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Geochemical, biological and clumped isotopologue evidence for substantial microbial methane production under carbon limitation in serpentinites of the Samail Ophiolite, Oman

Daniel B Nothaft, Alexis S Templeton, Jeemin H Rhim, David T Wang, Jabrane Labidi, Hannah M Miller, Eric S Boyd, Juerg M Matter, Shuhei Ono, Edward D Young, et al.

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Abstract

In hyperalkaline ($\text{pH} > 10$) fluids that have participated in low-temperature ($< 150^\circ\text{C}$) serpentinization reactions, the dominant form of C is often methane (CH_4), but the origin of this CH_4 is uncertain. To assess CH_4 origin in serpentinite aquifers within the Samail Ophiolite, Oman, we determined fluid chemical compositions, analyzed taxonomic profiles of fluid-hosted microbial communities, and measured isotopic compositions of hydrocarbon gases. We found that 16S rRNA gene sequences affiliated with methanogens were widespread in the aquifer. We measured clumped isotopologue ($^{13}\text{CH}_3\text{D}$ and $^{12}\text{CH}_2\text{D}_2$) relative abundances less than equilibrium, consistent with substantial microbial CH_4 production. Further, we observed an inverse relationship between dissolved inorganic C concentrations and $\delta^{13}\text{C}_{\text{CH}_4}$ across fluids bearing microbiological evidence of methanogenic activity, suggesting that the apparent C isotope effect of microbial methanogenesis is modulated by C availability. An additional source of CH_4 is evidenced by the presence of CH_4 -bearing fluid inclusions in the Samail Ophiolite and our measurement of high $\delta^{13}\text{C}$ values of ethane and propane, which are similar to those reported in studies of CH_4 -rich inclusions in rocks from the oceanic lithosphere. In addition, we observed 16S rRNA gene sequences affiliated with aerobic methanotrophs and, in lower abundance, anaerobic methanotrophs, indicating that microbial consumption of CH_4 in the ophiolite may further enrich CH_4 in ^{13}C . We conclude that substantial microbial CH_4 is produced under varying degrees of C limitation and mixes with abiotic CH_4 released from fluid inclusions. This study lends insight into the functioning of microbial ecosystems supported by water/rock reactions.

Plain Language Summary

Mantle rocks from beneath Earth's crust can be thrust to the surface, where they are exposed to rain and air containing carbon dioxide (CO_2). The groundwaters that become stored in these rocks often contain methane (CH_4 , a major component of "natural gas"), which can be formed from carbon dioxide in the subsurface. To investigate these methane-forming processes, we sampled water, gas, and suspended particles from groundwaters using wells previously drilled into the rocks. The particles contained microbes with the genetic ability to produce methane. We also precisely measured the amounts of combinations of C and H atoms of different masses (isotopes) in the natural gas to determine how it was formed. The results of these measurements suggest that microbes could actively produce a considerable amount of the methane, which mixes with methane from another source that was formed by non-biological processes, possibly long ago under different conditions than today's. Rocks like those studied here are widespread in the Solar System, so our finding that microbes live and produce methane in these rocks could help guide the search for life beyond Earth.

1 Introduction

At temperatures and pressures near the Earth's surface ($< 400^\circ\text{C}$, $< 100\text{ MPa}$), ultramafic rocks such as peridotite in contact with water are thermodynamically driven to hydrate and oxidize, forming variable amounts of serpentine, magnetite, brucite, hydrogen (H_2), and other phases (Evans, 1977; Frost, 1985; McCollom & Bach, 2009; Klein & Bach, 2009; Klein et al., 2009, 2019). This process, often called "serpentinization", can produce H_2 at temperatures at least as low as 55°C (Miller, Mayhew, et al., 2017). The resultant H_2 can be thermodynamically favored to reduce carbon dioxide (CO_2) to methane (CH_4) (Shock, 1992). The reduction of CO_2 by H_2 to form CH_4 can be catalyzed on mineral surfaces as in the Sabatier reaction (Etiope & Ionescu, 2015; Klein et al., 2019), or enzymatically through microbial methanogenesis (Whiticar, 1999).

In continental settings undergoing serpentinization, where fluid-rock reactions typically occur at low temperatures ($< 150^\circ\text{C}$), there is disagreement regarding the ori-

gin of CH₄. Three key potential CH₄ sources have been identified in these environments. One potential source is the abiotic reduction of CO₂ to CH₄ at warmer-than-present temperatures in fluid inclusions within crystals that can store CH₄ and subsequently release it. Another potential source is the abiotic, mineral-catalyzed reduction of CO₂ to CH₄ at the low temperatures that prevail in the present-day weathering environment. A third potential source is microbial methanogenesis.

