by abstraction from the 5'-deoxyadenosine or the PhnJ amino acids. A representation of the overall reaction that underscores the sources of hydrogen from medium water and the MPn methyl group is

In our labelled water experiments, the methane δD increased with the water δD (Figure 4). Moreover, because we used the same lot of MPn for all reactions, with one exception that was isotopically similar (see *Results*), the methyl-group D/H was the same for all our experiments, and the increase in the D/H of product methane solely reflects the addition of water derived hydrogen with different D/H ratios. The tight correlation between bulk water and methane δD is described by the linear regression with an R^2 value of 0.999 (Figure 4). The regression equation is

$$\delta D_{\text{CH}_4} = 0.05 \delta D_{\text{H}_2\text{O}} - 301.5.
 \tag{14}$$

383 The good correlation is consistent with the conclusion that methane is formed
 384 by transfer of a hydrogen from the medium water to the methyl group of
 385 MPn, and indicates that the transfer of hydrogen from water to the methyl
 386 group was consistent for all incubations in medium waters of different δD
 387 values. The slope in Equation 14 is the product of the fractionation factor
 388 between water and the hydrogen transferred to methane and the fraction of
 389 hydrogen in the methane derived from the water.

390 The good correlation described by Equation 14 is best explained as the
 391 result of constant fractions of hydrogen coming from the methyl group in
 392 MPn and water to produce methane. The most likely reason for this is
 393 that methane inherits 3/4 of its hydrogen directly from CH_3 , and that water
 394 contributes the last hydrogen. The hydrogen isotope conservation equation
 395 for the formation of methane is therefore

$$0.75(^D R_{\text{Methyl}}) + 0.25 ^D \alpha_{CH_4/H_2O} (^D R_{H_2O}) = ^D R_{CH_4} \quad (15)$$

396 where $^D R_i$ is the D/H ratio for species i . Using the known δD values for
 397 the product methane, medium water, and MPn methyl group from each
 398 experiment, the fractionation factor between methane and water, $^D \alpha_{CH_4/H_2O}$,
 399 for the one hydrogen transferred from water to make methane was determined
 400 by rearranging Equation 15, yielding:

$$^D \alpha_{CH_4/H_2O} = \frac{4(^D R_{CH_4} - (0.75 ^D R_{\text{Methyl}}))}{^D R_{H_2O}}. \quad (16)$$

401 The D/H fractionation factor between water and methane calculated from
 402 Equation 16 for the experiments with differing water δD values vary from
 403 0.203 to 0.219 with an average value of 0.209 ± 0.005 1σ . The consistency is
 404 a reflection of the good correlation between δD values for CH_4 and H_2O (Fig-
 405 ure 4). This fractionation factor corresponds to $^D \epsilon_{\text{methane/water}} = -790 \pm 5$
 406 ‰. The magnitude of fractionation between water and methane is relatively
 407 large, though not uncommon for processes involving hydrogen (Valentine
 408 et al., 2004; Kawagucci et al., 2014). However, the fractionation is not *a pri-*
 409 *ori* outside the range for classical effects given the likelihood that it reflects
 410 a convolution of steps (i.e., product of fractionation factors) involving D/H
 411 exchange on amino acids that accommodate the transfer of the last hydrogen
 412 that forms methane from water (Kamat et al., 2011). For this reason we do
 413 not attribute the magnitude of our derived fractionation factor to quantum
 414 tunneling. We note that because we know the D/H of the methyl group (ex-

415 changable hydrogen in MPn) and we can calculate the D/H of the hydrogen
 416 donated from water, we have characterized the D/H for the two sources of
 417 hydrogen drawn upon to construct the CH₄ molecules in each experiment.

