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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

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## 17 1. Introduction

18 Multiply-substituted ("clumped") isotopologues are powerful tracers of  
19 molecular formation reactions (Eiler and Schauble, 2004; Stolper et al., 2014;  
20 Wang et al., 2015; Yeung et al., 2015; Young et al., 2017; Yeung et al., 2017;  
21 Popa et al., 2019). Exploring variations in the relative abundance of the two  
22 doubly-substituted mass-18 isotopologues of methane,  $^{12}\text{CH}_2\text{D}_2$  and  $^{13}\text{CH}_3\text{D}$ ,  
23 have provided promising constraints on provenance, even where bulk C and  
24 H isotopic ratios ( $^{13}\text{C}/^{12}\text{C}$ , D/H) yielded ambiguous information as to source  
25 and process (Young et al., 2017; Haghnegahdar et al., 2017; Young, 2019;  
26 Giunta et al., 2019; Ash et al., 2019). The relative abundance of the two  
27 mass-18 clumped isotopologues in a given population of methane molecules  
28 formed at high temperature ( $\geq 1000$  K) in thermodynamic equilibrium, are  
29 approximated by a random distribution of all possible isotopologue combi-  
30 nations. This apportioning of isotopes is referred to as a "stochastic distri-  
31 bution," and provides the reference frame for reporting the relative abun-  
32 dances of clumped isotopologues (Eiler and Schauble, 2004). The abundance  
33 of a given multiply-substituted isotopologue relative to the most abundant  
34 isotopologue (e.g.  $^{13}\text{CH}_3\text{D}/^{12}\text{CH}_4$ ) is reported as a fractional difference from  
35 the stochastic relative abundances,  $\Delta_i$ , where subscript  $i$  refers to the specific  
36 clumped species, and  $\Delta_i$  are reported in permil (‰). This stochastic refer-  
37 ence frame highlights whether a process has favored or discriminated against  
38 the formation of molecules with bonds containing multiple heavy isotopes  
39 (e.g.,  $^{13}\text{CH}_3\text{D}$ ), rather than only differences in bulk isotope ratios. Under  
40 equilibrium conditions at low temperatures ( $< 300$  K), the stability associ-  
41 ated with bonds between heavy isotopes in a molecule increases, promoting  
42 the formation of the isotopically clumped molecular species. As tempera-  
43 ture approaches infinity, this effect diminishes and the relative abundance of  
44 multiply-substituted molecules decreases, approaching  $\Delta_i = 0$ . Under non-  
45 equilibrium conditions at low temperatures, departures from the stochastic  
46 distribution may provide insights into the kinetics and reaction(s) of forma-  
47 tion for a given methane population's natural history.

48 Methane produced by microorganisms is characterized by significant de-  
49 pletion or "anti-clumping" in the  $\Delta^{12}\text{CH}_2\text{D}_2$  values by more than 20 to 50‰,  
50 relative to thermodynamic equilibrium (Young et al., 2017). These  $\Delta^{12}\text{CH}_2\text{D}_2$  deficits  
51 are accompanied by negligible depletion in  $\Delta^{13}\text{CH}_3\text{D}$  values (Figure 1). Sev-  
52 eral hypotheses have been put forward to explain these observed departures  
53 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

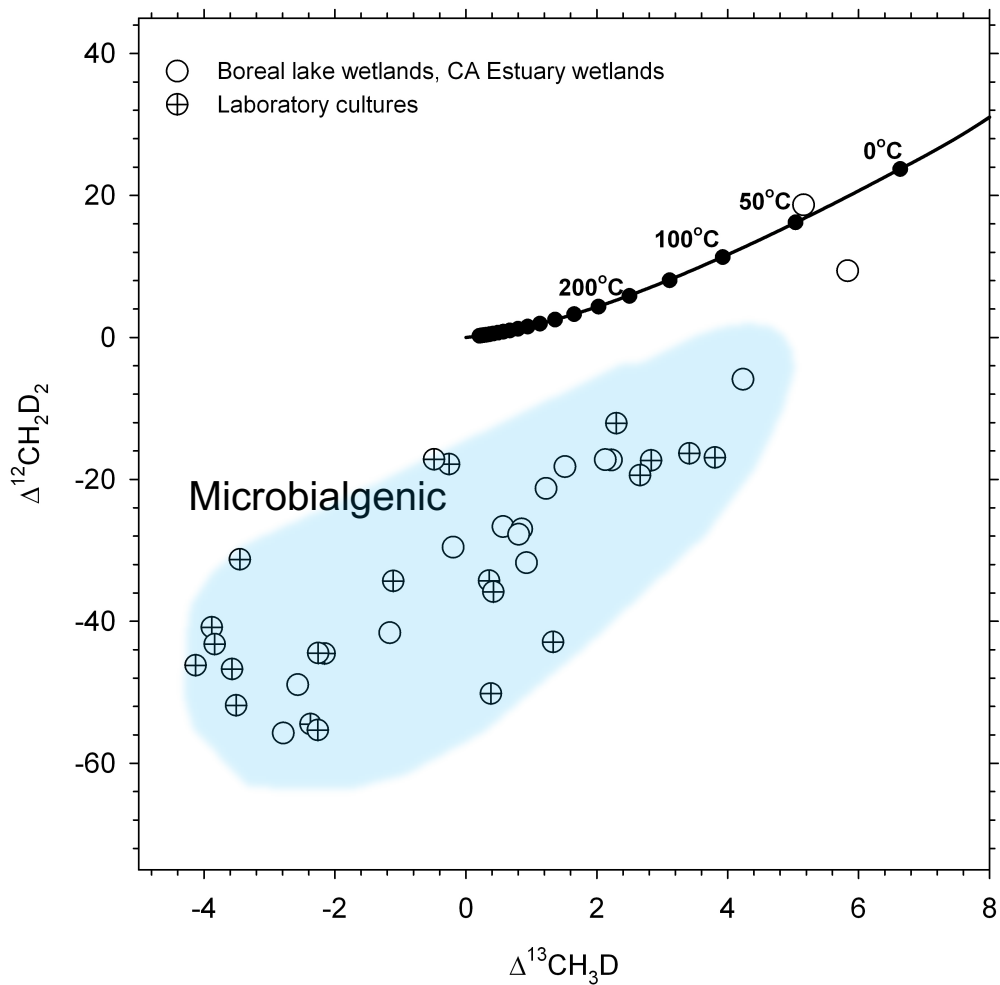


Figure 1: The distribution of laboratory axenic cultures (circles with crosses) and naturally-produced methane (open circles) by microbial methanogenesis in  $\Delta^{12}\text{CH}_2\text{D}_2$  vs.  $\Delta^{13}\text{CH}_3\text{D}$  space. The curve representing thermodynamic equilibrium is shown for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

92 between model predicted and experimentally generated isotopologue abun-  
 93 dances demonstrates that the combinatorial effect can yield markedly nega-  
 94 tive  $\Delta^{12}\text{CH}_2\text{D}_2$  values (below  $< -50\%$ ) with little influence on  $\Delta^{13}\text{CH}_3\text{D}$  values  
 95 ( $< 1\%$ ). Implications for the interpretation of experimental and natural methane  
 96 clumped isotopologue studies are discussed.