Storage of CH₄ produced at temperatures of 270 °C to 800 °C in fluid inclusions in minerals such as olivine and the release of this CH₄ through subsequent chemical/physical alteration are the dominant processes contributing to CH₄ fluxes from sediment-poor seafloor hydrothermal vents (Kelley, 1996; Kelley & Früh-Green, 1999; McDermott et al., 2015; D. T. Wang et al., 2018; Labidi et al., 2020). In continental, low-temperature serpentinizing settings, however, debate continues as to whether fluid inclusions can sustain observed CH₄ fluxes (Etiope & Whiticar, 2019; Grozeva et al., 2020).

Abiotic reduction of CO₂ to CH₄ can occur at temperatures at least as low as 20 °C when catalyzed by the transition metal ruthenium (Ru) (Etiope & Ionescu, 2015). Ru is present in considerable abundance in chromitite bodies in ultramafic rock accumulations (Etiope et al., 2018), but it has only been shown to catalyze CO₂ hydrogenation under conditions where free gas phases exist (Etiope & Ionescu, 2015). The prevalence of this process, particularly in aquifers whose fluid compositions appear to be dominantly influenced by aqueous reactions with harzburgite, is another matter of ongoing debate (Etiope, 2017; Miller, Matter, et al., 2017).

Low-temperature CH₄ production can also be mediated by microbes called “methanogens”. Microbial CH₄ has traditionally been viewed as a minor/negligible source of CH₄ in serpentinizing settings. This is due in large part to the relatively ¹³C-enriched composition of CH₄ in serpentinizing settings ($\delta^{13}\text{C}$ commonly -20‰ VPDB to 5‰ VPDB), which contrasts with the more ¹³C-depleted composition of CH₄ in sedimentary settings dominated by microbial methanogenesis ($\delta^{13}\text{C}$ commonly -90‰ VPDB to -50‰ VPDB) (Etiope, 2017; Milkov & Etiope, 2018; Etiope & Whiticar, 2019). However, cultures of methanogens can produce CH₄ with minimal C isotope fractionation in H₂-rich, CO₂-poor fluids simulating serpentinizing systems (Miller et al., 2018). In these cultures, it has been inferred that the net C isotope effect of methanogenesis was attenuated due to microbial conversion of a large proportion of available CO₂ to CH₄ when CO₂ was the limiting substrate. Such results illustrate that ¹³C-enriched CH₄ in natural serpentinizing settings does not necessarily derive from non-microbial sources. Still, the quantity and isotopic composition of microbial CH₄ in serpentinizing settings remains uncertain.

In this study, we assessed sources and sinks of CH₄ in the Samail Ophiolite of Oman, a site of active, low-temperature serpentinization and carbonation. Fluids and particulates in groundwaters accessed via wells in the Samail Ophiolite have been sampled for biogeochemical studies annually from 2014 through 2018 from January to March. Microbiological and geochemical data from sampling campaigns in 2014 through 2017 and a limited number of C and H bulk stable isotope analyses of CH₄ sampled in 2014 have been previously reported (Miller et al., 2016; Rempfert et al., 2017; Kraus et al., 2021; Fones et al., 2019, 2020) Here, we present new geochemical and 16S rRNA gene amplicon sequencing data from samples acquired in 2018. We also present new bulk stable isotope data on CH₄, ethane (C₂H₆), and propane (C₃H₈) from samples obtained from 2015 through 2018. Further, we report analyses of multiply-substituted “clumped” isotopologues of CH₄, ¹³CH₃D and ¹²CH₂D₂, for the first time on samples from this ophiolite. Leveraging one of the largest longitudinal data sets on CH₄ biogeochemistry in an ophiolite, we have identified robust trends across years and hydrogeologic settings. We observed a wide range of C isotopic compositions of CH₄ and short-chain alkanes, intramolecular isotopologue disequilibrium in CH₄, and widespread occurrence of gene sequences affiliated with methanogens, which collectively indicate that substantial quantities of microbial CH₄ are produced and mix with abiotic CH₄ released from fluid inclusions in the

127 Samail Ophiolite. Our finding that microbial methanogenesis proceeds even in hyper-
 128 alkaline fluids lends insight into the functioning of microbial ecosystems that leverage
 129 reactions between water and ultramafic rocks to power metabolic processes on Earth and
 130 perhaps on other rocky bodies of the Solar System (Ménez, 2020; Glein & Zolotov, 2020).