418 The large isotope fractionation during the transfer of water hydrogen to
 419 the methyl group ensures that two compositionally distinct hydrogen reser-
 420 voirs with distinct δD values contribute to product methane, one ultimately
 421 from medium water and the other from the methyl group in the MPn. Other
 422 mechanisms of methane formation that are analogous to the C-P lyase, such
 423 as microbial methylotrophic and acetotrophic methanogenesis (Penger et al.,
 424 2012), are likely influenced similarly. These metabolisms have been the target
 425 of recent investigations into their variance in $\Delta^{13}\text{CH}_3\text{D}$ in response to labelled
 426 substrate or water (Gruen et al., 2018), although $\Delta^{12}\text{CH}_2\text{D}_2$ values were not
 427 determined. In addition to these biogenic methane formation pathways, abi-
 428 otic methane formation pathways where hydrogenated sites on end-product
 429 methane derive from two or more different reservoirs or with different frac-
 430 tionations from the source reservoir may be similarly influenced, as has been
 431 suggested (Yeung, 2016; Young et al., 2017; Young, 2019). Evidence of ei-
 432 ther quantum tunneling or the combinatorial effect were captured in methane
 433 generated at low-temperature via the abiotic Sabatier reaction, where a clear
 434 anti-clumped signal was observed in the $\Delta^{12}\text{CH}_2\text{D}_2$ with minimal variance in
 435 $\Delta^{13}\text{CH}_3\text{D}$ (Young et al., 2017). To determine if this was the combinatorial
 436 effect, future experiments with isotopically enriched molecular hydrogen are
 437 needed.

438 5.2. Effect of multiple D/H reservoirs on $\Delta^{12}\text{CH}_2\text{D}_2$

439 The combination of multiple reservoirs with distinct D/H ratios has a
 440 clear influence on the $\Delta^{12}\text{CH}_2\text{D}_2$ value of product methane. There was no
 441 corresponding effect on $\Delta^{13}\text{CH}_3\text{D}$ values. The negative $\Delta^{12}\text{CH}_2\text{D}_2$ values re-
 442 sult from the nature of the clumped isotope reference frame. In general,
 443 when the stochastic distribution is calculated from a sample gas that av-
 444 erages different isotopic pools, negative Δ_i values result. By altering the
 445 isotopic composition of one of the two pools of hydrogen in the C-P lyase
 446 pathway, the medium water, we have apparently isolated this combinatorial
 447 effect on $\Delta^{12}\text{CH}_2\text{D}_2$.

448 In Figure 6, we illustrate the expected combinatorial effect on prod-
 449 uct methane for experiments like those reported here. When calculated
 450 $\Delta^{12}\text{CH}_2\text{D}_2$ values are plotted against the D/H isotopic composition of the
 451 water hydrogen isotopic composition where the methyl D/H is unchanged,

452 a parabolic trend emerges with an $\Delta^{12}\text{CH}_2\text{D}_2$ value near zero at the apex,
 453 whereas the $\Delta^{13}\text{CH}_3\text{D}$ is unchanged. This topology is predicted by the in-
 454 equality in Equation 4 and has been demonstrated mathematically (Yeung
 455 et al., 2015; Yeung, 2016; Röckmann et al., 2016). As the isotopic com-
 456 position of the hydrogens originating from the water and the methyl group
 457 diverge, the $\Delta^{12}\text{CH}_2\text{D}_2$ values become exponentially more negative. Negative
 458 values for $\Delta^{13}\text{CH}_3\text{D}$ do not emerge because of the unambiguous distinction
 459 between the $^{13}\text{C}/^{12}\text{C}$ and D/H ratios, yielding no ambiguity in the stochastic
 460 distribution (Figure 6).

461 5.3. Model for the observed $\Delta^{12}\text{CH}_2\text{D}_2$ effect

462 Our results resemble those expected for the combinatorial effect (com-
 463 pare Figures 5 and 6) and suggest that a combinatorial effect in $\Delta^{12}\text{CH}_2\text{D}_2$ is
 464 indeed manifest in at least one pathway for microbialgenic methane for-
 465 mation. In order to determine the extent to which the range of observed
 466 $\Delta^{12}\text{CH}_2\text{D}_2$ values is explained solely by the combinatorial effect we applied a
 467 model in which we calculate methane isotopologue abundances produced by
 468 a network of bi-molecular reactions between the isotopologues of the MPn
 469 methyl group and water, including the fractionation factor derived from
 470 Equation 16. The relative rates of the reactions were taken to be the product
 471 of the reactant relative concentrations. The eleven reactions comprising the
 472 model are