## 97 2. Principles of the Combinatorial Effect

98 The stochastic distribution is the reference frame for reporting multiply  
 99 substituted (clumped) isotopologue abundances. It is defined by the most  
 100 probable abundance of the multiply-substituted isotopologue of interest as-  
 101 suming that the isotopes comprising the molecules were randomly distributed  
 102 among all possible isotopologues. The stochastic reference is calculated by  
 103 equating fractional isotopic abundances with probabilities. To illustrate the  
 104 essence of the combinatorial effect and for simplicity we examine cases for the  
 105 formation of two diatomic molecules, carbon monoxide (CO) and molecular  
 106 oxygen ( $\text{O}_2$ ).

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

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

110 where  $x^{13}\text{C}$  is the fractional abundance of  $^{13}\text{C}$  relative to all carbon iso-  
 111 topes by number (i.e.,  $^{13}\text{C}/(^{12}\text{C} + ^{13}\text{C})$ ),  $x^{18}\text{O}$  is the fractional abundance  
 112 of  $^{18}\text{O}$  relative to all oxygen isotopes (i.e.,  $^{18}\text{O}/(^{16}\text{O} + ^{17}\text{O} + ^{18}\text{O})$ ), and  
 113  $x(^{13}\text{C}^{18}\text{O})$  is the fractional abundance of the  $^{13}\text{C}^{18}\text{O}$  isotopologue relative  
 114 to all CO molecules. The ratio of this multiply-substituted isotopologue  
 115 to the most abundant isotopic species is therefore  $x(^{13}\text{C}^{18}\text{O})/x(^{12}\text{C}^{16}\text{O})$ . It  
 116 is common to express ratios of relative isotopologue fractions in terms of  
 117 the isotope ratios of their constituent elements. In this case,  $x(^{13}\text{C}^{18}\text{O})/$   
 118  $x(^{12}\text{C}^{16}\text{O}) = (^{13}\text{C}/^{12}\text{C})(^{18}\text{O}/^{16}\text{O}) = {}^{13}R^{18}R$ . Similarly, the stochastic frac-  
 119 tional abundance of the multiply-substituted isotopologue of molecular oxy-  
 120 gen ( $\text{O}_2$ ) composed of two  $^{18}\text{O}$  atoms is

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

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

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 O<sub>2</sub> 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 O<sub>2</sub>, 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 O<sub>2</sub> 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 O<sub>2</sub> 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 O<sub>2</sub> 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

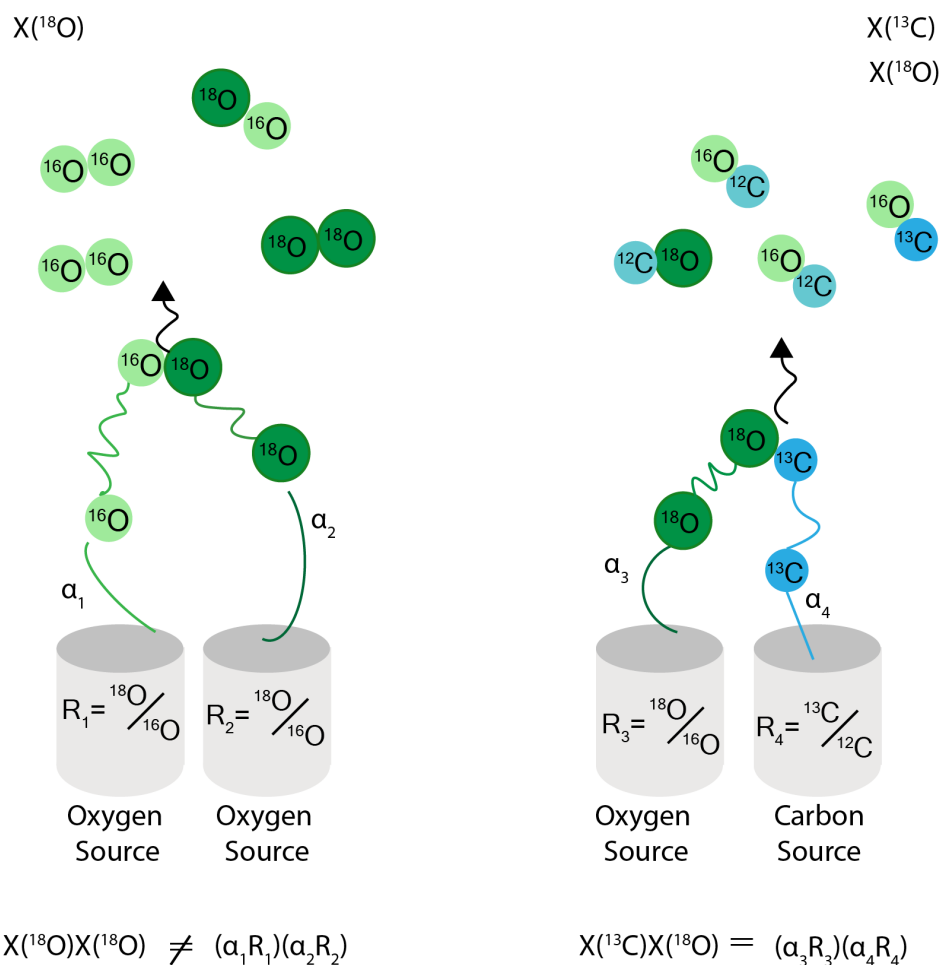


Figure 2: Schematic illustrations showing the pairing of oxygen-oxygen or oxygen-carbon isotopes when molecules of  $\text{O}_2$  or  $\text{CO}$  are formed, respectively. The potential for two separate reactant reservoirs to contribute atoms from pools of distinct isotopic composition is illustrated for each case. For  $\text{CO}$ , 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  $\text{CO}$ , offset by any fractionation, which can be individually traced. On the contrary, in the case of two oxygen reservoirs contributing to  $\text{O}_2$ , only a single average isotopic composition can be determined for the product  $\text{O}_2$  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  $\text{O}_2$  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  $\Delta_i$  value.  
169 The spuriously low and even negative  $\Delta_i$  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  $\text{N}_2$ ,  $\text{O}_2$ ,  $\text{H}_2$ ,  $\text{CH}_4$ , and  $\text{N}_2\text{O}$ . Despite the established theoretical