131 2 Geologic Setting

132 The Samail Ophiolite (Figure 1) consists of pelagic sedimentary rocks (< 0.1 km),
 133 volcanic rocks (0.5 km to 2.0 km), sheeted dikes (1 km to 1.5 km), gabbro and igneous peri-
 134 dotite (0.5 km to 6.5 km), residual mantle peridotites, (8 km to 12 km), and a metamor-
 135 phic sole of greenschist- to granulite-facies metamorphic rocks (< 0.5 km) (Glennie et
 136 al., 1973; Coleman & Hopson, 1981; Lippard et al., 1986; Nicolas, 1989; Nicolas et al.,
 137 2000). The ophiolite crust formed from 96.12 Ma to 95.50 Ma, and convergence began
 138 at about the same time (Rioux et al., 2016), or up to 10 Myr earlier (Guilmette et al.,
 139 2018; Soret et al., 2020). Ophiolite emplacement continued until 78 Ma to 71 Ma (Rabū
 140 et al., 1993). Part of the ophiolite was subaerially eroded in the Late Cretaceous, then
 141 became covered in parts by Maastrichtian to Eocene limestones due to subsidence and trans-
 142 gression (Nolan et al., 1990; Skelton et al., 1990).

143 The mantle section of the ophiolite is mainly composed of highly depleted, resid-
 144 ual mantle harzburgites, together with 5% to 15% dunite, which both contain a few per-
 145 cent chromian spinel (Godard et al., 2000; Hanghøj et al., 2010; Boudier & Coleman, 1981;
 146 Collier, 2012). The extent of serpentinization is typically 30% to 60%, reaching 100%
 147 in some cases (Dewandel et al., 2003; Boudier et al., 2009; Miller et al., 2016; P. Kele-
 148 men et al., 2020). Chromitites are most often found in association with dunites near the
 149 crust-mantle transition, possibly representing bases of cumulate piles, but are also found
 150 dispersed throughout the mantle section (Rollinson, 2005).

151 Geologic reservoirs of C underlying the ophiolite include Mid Permian to Late Cre-
 152 taceous shallow marine carbonates, which host oil and gas fields in parts of northern Oman
 153 and the United Arab Emirates (Terken, 1999; Alsharhan, 1989; Etiope et al., 2015). Maas-
 154 trichtian to Eocene limestones that partially overly the ophiolite have been shown to trans-
 155 fer inorganic C to peridotites where they are in contact (de Obeso & Kelemen, 2018).
 156 C is also stored within the ophiolite, primarily in the form of carbonate minerals (Neal
 157 & Stanger, 1985; P. B. Kelemen & Matter, 2008; P. B. Kelemen et al., 2011; Noël et al.,
 158 2018). Hydration and carbonation of $> 20\,000$ km³ of peridotite continue today in the
 159 Samail Ophiolite, largely at < 60 °C (Neal & Stanger, 1983, 1985; P. B. Kelemen & Mat-
 160 ter, 2008; P. B. Kelemen et al., 2011; Streit et al., 2012; A. N. Paukert et al., 2012; Chav-
 161 agnac, Ceuleneer, et al., 2013; Chavagnac, Monnin, et al., 2013; Mervine et al., 2014; Falk
 162 et al., 2016; Miller et al., 2016; Paukert Vankeuren et al., 2019).

163 3 Methods

164 3.1 Fluid sampling and field measurements

165 Wells were drilled into the Samail Ophiolite by the Ministry of Regional Munic-
 166 ipalities and Water Resources of the Sultanate of Oman prior to 2006 (“WAB” and “NSHQ”
 167 wells in this study) and by the Oman Drilling Project in 2016 through 2018 (“CM”) (Parsons
 168 International & Co., 2005; P. Kelemen et al., 2013). Information on well location, con-
 169 struction, and water level are given in Table 1. In sampling campaigns in 2014 and 2015,
 170 a 12 V submersible Typhoon ® pump (Proactive Env. Products, Bradenton, FL, USA)
 171 with typical flow rates of 5 L·min⁻¹ was used. This pump was used in all years of sam-
 172 pling at well NSHQ04 due to partial obstruction of this well. In all other sampling from
 173 2016 onwards, a larger submersible pump (Grundfos SQ 2-85) with typical flow rates of
 174 20 L·min⁻¹ was used. The pumping depths are reported in Tables 1 and 2. For fluids sam-
 175 pled in 2018, temperature, conductivity, and pH were measured using a ColeParmer PC100