Figure 6: An illustration of the difference between the stochastic abundances of $\Delta^{12}\text{CH}_2\text{D}_2$ and $\Delta^{13}\text{CH}_3\text{D}$ predicted when using average isotopic compositions of the sample gas versus the isotopic compositions of the specific reactants. The abundance of the clumped isotopologues that results from the δD and $\delta^{13}\text{C}$ in any given methane population is shown by the white bars, whereas, the abundance of the clumped isotopologues predicted from using the isotopic composition of the specific reservoirs, water and MPn, is given in the black bars, and correspond to the left Y-axes. The clumped isotope values are depicted by the blue dashed line, and correspond to the right Y-axes. If the isotopic composition of the source reservoirs that contribute to product methane have the same δD values, then the clumped values will not vary, shown by the horizontal blue bar at zero in each panel. In reality, the clumped isotopic compositions are negative across most water δD values (assuming an invariant methyl-group), because the stochastic isotopologue abundance using the average C and H isotopic composition of the sample gas is greater than that given by the isotopic composition of the methyl group and water. All points in this plot are illustrative and not real data.

473 We also used an analogous set of reactions in which the ^{13}C -bearing isotopo-
 474 logues of the methyl group were included. The rate of reaction was modified
 475 by the fractionation factor from Equation 16 where a D in CH_4 comes from
 476 water. We performed the calculations for values of $^{\text{D}}\alpha_{\text{CH}_4/\text{H}_2\text{O}}$ ranging from
 477 0.19 to 0.23 in order to allow for uncertainties in the precise value.

478 We performed the calculations in two ways. In the first calculation, we as-
 479 summed that the reactants had stochastic relative abundances of isotopologues
 480 calculated from their measured bulk $\delta^{13}\text{C}$ and δD values. From the relative
 481 rates of reaction we calculate the resulting abundances of the methane iso-
 482 topologues and the $\Delta^{12}\text{CH}_2\text{D}_2$ and $\Delta^{13}\text{CH}_3\text{D}$ values expected for each incu-
 483 bation experiment.

484 The fit of the model curves obtained using purely stochastic reactant
 485 isotopologue abundances is sufficiently good (Figure 7) that we conclude
 486 that the combinatorial effect is the dominant process governing the values
 487 for $\Delta^{12}\text{CH}_2\text{D}_2$ in the product methane. However, there is a marked offset
 488 between the model curves and the measured values at higher water δD values.
 489 As expected, the calculations, produce $\Delta^{13}\text{CH}_3\text{D}$ within $< 0.2\%$, signifying
 490 no effect on $\Delta^{13}\text{CH}_3\text{D}$, as observed in our experiments.

491 In our second calculation method, we obtained an improved fit to the data
 492 by considering that the reactant methyl group did not have a purely stochas-
 493 tic distribution of isotopologues. Specifically, we allowed for a higher concen-
 494 tration of CHD_2 relative to stochastic. In order to determine a best-fit value
 495 for the reactant methyl clumping, we adjusted the ratio $\text{CHD}_2/(\text{CHD}_2)_{\text{stochastic}}$
 496 to minimize the chi-square calculated from the deviation in $\Delta^{12}\text{CH}_2\text{D}_2$ values
 497 between the data and the model. For this minimization we adopted the mean
 498 $^{\text{D}}\alpha_{\text{CH}_4/\text{H}_2\text{O}}$ value of 0.209. The results yield a best-fit $\text{CHD}_2/(\text{CHD}_2)_{\text{stochastic}}$
 499 ratio of 1.0114 that can be expressed as a $\Delta^{12}\text{CHD}_2$ value of 11.4% (Figure
 500 7). For a nominal reproducibility of $\pm 2\%$ for $\Delta^{12}\text{CH}_2\text{D}_2$ values implied by
 501 some of the replicate experimental results in Table 1, the reduced chi-square
 502 value for the fit of this model is 2.5 (for $n = 12$ experiments). This value is
 503 sufficiently close to unity to suggest that the vast majority of the variation
 504 exhibited by our data is due to the combinatorial effect that includes inher-
 505 itance of a modest amount of DD clumping from the reactant methyl group
 506 in MPn.