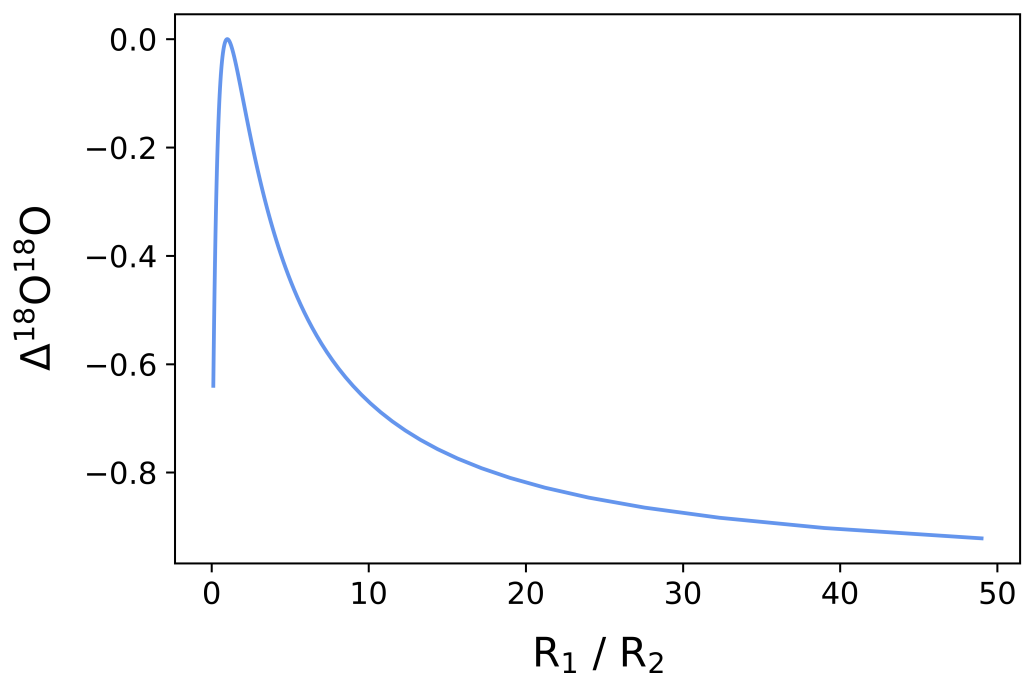


Figure 3: An illustration of the combinatorial effect seen when plotting  $\Delta^{18}\text{O}^{18}\text{O}$  as a function of the ratio of the isotopic compositions,  $R_1$  and  $R_2$ , of the distinct contributing oxygen reservoirs.

185 impact of combinatorics in influencing clumped isotopologue  $\Delta_i$  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 ( $\text{NaNO}_3$ ) 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  $\text{NaNO}_3$  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  $\text{D}_2\text{O}$  (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&Etebau 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 ( $\delta\text{D}$ ) 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 ( $\delta\text{D}$ ) 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-CH<sub>3</sub>)  
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 ( $\delta\text{D}$ ) 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  $1\sigma$  uncertainty  
260 is 0.5‰ for  $\delta D$ , 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  $4.0 \times 10^{-7}$  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

293 gel as previously described (Young et al., 2017). Due to the significant partial  
 294 pressure of N<sub>2</sub> relative to methane, and because complete separation of  
 295 methane from N<sub>2</sub> was not achieved on the first pass through the GC, multiple  
 296 aliquots were first passed through the GC, cryofocused, and co-trapped  
 297 onto the same silica gel in a single glass cold-finger (6 to 7 GC-passes per  
 298 sample bag). Once the requisite sample size of methane was captured (> 40  
 299 micromoles), the combined sample was once again cryofocused and passed  
 300 through the GC a final time to remove trace N<sub>2</sub>. The purified methane was  
 301 then transferred to the Panorama inlet system for the bulk and clumped  
 302 isotope ratio analyses following published protocols (Young et al., 2017).

### 303 3.4. Isotope notation

304 The isotopic compositions of carbon and hydrogen are reported as deviations  
 305 from the carbon and hydrogen reference materials Vienna Peedee  
 306 Belemnite and Vienna Standard Mean Ocean Water (VPDB and VSMOW,  
 307 respectively). Standard delta notation is used to express the fractional differences:  
 308

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

309 and

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

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

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

314 and

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

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

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

318 and

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

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  $\delta\text{D}$  values in the range from  
324  $-376$  to  $+6800\%$  are given in Table 1. The  $\delta\text{D}$  of product methane correlates  
325 closely with the  $\delta\text{D}$  of water (Figure 4). The methane  $\delta^{13}\text{C}$  values vary little  
326 between  $-97$  to  $-101\%$  (mean =  $-99.9 \pm 1.2\%$ ,  $1\sigma$ ), 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  $\delta\text{D}$  of  $-138 \pm 5\%$  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  $\delta\text{D}$  being  $-132 \pm 5\%$  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  $0.35 \pm 0.40\ 1\sigma$  (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  $\delta\text{D}$ , despite the significant contribution of water sourced from the medium  
342 with a range of  $\delta\text{D}$  values from  $-376$  to  $+6800\%$ . In sharp contrast, the  
343  $\Delta^{12}\text{CH}_2\text{D}_2$  values varied from  $-69.2$  to  $+7.1\%$ , a spread much greater than  
344 measurement uncertainty of  $< 1\%$  (Figure 5). The methane  $\Delta^{12}\text{CH}_2\text{D}_2$   
345 values correlated closely with the medium water  $\delta\text{D}$  in a non-linear, semi-  
346 parabolic relationship (Figure 5).