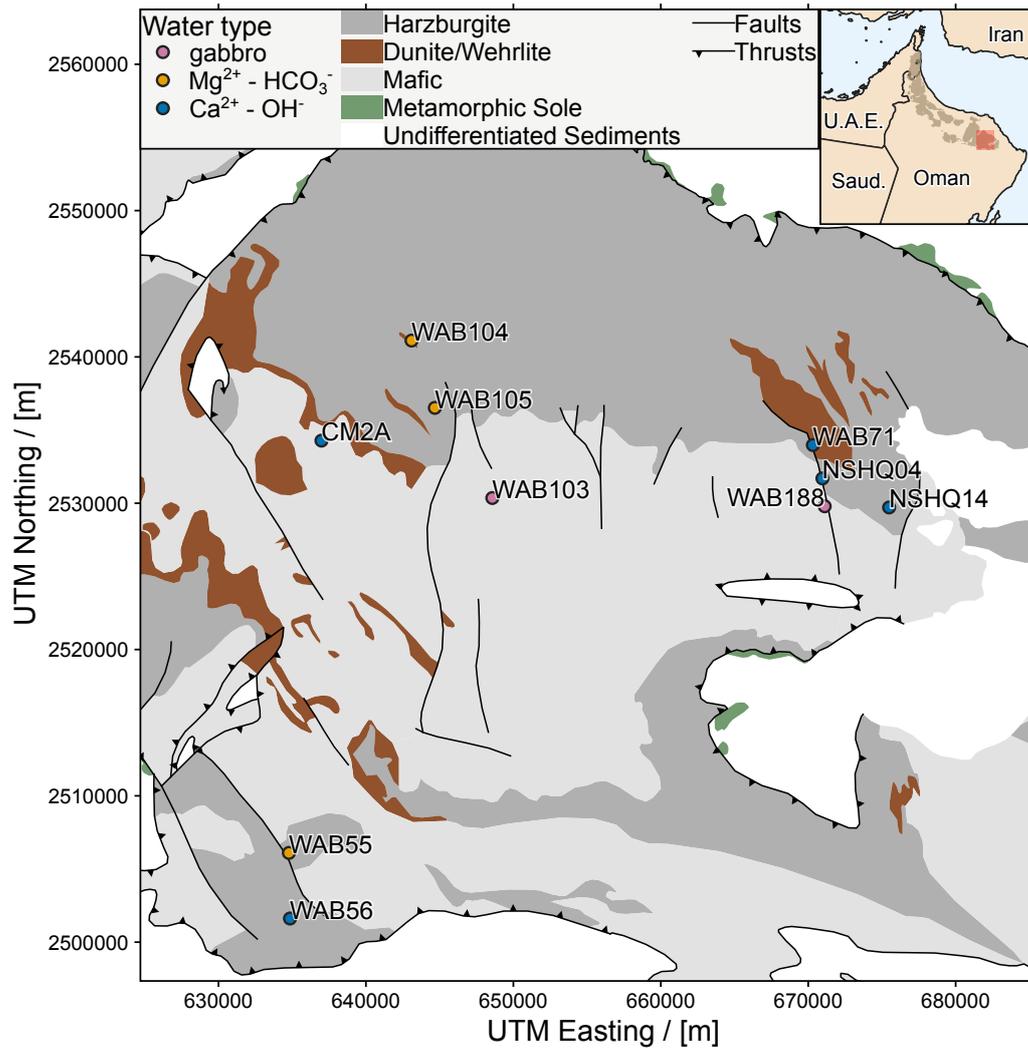


Figure 1. Study area in Samail Ophiolite, Sultanate of Oman. Geologic map data from Nicolas et al. (2000). Inset: overview of Samail Ophiolite (shaded in brown) with study area (larger map) indicated by the red shaded box. A topographic map of the study area is provided in Supporting Information Figure S1.

176 Meter, while Eh was measured using a Mettler Toledo SG2 SevenGo meter. The analytical uncertainties for temperature, conductivity, pH, and Eh are 0.5 °C, 1.0 % of measured value, 0.01 $\mu\text{S}\cdot\text{cm}^{-1}$, and 1 mV, respectively. Each well was pumped for ≥ 20 min prior to sampling. Sampling commenced once fluid pH and conductivity measurements stabilized.

181 3.2 Chemical and isotopic analyses of fluids

182 To analyze aqueous concentrations (c) of non-carbonaceous chemical species, samples were collected by passing groundwater through a 0.2 μm filter into polypropylene conical tubes. Aqueous concentrations of $\sum \text{Na}$, $\sum \text{Ca}$, $\sum \text{Mg}$, $\sum \text{Al}$, $\sum \text{Fe}$, and $\sum \text{Si}$ were measured by inductively coupled plasma (ICP) atomic emission spectroscopy on a PerkinElmer Optima 5300 (repeatability as median relative standard deviation of 3%). Aqueous concentrations of Cl^- , Br^- , F^- , and SO_4^{2-} were measured on a Dionex IC25

Table 1. Well data and field measurements.