507 In order to assess the importance of the analytical uncertainties in the
 508 two input parameters for our calculations, we performed Monte Carlo (MC)
 509 simulations of the experiments by randomly varying the MPn methyl-group
 510 δD values and the $^{\text{D}}\alpha_{\text{CH}_4/\text{H}_2\text{O}}$ values in our model calculations at the water

Figure 7: Plots of $\Delta^{12}\text{CH}_2\text{D}_2$ vs. water δD showing our experimental results (triangles) and the two model calculations described in the text (curves). The model curves in panel A assume stochastic distributions for all of the reactant isotopologues. The model curves in panel B are obtained using a best-fit $\Delta^{12}\text{CHD}_2$ value of 11.4‰ for the reactant methyl group (see text). Curves for a range of $\alpha_{\text{CH}_4/\text{H}_2\text{O}}$ values from 0.19 to 0.23 are shown for reference. Monte Carlo simulations of the effects of uncertainties in MPn methyl δD and our derived $\alpha_{\text{CH}_4/\text{H}_2\text{O}}$ values are shown by the circles. Means and standard deviations for the Monte Carlo simulations are shown by the filled circle and 1σ vertical error bars.

511 δD values corresponding to the experiments. We drew 100 random instances
 512 of these values from parent Gaussian distributions based on the measured
 513 means and standard deviations for these parameters and used these as inputs
 514 to the calculations. The resulting MC estimates for the uncertainties in the
 515 models are shown in both panels in Figure 7. For the 7 groups of experiments
 516 representing 7 distinct δD water values, 6 have measured $\Delta^{12}\text{CH}_2\text{D}_2$ values
 517 that are within the 4σ uncertainty in our best-fit model with $\Delta^{12}\text{CHD}_2 =$
 518 11.4‰ (panel B, Figure 7).

519 Our best-fit model allows us to not only simulate the combinatorial effect
 520 on the product methane, but it also affords an estimate of the DD clumping in
 521 the MPn methyl group that produced the methane. The value for $\Delta^{12}\text{CHD}_2$
 522 in this instance reflects the process of forming reagent MPn. Experiments
 523 similar to these might be useful in exploring the DD ordering in naturally
 524 occurring MPn methyl groups.

525 5.4. *Implications for applications of $\Delta^{12}\text{CH}_2\text{D}_2$*

526 Negative $\Delta^{12}\text{CH}_2\text{D}_2$ values have been attributed to either combinatorial
 527 effects or quantum tunneling effects (Young et al., 2017; Young, 2019). The
 528 results of this study lend support to the interpretation that combinatorial
 529 effects dominate. These effects may arise from enzymatically-mediated ki-
 530 netic steps within a cell or surface catalyzed steps during abiotic synthesis.
 531 In both cases we refer to these as endogenous combinatorial effects because
 532 they are the result of distinct pools of hydrogen created by the kinetic steps
 533 leading to methane formation. In the present study, the pools of contrasting
 534 D/H ratios arise in part because of the isotopic composition of the growth
 535 medium, comprising what we refer to as an example of exogenous combi-
 536 natorial effects to emphasize that the pools of hydrogen with distinct D/H
 537 ratios include an external reservoir. Endogenous combinatorial effects may
 538 explain the very low $\Delta^{12}\text{CH}_2\text{D}_2$ values seen in microbial methanogenesis and
 539 in abiotic Sabatier reaction products (Young et al., 2017; Young, 2019).