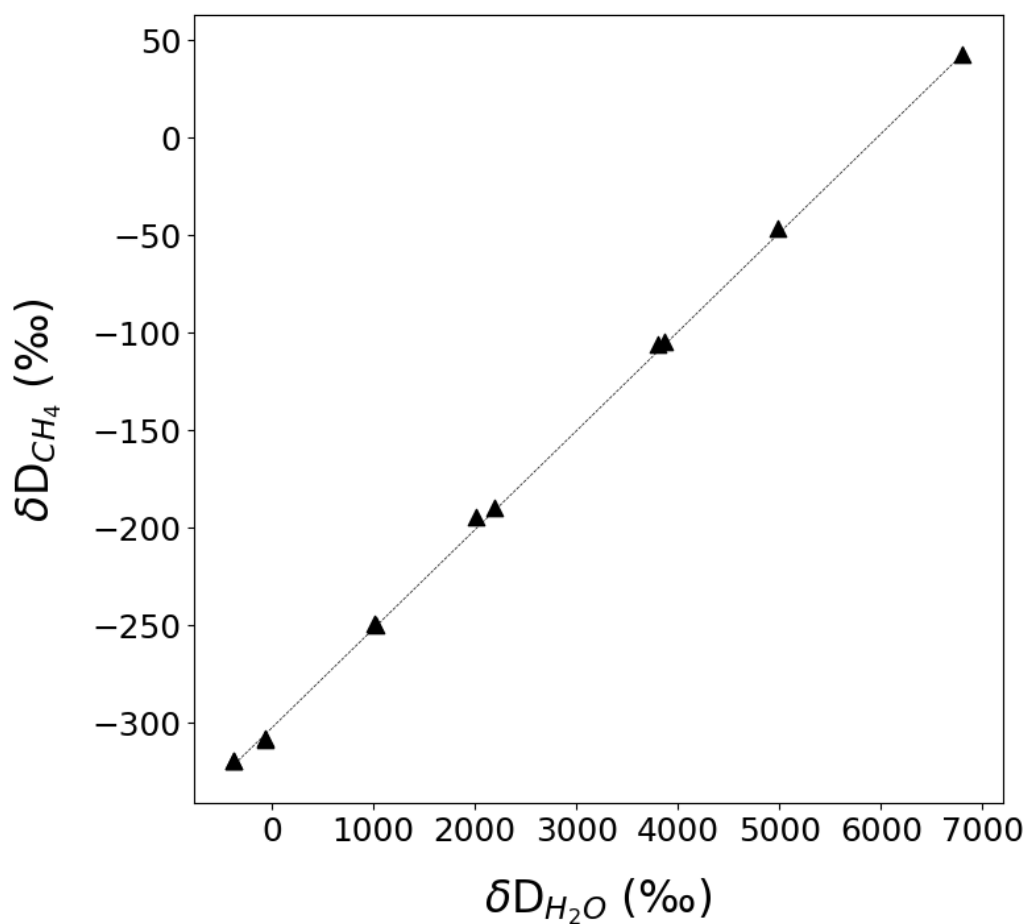


Figure 4: The D/H ratios of product methane vs. the D/H of medium water expressed in per mil deviations from VSMOW. Linear regression yields  $\delta D_{CH_4} = 0.05 \delta D_{H_2O} - 301.5$ , with an  $R^2$  of 0.999. The strong correlation between the isotopic ratios demonstrates addition of a consistent 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).



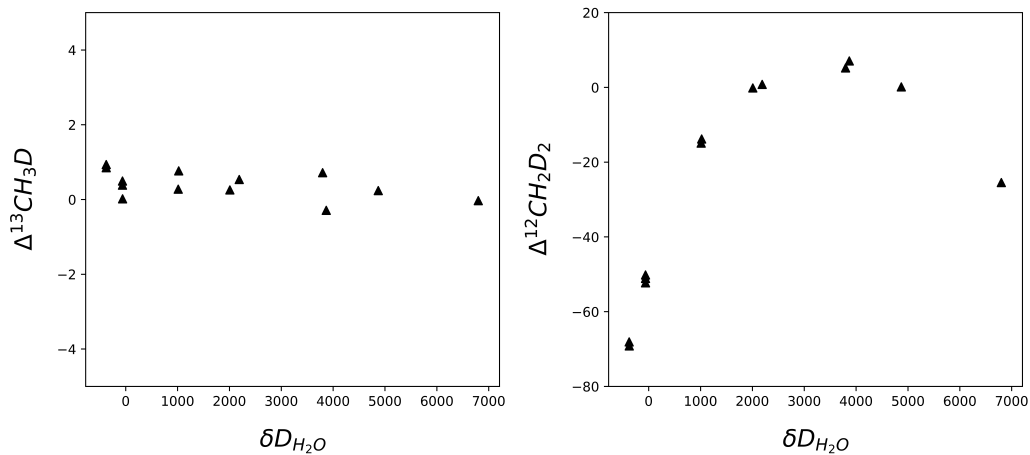


Figure 5: The measured (left)  $\Delta^{13}\text{CH}_3\text{D}$  and (right)  $\Delta^{12}\text{CH}_2\text{D}_2$  of microbialgenic methane produced in experiments with water of varying  $\delta\text{D}$  water for methane produced in various D-spiked experiments. Errors are smaller than the symbols.

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

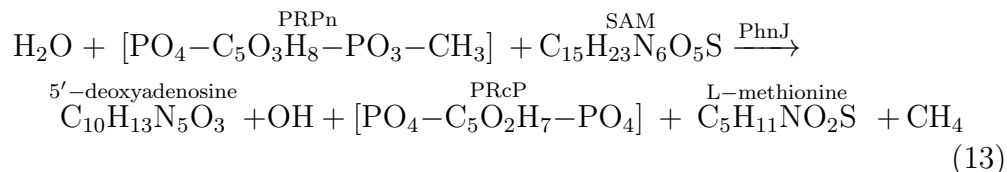
Water			Methane							
Sample ID	$\delta\text{D}$	1se	$\delta^{13}\text{C}$	1se	$\delta\text{D}$	1se	$\Delta^{13}\text{CH}_3\text{D}$	1se	$\Delta^{12}\text{CH}_2\text{D}_2$	1se
-400 B1	-376	0.5	-100.388	0.012	-319.26	0.04	0.93	0.35	-69.18	1.15
-400 B2	-376	0.5	-98.950	0.013	-319.22	0.04	0.85	0.44	-69.10	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