Well	UTM coordinates (WGS-84)		Geologic description	Well depth / [mbgl]	Screen interval / [mbct]	Water level / [mbct]	Pump depth / [mbct]	Conductivity / [$\mu\text{S} \cdot \text{cm}^{-1}$]	Temperature / [$^{\circ}\text{C}$]	pH	E/h / [mV]	f_{O_2} / [bar] ^b
	easting	northing										
WAB103	648 577	2 530 362	Gabbro	101	90 – 98	15	70.	1410	34.9	8.51	167 ^a	$2.99 \cdot 10^{-36}$
WAB188	671 123	2 529 798	Gabbro, near contact with harzburgite	78	34.5 – 51	9.5	50.	1120	35.6	8.16	214 ^a	$2.01 \cdot 10^{-34}$
WAB104	643 099	2 541 124	Harzburgite	120.4	100.8 – 104	40.	85	548	33.7	8.79	133	$1.23 \cdot 10^{-37}$
WAB105	644 678	2 536 524	Harzburgite	120.5	110 – 117	16.5	60.	498	33.7	8.66	162	$2.99 \cdot 10^{-36}$
WAB55	634 777	2 506 101	Harzburgite with abundant carbonate veins, near contact with gabbro	102	8 – 97	7.5 ^a	50. ^a	1183 ^a	36.2 ^a	9.62 ^a	269 ^a	$7.17 \cdot 10^{-25}$
WAB56	634 851	2 501 617	Harzburgite	106	7 – 27	7.62 ^a	30. ^a	930. ^a	35.6 ^a	10.61 ^a	20.2 ^a	$2.81 \cdot 10^{-37}$
NSHQ04	670 971	2 531 699	Harzburgite, near fault with gabbro	304	open > 5.8	4.7	8	3350	33.4	10.51 ^a	-174	$5.14 \cdot 10^{-51}$
WAB71	670 322	2 533 981	Dumite, near fault with harzburgite	136.5	128 – 131	8.3	70.	1970	34.9	11.22	-229	$2.52 \cdot 10^{-51}$
CM2A	636 988	2 534 284	Mostly dumite with occasional gabbro and harzburgite	400.	open > 23.7	13.4	75	2860	33.6	11.32	n.d.	n.d.
NSHQ14	675 495	2 529 716	Harzburgite	304	open > 5.8	9.2	85	2670	36.7	11.39	-253 ^a	$1.19 \cdot 10^{-51}$

Measurements refer to sampling February–March, 2018, unless noted. Well elevations are given in Supporting Information Figure S1. *Abbreviations:* n.d., not determined; mbgl, meters below ground level; mbct, meters below casing top. Casings extend ~ 1 m above ground level.

^aNot determined during 2018 sampling, so most recent prior data is reported (2015 to 2017; (Rempfert et al., 2017; Fones et al., 2019)).

^bCalculated from temperature, pH, and E/h . Where one or more of these parameters were obtained during different sampling years, f_{O_2} should be considered a representative estimate.

Table 2. Isotopic compositions of CH₄, C₂H₆, and C₃H₈.

Well	Sample year	Pump depth / [mbct]	laboratory	$\delta^{13}\text{C}_{\text{CH}_4}$	$\delta\text{D}_{\text{CH}_4}$	$\Delta^{13}\text{CH}_3\text{D}$	$\Delta^{12}\text{CH}_2\text{D}_2$	$\delta^{13}\text{C}_{\text{C}_2\text{H}_6}$	$\delta^{13}\text{C}_{\text{C}_3\text{H}_8}$
WAB188	2018	50.	CUB	-86.7	n.d.	n.d.	n.d.	n.d.	n.d.
	2017	78	CUB	-60.8	n.d.	n.d.	n.d.	n.d.	n.d.
	2015	20.	LBNL	-71.3	n.d.	n.d.	n.d.	n.d.	n.d.
WAB56	2015	12	LBNL	-83.2	n.d.	n.d.	n.d.	n.d.	n.d.
NSHQ04	2018	8	CUB	4.7	-229	n.d.	n.d.	n.d.	n.d.
			UCLA	4.177	-227.396	0.229 ± 0.288	-24.502 ± 0.944	n.d.	n.d.
	2017	5.8	CUB	6.8	-225	n.d.	n.d.	n.d.	n.d.
			MIT	3.59	-229.67	0.12 ± 0.17	n.d.	n.d.	n.d.
	2015	22	LBNL	0.8	-209	n.d.	n.d.	n.d.	n.d.
			MIT	1.60	-230.00	0.72 ± 0.29	n.d.	n.d.	n.d.
2014	18	LBNL	2.4	-205	n.d.	n.d.	n.d.	n.d.	
WAB71	2018	70.	CUB	3.6	-307	n.d.	n.d.	n.d.	n.d.
	2017	50.	CUB	3.9	-313	n.d.	n.d.	n.d.	n.d.
	2016	50.	LBNL	3.0	n.d.	n.d.	n.d.	n.d.	n.d.
	2015	18	LBNL	2.9	n.d.	n.d.	n.d.	n.d.	n.d.
CM2A	2018	75	CUB	-4.3	-206	n.d.	n.d.	n.d.	n.d.
			MIT	-3.83	-190.32	2.87 ± 0.57	n.d.	n.d.	n.d.
			UCLA	-4.710	-197.73	2.638 ± 0.284	-1.267 ± 0.886	n.d.	n.d.
NSHQ14	2018	85	CUB	-2.3	-314	n.d.	n.d.	n.d.	n.d.
			MIT	-5.02	-311.73	0.77 ± 0.44	n.d.	n.d.	n.d.
			UCLA	-3.352	-293.58	2.074 ± 0.298	-0.204 ± 1.358	n.d.	n.d.
	2017	85	CUB	0.2	-271	n.d.	n.d.	-6.0	+3.3
			MIT	-0.08	-268.82	0.69 ± 0.23	n.d.	n.d.	n.d.
	2016	70.	LBNL	1.8	-273	n.d.	n.d.	n.d.	n.d.
MIT			-6.89	-308.52	0.69 ± 0.17	n.d.	n.d.	n.d.	
2015	20.	LBNL	3.7	n.d.	n.d.	n.d.	n.d.	n.d.	
2014	260.	LBNL	3.0	-232	n.d.	n.d.	n.d.	n.d.	