540 A critical advantage of using $\Delta^{12}\text{CH}_2\text{D}_2$ and $\Delta^{13}\text{CH}_3\text{D}$ values to charac-
 541 terize the provenance of methane molecules is their relative insensitivity to
 542 substrate bulk isotopic compositions (Young et al., 2017; Young, 2019). For
 543 example, the very negative $\Delta^{12}\text{CH}_2\text{D}_2$ values that are characteristic of, or
 544 even diagnostic of, microbialgenic methane appear to be insensitive to the
 545 δD of the local aqueous environs (Young et al., 2017; Young, 2019; Ash et al.,
 546 2019). However, the strong response of $\Delta^{12}\text{CH}_2\text{D}_2$ values to the isotopic com-
 547 position of water in these experiments raises the specter of substrate bulk

isotope effects on methane $\Delta^{12}\text{CH}_2\text{D}_2$ values. Though some of the experimental water values were not representative of natural systems (e.g. $\delta\text{D}_{\text{H}_2\text{O}} > 0\text{‰}$), both the Hanover, NH, USA (house) and South Pole, Antarctica waters represent values for reservoirs in nature that can contribute hydrogen to methane. We note that the more than 300‰ difference in the δD of the two waters resulted in a more than 15‰ shift in methane $\Delta^{12}\text{CH}_2\text{D}_2$ values at a fixed substrate MPn isotopic composition (Figure 5). The likelihood for such effects in nature depends on the variability of D/H in natural waters and variability in fractionation factors between water and the natural methyl-type substrates used in methanogenesis. For the step-wise addition of hydrogen to carbon during hydrogenotrophic ($\text{H}_2 + \text{CO}_2$) methanogenesis, where all of the hydrogen for the product methane ultimately originates from water, endogenous combinatorial effects likely dominate due to the different D/H fractionation factors associated with each hydrogen addition step (Young, 2019). This said, if some of the hydrogens on methane come from molecular hydrogen (Valentine et al., 2004; Kawagucci et al., 2014), the opportunity for combinatorial influence arises. Indeed, the other canonical microbial pathways of methane formation, methylotrophic or acetotrophic, are clear analogs to the C-P lyase pathway, in that different D/H pools are accessed in the form of water and preexisting methyl groups, suggesting that exogenous combinatorial effects are at play. This is especially true given the likelihood for large D/H fractionations between water and C-H bearing organic sources of H in nature (Hattori et al., 2010), without which reservoir (exogenous) combinatorial effects are unlikely. More work is required to understand whether or not differences in $\Delta^{12}\text{CH}_2\text{D}_2$ values among different methanogenesis pathways (Giunta et al., 2019; Young et al., 2017) could in part reflect the different magnitudes of endogenous and exogenous combinatorial effects.

6. Conclusions

The source of depleted $\Delta^{12}\text{CH}_2\text{D}_2$ values in methane produced by microbes is evidenced in the aerobic C-P lyase pathway. This metabolism for methane production incorporates hydrogen from two distinct reservoirs: a methyl group, contributing three hydrogenated sites, and water that donates a single hydrogen. By varying the D/H composition of the water medium in which the bacteria generate methane, we observed the effect of combinatorics on $\Delta^{12}\text{CH}_2\text{D}_2$ values. Our results indicate that variations in the $\Delta^{12}\text{CH}_2\text{D}_2$ of

methane produced by C-P lyase is primarily the result of the difference in D/H between the water and the reactant methyl group. The consistency between predictions for the purely combinatorial reservoir effect and the measured isotopologue abundances demonstrates that in this case, combinatorics are the main cause of the negative $\Delta^{12}\text{CH}_2\text{D}_2$ values with no resolvable effect on $\Delta^{13}\text{CH}_3\text{D}$ values for the product methane. These results lend credence to the interpretation that combinatorial effects dominate the depleted microbialgenic methane $\Delta^{12}\text{CH}_2\text{D}_2$ values as well as those arising from some surface catalyzed abiotic methane production pathways. This work raises the prospect that differential water H-isotopic compositions or fractionations in nature may induce the combinatorial effect in some microbial methane production pathways.

Acknowledgements

Funding was provided by the Simons Foundation (WDL), the NASA NH Space Grant NNX15AH79 (WDL, LT), and the Sloan Foundation Deep Carbon Observatory (EDY); we thank Beverly Chiu and Alec Cobban for laboratory assistance.

Research Data

All code for model and figures is posted in a permanent archive FigShare DOI: 10.6084/m9.figshare.11676357

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