## 347 5. Discussion

### 348 5.1. D/H fractionation between MPn and water

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

354 are ultimately derived from the water in which MPn is biosynthesized, offset  
 355 by an unknown isotope effect. The hydrogen isotope fractionation between  
 356 methyl-group hydrogens and water in more thoroughly studied processes,  
 357 such as acetogenesis, show offsets from bulk water  $\delta D$  of hundreds of per-  
 358 mil (Hattori et al., 2010). Here we utilized synthetic MPn with an average  
 359  $\delta D$  value distinct from all medium waters utilized (see Results). Phosphorus-  
 360 starved microbes that encode the C-P lyase enzyme are able to grow on MPn  
 361 as their sole source of phosphorus by breaking down MPn (Repeta et al., 2016;  
 362 Sosa et al., 2019; Carini et al., 2014), and as a by-product, release methane  
 363 (Kamat et al., 2011). The carbon-phosphorus bond in the methyl phos-  
 364 phonate intermediate, ribose-1-methylphosphonate 5-phosphate (PRPn), is  
 365 attacked by the C-P lyase enzyme subunit PhnJ, producing ribose-1,2-cyclic-  
 366 phosphate-5-phosphate (PRcP), and releasing the methyl group used to form  
 367 methane. This reaction breaks the methyl group from the rest of the phospho-  
 368 nate compound. In vitro experiments with PhnJ showed that the hydrogen  
 369 added to the methyl group originates from the bulk solvent (water) via a sol-  
 370 vent exchangeable site on an amino acid in PhnJ (Kamat et al., 2011). The  
 371 medium-derived hydrogen is delivered to form methane either by abstraction  
 372 from the 5'-deoxyadenosine or the PhnJ amino acids. A representation of  
 373 the overall reaction that underscores the sources of hydrogen from medium  
 374 water and the MPn methyl group is



375 In our labelled water experiments, the methane  $\delta D$  increased with the water  
 376  $\delta D$  (Figure 4). Moreover, because we used the same lot of MPn for all  
 377 reactions, with one exception that was isotopically similar (see *Results*), the  
 378 methyl-group D/H was the same for all our experiments, and the increase  
 379 in the D/H of product methane solely reflects the addition of water derived  
 380 hydrogen with different D/H ratios. The tight correlation between bulk water  
 381 and methane  $\delta D$  is described by the linear regression with an  $R^2$  value of 0.999  
 382 (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  $\delta D$   
 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  $\text{CH}_3$ , and that water  
 394 contributes the last hydrogen. The hydrogen isotope conservation equation  
 395 for the formation of methane is therefore

$$0.75({}^D R_{\text{Methyl}}) + 0.25 {}^D \alpha_{\text{CH}_4/\text{H}_2\text{O}} ({}^D R_{\text{H}_2\text{O}}) = {}^D R_{\text{CH}_4} \quad (15)$$

396 where  ${}^D R_i$  is the D/H ratio for species  $i$ . Using the known  $\delta D$  values for  
 397 the product methane, medium water, and MPn methyl group from each  
 398 experiment, the fractionation factor between methane and water,  ${}^D \alpha_{\text{CH}_4/\text{H}_2\text{O}}$ ,  
 399 for the one hydrogen transferred from water to make methane was determined  
 400 by rearranging Equation 15, yielding:

$${}^D \alpha_{\text{CH}_4/\text{H}_2\text{O}} = \frac{4({}^D R_{\text{CH}_4} - (0.75 {}^D R_{\text{Methyl}}))}{{}^D R_{\text{H}_2\text{O}}}. \quad (16)$$

401 The D/H fractionation factor between water and methane calculated from  
 402 Equation 16 for the experiments with differing water  $\delta D$  values vary from  
 403 0.203 to 0.219 with an average value of  $0.209 \pm 0.005$   $1\sigma$ . The consistency is  
 404 a reflection of the good correlation between  $\delta D$  values for  $\text{CH}_4$  and  $\text{H}_2\text{O}$  (Fig-  
 405 ure 4). This fractionation factor corresponds to  ${}^D \varepsilon_{\text{methane}/\text{water}} = -790 \pm 5$   
 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 CH<sub>4</sub> 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  $\delta D$  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 $\Delta^{12}\text{CH}_2\text{D}_2$

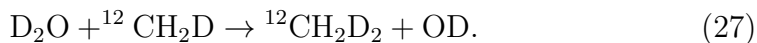
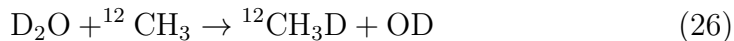
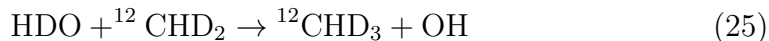
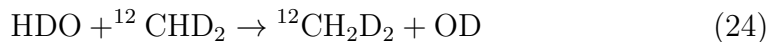
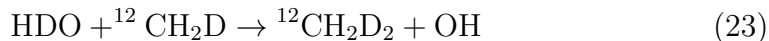
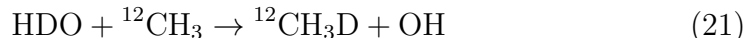
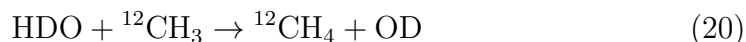
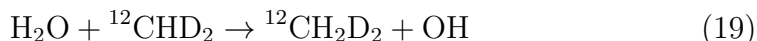
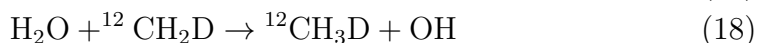
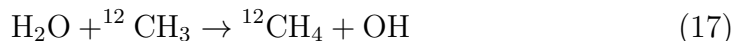
439 The combination of multiple reservoirs with distinct D/H ratios has a  
440 clear influence on the  $\Delta^{12}\text{CH}_2\text{D}_2$  value of product methane. There was no  
441 corresponding effect on  $\Delta^{13}\text{CH}_3\text{D}$  values. The negative  $\Delta^{12}\text{CH}_2\text{D}_2$  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  $\Delta_i$  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