All isotopic values reported in ‰ units. $\delta^{13}\text{C}$ and δD reported in the VPDB and VSMOW reference frames, respectively. Data from 2014 previously reported by Miller et al. (2016). *Abbreviations:* n.d., not determined; mbct, meters below casing top.

188 ion chromatograph with an AS9-HC IonPac column, with the exception of NO_3^- , which
 189 was measured on a Dionex 4500I ion chromatograph with an IonPac AS14 column us-
 190 ing EPA method 300.0 (analytical uncertainty of 2%).

191 The concentration and $\delta^{13}\text{C}$ of dissolved inorganic C ($\sum \text{CO}_2$) were measured by
 192 acidification of water samples and transfer of resultant CO_2 (g) via a Thermo Fisher Gas-
 193 Bench II to a Thermo Delta V Plus isotope ratio mass spectrometer. We optimized the
 194 methods of Assayag et al. (2006) for the wide range of $c_{\sum \text{CO}_2}$ observed in ophiolite ground-
 195 waters. Complete methodological details are available in D. B. Nothaft (2019b). Sam-
 196 ple $\delta^{13}\text{C}$ values were converted to the VPDB reference frame using measured $\delta^{13}\text{C}$ val-
 197 ues of international reference materials (Harding Iceland Spar and LSVEC). Isotopic ref-
 198 erence frame calculations were performed using the Isoverse suite of packages (Kopf et
 199 al., 2021) for the statistical programming language, R (R Core Team, 2019).

200 Water $\delta^{18}\text{O}$ and δD were measured on a Picarro L2120-i cavity ring down spectrom-
 201 eter. The instrument analyzed each sample six times, excluding the first three analyses
 202 to avoid memory effects. Reported precision is the standard deviation of the last three
 203 measurements. Reported accuracy is the mean difference between accepted values and
 204 measured values of standards. Mean precision in the run was 0.06 ‰ for $\delta^{18}\text{O}$ and 0.23 ‰
 205 for δD ; mean accuracy was 0.04 ‰ for $\delta^{18}\text{O}$ and 0.47 ‰ for δD .

206 Gases dissolved in pumped groundwaters were sampled by injecting water into N_2
 207 purged vials for headspace gas analysis using methods described by Miller et al. (2016)
 208 in field campaigns occurring from 2014 to 2017. In addition, the bubble strip method
 209 (modified from (Kampbell et al., 1998)) was used from 2016 to 2018. Details on bub-
 210 ble strip gas sampling are available in D. B. Nothaft (2019a). The gas concentrations
 211 reported in this study were determined from bubble strip samples. These concentrations
 212 were measured on an SRI 8610C gas chromatograph (GC) with N_2 as the carrier gas.
 213 H_2 , CO , CH_4 , and CO_2 were separated with a 2 mm by 1 mm ID micropacked ShinCar-
 214 bon ST column, whereas alkanes of 2 to 6 C atoms (“ $\text{C}_2\text{--C}_6$ short-chain alkanes”) were
 215 separated with a PORAPAK Q 6 ft by 0.085 in ID column. Peak intensities were mea-
 216 sured concurrently using a thermal conductivity detector (TCD) and a flame ionization
 217 detector (FID) and calibrated with standard gas mixes (Supelco Analytical, Bellefonte,
 218 PA, USA; accuracy of $\pm 2\%$ of reported concentration). Measurement repeatability ex-
 219 pressed as relative standard deviation was 5% over most of the calibrated range. The
 220 limit of quantitation was defined as the signal at which the relative standard deviation
 221 increased to 20%. In 2018, H_2 and CO were analyzed on a Peak Performer 1 gas chro-
 222 matograph equipped with a reducing compound photometer (RCP). Due to the high sen-
 223 sitivity of the RCP, the signal at limit of quantitation (S_{LQ}) for these analyses was de-
 224 fined as $S_{\text{LQ}} = S_{\text{b}} + 10 \cdot \sigma_{\text{b}}$, where S_{mb} is the mean signal of blanks prepared in field
 225 and σ_{b} is the population standard deviation of these blanks, in accordance with Amer-
 226 ican Chemical Society guidelines (MacDougall et al., 1980). Gaseous concentrations were
 227 converted to aqueous concentrations using gas solubilities (Sander, 2015) and corrected
 228 for temperature and volume changes between sampling and analysis.