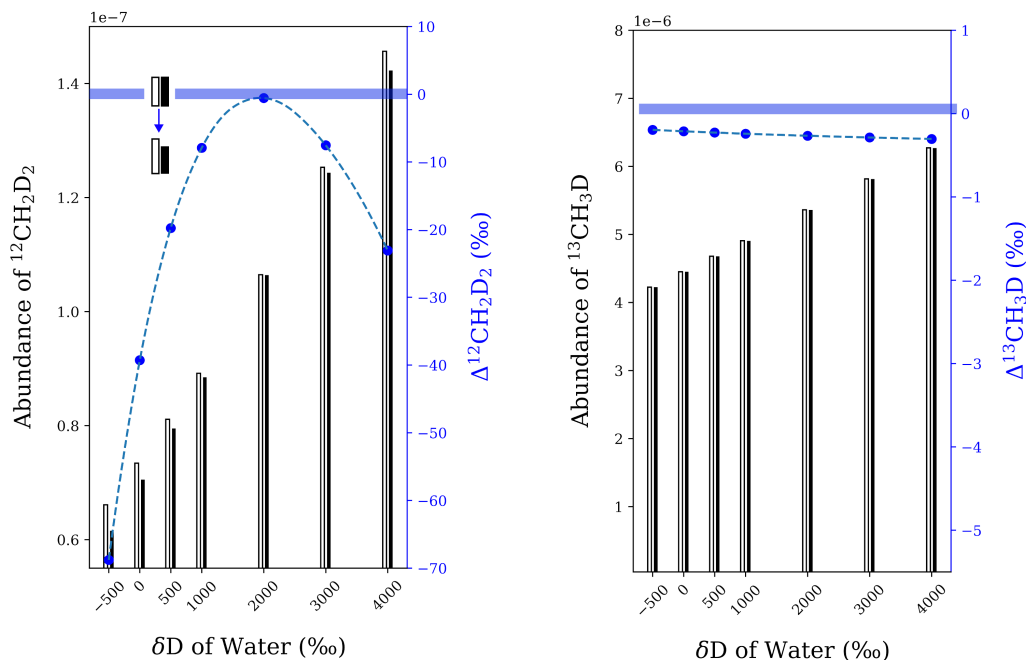


Figure 6: An illustration of the difference between the stochastic abundances of  $\Delta^{12}\text{CH}_2\text{D}_2$  and  $\Delta^{13}\text{CH}_3\text{D}$  predicted when using average isotopic compositions of the sample gas versus the isotopic compositions of the specific reactants. The abundance of the clumped isotopologues that results from the  $\delta\text{D}$  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  $\delta\text{D}$  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  $\delta\text{D}$  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  $^{13}\text{C}$ -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  $\text{CH}_4$  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 sumed that the reactants had stochastic relative abundances of isotopologues  
480 calculated from their measured bulk  $\delta^{13}\text{C}$  and  $\delta\text{D}$  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  $\delta\text{D}$  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  $\text{CHD}_2$  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  $\delta\text{D}$  values and the  $^{\text{D}}\alpha_{\text{CH}_4/\text{H}_2\text{O}}$  values in our model calculations at the water

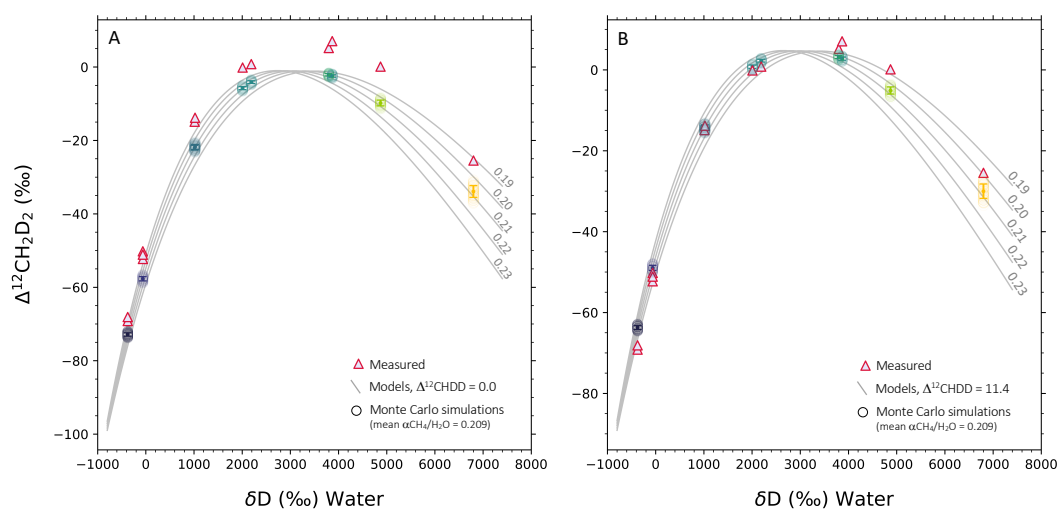


Figure 7: Plots of  $\Delta^{12}\text{CH}_2\text{D}_2$  vs. water  $\delta\text{D}$  showing our experimental results (triangles) and the two model calculations described in the text (curves). The model curves in panel A assume stochastic distributions for all of the reactant isotopologues. The model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



511  $\delta D$  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  $\delta D$  water values, 6 have measured  $\Delta^{12}\text{CH}_2\text{D}_2$  values  
517 that are within the  $4\sigma$  uncertainty in our best-fit model with  $\Delta^{12}\text{CHD}_2 =$   
518  $11.4\text{‰}$  (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  $\delta D$  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

548 isotope effects on methane  $\Delta^{12}\text{CH}_2\text{D}_2$  values. Though some of the experi-  
549 mental water values were not representative of natural systems (e.g.  $\delta\text{D}_{\text{H}_2\text{O}}$   
550  $> 0\text{‰}$ ), both the Hanover, NH, USA (house) and South Pole, Antarctica  
551 waters represent values for reservoirs in nature that can contribute hydrogen  
552 to methane. We note that the more than 300‰ difference in the  $\delta\text{D}$  of the  
553 two waters resulted in a more than 15‰ shift in methane  $\Delta^{12}\text{CH}_2\text{D}_2$  values  
554 at a fixed substrate MPn isotopic composition (Figure 5). The likelihood  
555 for such effects in nature depends on the variability of D/H in natural wa-  
556 ters and variability in fractionation factors between water and the natural  
557 methyl-type substrates used in methanogenesis. For the step-wise addition  
558 of hydrogen to carbon during hydrogenotrophic ( $\text{H}_2 + \text{CO}_2$ ) methanogene-  
559 sis, where all of the hydrogen for the product methane ultimately originates  
560 from water, endogenous combinatorial effects likely dominate due to the dif-  
561 ferent D/H fractionation factors associated with each hydrogen addition step  
562 (Young, 2019). This said, if some of the hydrogens on methane come from  
563 molecular hydrogen (Valentine et al., 2004; Kawagucci et al., 2014), the op-  
564 portunity for combinatorial influence arises. Indeed, the other canonical  
565 microbial pathways of methane formation, methylotrophic or acetotrophic,  
566 are clear analogs to the C-P lyase pathway, in that different D/H pools are  
567 accessed in the form of water and preexisting methyl groups, suggesting that  
568 exogenous combinatorial effects are at play. This is especially true given  
569 the likelihood for large D/H fractionations between water and C-H bearing  
570 organic sources of H in nature (Hattori et al., 2010), without which reser-  
571 voir (exogenous) combinatorial effects are unlikely. More work is required to  
572 understand whether or not differences in  $\Delta^{12}\text{CH}_2\text{D}_2$  values among different  
573 methanogenesis pathways (Giunta et al., 2019; Young et al., 2017) could in  
574 part reflect the different magnitudes of endogenous and exogenous combina-  
575 torial effects.

## 576 6. Conclusions

577 The source of depleted  $\Delta^{12}\text{CH}_2\text{D}_2$  values in methane produced by mi-  
578 crobes is evidenced in the aerobic C-P lyase pathway. This metabolism for  
579 methane production incorporates hydrogen from two distinct reservoirs: a  
580 methyl group, contributing three hydrogenated sites, and water that donates  
581 a single hydrogen. By varying the D/H composition of the water medium in  
582 which the bacteria generate methane, we observed the effect of combinatorics  
583 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

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

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## 601 **Research Data**

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

## 604 **References**

- 605 Ash, J., M. Egger, T. Treude, I. Kohl, B. Cragg, R. Parkes, C. Slomp,  
606 B. Sherwood Lollar, and E. Young (2019). Exchange catalysis during  
607 anaerobic methanotrophy revealed by  $^{12}\text{CH}_2\text{D}_2$  and  $^{13}\text{CH}_3\text{D}$  in methane.  
608 *Geochemical Perspectives Letters*, 26–30.
- 609 Cao, X., H. Bao, and Y. Peng (2019). A kinetic model for isotopologue  
610 signatures of methane generated by biotic and abiotic  $\text{CO}_2$  methanation.  
611 *Geochimica et Cosmochimica Acta* 249, 59–75.
- 612 Carini, P., A. E. White, E. O. Campbell, and S. J. Giovannoni (2014).  
613 Methane production by phosphate-starved SAR11 chemoheterotrophic ma-  
614 rine bacteria. *Nature Communications* 5, 1–7.

- 615 Eiler, J. M. and E. Schauble (2004).  $^{18}\text{O}^{13}\text{C}^{16}\text{O}$  in Earths atmosphere.  
616 *Geochimica et Cosmochimica Acta* 68, 4767–4777.
- 617 Giunta, T., E. D. Young, O. Warr, I. Kohl, J. L. Ash, A. Martini, S. O. Munde,  
618 D. Rumble, I. Pérez-Rodríguez, M. Wasley, D. E. LaRowe, A. Gilbert,  
619 and B. Sherwood Lollar (2019). Methane sources and sinks in continental  
620 sedimentary systems: New insights from paired clumped isotopologues  
621  $^{13}\text{CH}_3\text{D}$  and  $^{12}\text{CH}_2\text{D}_2$ . *Geochimica et Cosmochimica Acta* 245, 327–351.
- 622 Gruen, D. S., D. T. Wang, M. Könneke, B. D. Topçuoğlu, L. C. Stewart,  
623 T. Goldhammer, J. F. Holden, K.-U. Hinrichs, and S. Ono (2018). Ex-  
624 perimental investigation on the controls of clumped isotopologue and hy-  
625 drogen isotope ratios in microbial methane. *Geochimica et Cosmochimica*  
626 *Acta* 237, 339–356.
- 627 Haghnegahdar, M. A., E. A. Schauble, and E. D. Young (2017). A model  
628 for  $^{12}\text{CH}_2\text{D}_2$  and  $^{13}\text{CH}_3\text{D}$  as complementary tracers for the budget of  
629 atmospheric  $\text{CH}_4$ . *Global Biogeochemical Cycles* 31(9), 1387–1407.
- 630 Hattori, R., K. Yamada, H. Shibata, S. Hirano, O. Tajima, and N. Yoshida  
631 (2010). Measurement of the isotope ratio of acetic acid in vinegar by hs-  
632 spme-gc-tc/c-irms. *Journal of Agricultural and Food Chemistry* 58(12),  
633 7115–7118. PMID: 20504023.
- 634 Kamat, S. S., H. J. Williams, L. J. Dangott, M. Chakrabarti, and F. M.  
635 Raushel (2013). The catalytic mechanism for aerobic formation of methane  
636 by bacteria. *Nature* 497(7447), 132–136.
- 637 Kamat, S. S., H. J. Williams, and F. M. Raushel (2011). Intermediates  
638 in the transformation of phosphonates to phosphate by bacteria. *Nature*  
639 480(7378), 570–573.
- 640 Kawagucci, S., M. Kobayashi, S. Hattori, K. Yamada, Y. Ueno, K. Takai, and  
641 N. Yoshida (2014). Hydrogen isotope systematics among  $\text{h}_2\text{-h}_2\text{o-ch}_4$  dur-  
642 ing the growth of the hydrogenotrophic methanogen methanothermobac-  
643 ter thermotrophicus strain  $\delta\text{h}$ . *Geochimica et Cosmochimica Acta* 142,  
644 601–614.
- 645 Kopec, B., X. Feng, E. Posmentier, and L. Sonder (2019). Seasonal deuterium  
646 excess variations of precipitation at summit, greenland, and their climato-

- 647 logical significance. *Journal of Geophysical Research: Atmospheres* 124(1),  
648 72–91.
- 649 Metcalf, W. W., B. M. Griffin, R. M. Cicchillo, J. Gao, S. C. Janga, H. A.  
650 Cooke, B. T. Circello, B. S. Evans, W. Martens-Habbena, D. A. Stahl, and  
651 W. A. van der Donk (2012). Synthesis of Methylphosphonic Acid by Marine  
652 Microbes: A Source for Methane in the Aerobic Ocean. *Science* 337(6098),  
653 1104–1107.
- 654 Penger, J., R. Conrad, and M. Blaser (2012). Stable Carbon Isotope Frac-  
655 tionation by Methylotrophic Methanogenic Archaea. *Applied and Envi-  
656 ronmental Microbiology* 78(21), 7596–7602.
- 657 Popa, M. E., D. Paul, C. Janssen, and T. Röckmann (2019). H<sub>2</sub> clumped  
658 isotope measurements at natural isotopic abundances. *Rapid Communica-  
659 tions in Mass Spectrometry* 33(3), 239–251.
- 660 Qi, H. and T. B. Coplen (2011). Investigation of preparation techniques for  
661  $\delta^2\text{H}$  analysis of keratin materials and a proposed analytical protocol. *Rapid  
662 Communications in Mass Spectrometry* 25(15), 2209–2222.
- 663 Repeta, D. J., S. Ferrón, O. A. Sosa, C. G. Johnson, L. D. Repeta, M. Acker,  
664 E. F. DeLong, and D. M. Karl (2016). Marine methane paradox ex-  
665 plained by bacterial degradation of dissolved organic matter. *Nature Geo-  
666 science* 9(12), 884–887.
- 667 Röckmann, T., M. E. Popa, M. C. Krol, and M. E. G. Hofmann (2016).  
668 Statistical clumped isotope signatures. *Scientific Reports* 6(1), 31947.
- 669 Schimmelmann, A. (1991). Determination of the concentration and stable  
670 isotopic composition of nonexchangeable hydrogen in organic matter. *An-  
671 alytical chemistry* 63(21), 2456–2459.
- 672 Sosa, O. A., D. J. Repeta, E. F. DeLong, M. D. Ashkezari, and D. M. Karl  
673 (2019, may). Phosphate limited ocean regions select for bacterial popu-  
674 lations enriched in the carbon–phosphorus lyase pathway for phosphonate  
675 degradation. *Environmental Microbiology*, 1462–2920.14628.
- 676 Stolper, D. A., M. Lawson, C. L. Davis, A. A. Ferreira, E. V. S. Neto, G. S.  
677 Ellis, M. D. Lewan, A. M. Martini, Y. Tang, M. Schoell, A. L. Sessions, and

- 678 J. M. Eiler (2014). Formation temperatures of thermogenic and biogenic  
679 methane. *Science* 344(6191), 1500–1503.
- 680 Taenzer, L., P. Carini, A. Masterson, B. Bourque, J. Gaube, and W. Leavitt  
681 (2020). Microbial methane from methylphosphonate isotopically records  
682 source. *Geophysical Research Letters* 47(1), e2019GL085872.
- 683 Valentine, D. L. (2011). Emerging Topics in Marine Methane Biogeochem-  
684 istry. *Annual Review of Marine Science* 3(1), 147–171.
- 685 Valentine, D. L., A. Chidthaisong, A. Rice, W. S. Reeburgh, and S. C. Tyler  
686 (2004). Carbon and hydrogen isotope fractionation by moderately ther-  
687 mophilic methanogens 1 Associate editor: N. E. Ostrom. *Geochimica et*  
688 *Cosmochimica Acta* 68(7), 1571–1590.
- 689 Wang, D. T., D. S. Gruen, B. S. Lollar, K.-U. Hinrichs, L. C. Stewart, J. F.  
690 Holden, A. N. Hristov, J. W. Pohlman, P. L. Morrill, M. Konneke, K. B.  
691 Delwiche, E. P. Reeves, C. N. Sutcliffe, D. J. Ritter, J. S. Seewald, J. C.  
692 McIntosh, H. F. Hemond, M. D. Kubo, D. Cardace, T. M. Hoehler, and  
693 S. Ono (2015, apr). Nonequilibrium clumped isotope signals in microbial  
694 methane. *Science* 348(6233), 428–431.
- 695 White, A. K. and W. W. Metcalf (2004). Two C-P Lyase Operons in Pseu-  
696 domonas stutzeri and Their Roles in the Oxidation of Phosphonates, Phos-  
697 phite, and Hypophosphite. *Journal of Bacteriology* 186(14), 4730–4739.
- 698 Yeung, L. Y. (2016). Combinatorial effects on clumped isotopes and their  
699 significance in biogeochemistry. *Geochimica et Cosmochimica Acta* 172,  
700 22–38.
- 701 Yeung, L. Y., J. L. Ash, and E. D. Young (2015). Biological signatures in  
702 clumped isotopes of O<sub>2</sub>. *Science* 348(6233), 431–434.
- 703 Yeung, L. Y., S. Li, I. E. Kohl, J. A. Haslun, N. E. Ostrom, H. Hu, T. P.  
704 Fischer, E. A. Schauble, and E. D. Young (2017). Extreme enrichment in  
705 atmospheric <sup>15</sup>N<sub>2</sub>. *Science advances* 3(11), eaao6741.
- 706 Young, E. (2019). A two-dimensional perspective on ch<sub>4</sub> isotope clumping:  
707 Distinguishing process from source. In O. B. N., I. Daniel, and R. Dasgupta  
708 (Eds.), *Deep Carbon Past to Present*, pp. 388–414. Cambridge: Cambridge  
709 University Press.

- 710 Young, E., I. Kohl, B. S. Lollar, G. Etiope, D. Rumble, S. Li, M. Hagh-  
711 negahdar, E. Schauble, K. McCain, D. Foustoukos, C. Sutcliffe, O. Warr,  
712 C. Ballentine, T. Onstott, H. Hosgormez, A. Neubeck, J. Marques, I. Pérez-  
713 Rodríguez, A. Rowe, D. LaRowe, C. Magnabosco, L. Yeung, J. Ash, and  
714 L. Bryndzia (2017). The relative abundances of resolved  $^{12}\text{CH}_2\text{D}_2$  and  
715  $^{13}\text{CH}_3\text{D}$  and mechanisms controlling isotopic bond ordering in abiotic and  
716 biotic methane gases. *Geochimica et Cosmochimica Acta* 203, 235–264.
- 717 Young, E. D., D. Rumble, P. Freedman, and M. Mills (2016). A large-radius  
718 high-mass-resolution multiple-collector isotope ratio mass spectrometer for  
719 analysis of rare isotopologues of  $\text{O}_2$ ,  $\text{N}_2$ ,  $\text{CH}_4$  and other gases. *International*  
720 *Journal of Mass Spectrometry* 401, 1–10.
- 721 Yu, X., J. R. Doroghazi, S. C. Janga, J. K. Zhang, B. Circello, B. M. Griffin,  
722 D. P. Labeda, and W. W. Metcalf (2013). Diversity and abundance of  
723 phosphonate biosynthetic genes in nature. *Proceedings of the National*  
724 *Academy of Sciences* 110(51), 20759–20764.
- 725 Zhang, X., A. L. Gillespie, and A. L. Sessions (2009). Large D/H variations  
726 in bacterial lipids reflect central metabolic pathways. *Proceedings of the*  
727 *National Academy of Sciences* 106(31), 12580–12586.