229 Prior to 2017, bulk stable isotope analyses of CH_4 were conducted at the Center
 230 for Isotope Geochemistry at the Lawrence Berkeley National Laboratory (LBNL) by gas
 231 chromatography/combustion/pyrolysis isotope-ratio mass spectrometry (GC/C/Pyr/IRMS)
 232 using methods described by Miller et al. (2016). The measurement repeatability expressed
 233 as 1 sample standard deviation (s) for these analyses is $\pm 0.2\%$ for $\delta^{13}\text{C}$ and $\pm 5\%$ for
 234 δD .

235 From 2017 onwards, bulk stable isotope analyses of CH_4 and co-occurring alkane
 236 gases were conducted at the University of Colorado - Boulder (CUB) by GC/C/Pyr/IRMS
 237 using a Trace 1310 GC equipped with an Agilent J & W GS-CarbonPLOT column (30 m
 238 length, 0.32 mm ID, 3.0 μm film) coupled to a Thermo Scientific MAT253 IRMS. CH_4
 239 isotope standards purchased from Airgas (uncertainties of $\pm 0.3\%$ for $\delta^{13}\text{C}$ and $\pm 5\%$

240 for δD) were used for calibration. Over the range of peak amplitudes of analyses reported
 241 here, the repeatability expressed as 1 s on analyses of standards is $\pm 0.6\text{‰}$ for $\delta^{13}\text{C}$ and
 242 $\pm 7\text{‰}$ for δD . The analytical uncertainty (accuracy) expressed as 1 standard error on
 243 a 3-point calibration was $< 0.3\text{‰}$ for $\delta^{13}\text{C}$ and $< 9\text{‰}$ for δD (Supporting Information
 244 Section S1).

245 The relative abundances of CH_4 isotopologues, including the doubly-substituted
 246 isotopologue, $^{13}\text{CH}_3\text{D}$, were measured at the Massachusetts Institute of Technology (MIT)
 247 by tunable infrared laser direct absorption spectroscopy following the methods described
 248 by Ono et al. (2014). Abundances of CH_4 isotopologues, including both $^{13}\text{CH}_3\text{D}$ and $^{12}\text{CH}_2\text{D}_2$,
 249 were measured at the University of California, Los Angeles (UCLA) by high-mass-resolution
 250 gas-source isotope ratio mass spectrometry following the procedure of E. D. Young et
 251 al. (2016). The abundance of $^{13}\text{CH}_3\text{D}$ relative to a random (stochastic) distribution of
 252 isotopes among the isotopologues in a CH_4 sample is described by its $\Delta^{13}\text{CH}_3\text{D}$ value,
 253 which is defined as: $\Delta^{13}\text{CH}_3\text{D} = \ln Q$, where Q is the reaction quotient of the isotope
 254 exchange reaction:



255 Analogous expressions can be written for doubly-deuterated CH_4 , $^{12}\text{CH}_2\text{D}_2$.

256 3.3 16S rRNA gene sequencing and analysis

257 Biomass for DNA extraction was concentrated by pumping 5 L to 20 L of ground-
 258 water through Millipore polycarbonate inline filters (0.45 μm pore diameter, 47 mm fil-
 259 ter diameter). At well NSHQ04, a 0.22 μm pore diameter polyethersulfone Millipore Sterivex
 260 filter was used instead due to the lower-flow pump used at this well (Section 3.1). Fil-
 261 ters were placed in cryovials, transported frozen in liquid N_2 , and stored in a -70 °C freezer
 262 until extraction. DNA was extracted from one quarter subsamples of each filter using
 263 a Qiagen PowerSoil DNA extraction kit. The V4 hypervariable region of the 16S rRNA
 264 gene was amplified by PCR in duplicate reactions using the 515 (Parada) - 806R (Ap-
 265 prill) primer pair modified to include Illumina adapters and the appropriate error-correcting
 266 barcodes. Each 25- μL reaction mixture included 12.5 μL of Promega HotStart Master-
 267 mix, 10.5 μL of PCR-grade water, 1 μL of PCR primers (combined at 10 μM), and 1 μL
 268 of purified genomic DNA. PCR consisted of an initial step at 94 °C for 3 min followed
 269 by 35 cycles of 94 °C for 45 s, 50 °C for 1 min, and 72 °C for 1.5 min. PCR concluded with
 270 a final elongation step at 72 °C for 10 min. No-template controls and DNA extraction
 271 controls were subjected to PCR to check for potential contamination in our PCR and
 272 DNA extraction reagents, respectively. Amplification was evaluated via electrophoresis
 273 in a 2% agar gel. Amplicons from duplicate reactions were pooled, cleaned, and their
 274 concentrations normalized using a Thermo Fisher SequelPrep normalization plate kit.
 275 Amplicons were sequenced on an Illumina MiSeq at the CUB Next-Generation Sequenc-
 276 ing Facility with 2-by-150 bp paired-end chemistry.

277 Sequences were demultiplexed with idemp (Wu, 2017). The resultant fastq files were
 278 quality filtered using Figaro v1.1.1 (Weinstein, 2019) and the DADA2 v1.16 R package
 279 (Callahan et al., 2016). Amplicon sequence variants were assigned taxonomy to the genus
 280 level using the RDP classifier (Q. Wang et al., 2007) trained on the Silva SSU 138 ref-
 281 erence database (Quast et al., 2012) using the DADA2 assignTaxonomy function. Species
 282 level assignments were based on exact matching between amplicon sequence variants and
 283 sequenced reference strains using the DADA2 addSpecies function. Sequences assigned
 284 to mitochondria, chloroplast, and Eukaryota, or not assigned at the domain level (col-
 285 lectively $< 1\%$ of sequences), were removed. After all of the above filtering, 24 000 to
 286 40 000 reads per sample remained for the samples presented here obtained in 2018. In
 287 addition, 16S rRNA gene sequencing data from previous Oman sampling campaigns (2014
 288 through 2017; (Miller et al., 2016; Rempfert et al., 2017; Kraus et al., 2021)) were re-

289 processed in accordance with the methods outlined here to facilitate comparisons across
 290 the data sets. The complete data processing pipeline for samples across all years, from
 291 raw data provided by the sequencing facility through to taxonomic assignment, are avail-
 292 able in D. B. Nothhaft, Rempfert, and Kraus (2021). Additional analyses and plotting
 293 can be found in D. B. Nothhaft, Templeton, et al. (2021). For samples presented in this
 294 study, demultiplexed fastq files (without additional processing) are also accessible on the
 295 NCBI Short Read Archive under accession PRJNA655565.

296 3.4 Thermodynamic calculations

297 Oxidation-reduction potential, pH, and concentrations of major ions and $\sum \text{CO}_2$
 298 were used as inputs for the modeling software PHREEQC (Charlton & Parkhurst, 2011;
 299 Parkhurst & Appelo, 2013), with which fluids were speciated using the LLNL database.
 300 Activities of formate and acetate were separately calculated according to the Debye-Hückel
 301 equation. Activities of the aqueous gases were assumed equivalent to their concentra-
 302 tions, which is reasonable for neutral species in low ionic strength solutions. Standard
 303 Gibbs free energies (ΔG_r°) of the CH_4 -forming reactions were calculated using the pro-
 304 gram SUPCRTBL (Johnson et al., 1992; Zimmer et al., 2016) using conditions of 1 bar
 305 and 35°C to approximate *in situ* conditions. Gibbs free energies were then calculated
 306 as $\Delta G_r = \Delta G_r^\circ + RT \ln Q_r$, where R is the universal gas constant, T is temperature,
 307 and Q_r is the reaction quotient. All of the above calculations and software inputs and
 308 outputs can be found in D. B. Nothhaft, Templeton, et al. (2021).

309 4 Results and discussion

310 4.1 Controls on groundwater chemistry

311 To assess the source and reaction histories of Samail Ophiolite groundwaters, we
 312 measured their stable isotopic compositions and solute concentrations. Groundwater δD
 313 and $\delta^{18}\text{O}$ plotted near local and global meteoric water lines (Weyhenmeyer et al., 2002;
 314 Terzer et al., 2013), indicating that the groundwaters derive from rain (Table 3; Support-
 315 ing Information Figure S2; (Matter et al., 2006; Miller et al., 2016; Paukert Vankeuren
 316 et al., 2019)). The sampled groundwaters included oxidized and moderately alkaline Mg^{2+} -
 317 HCO_3^- waters, typical of reaction with peridotite in communication with the atmosphere,
 318 and reduced and hyperalkaline Ca^{2+} - OH^- waters, typical of extensive hydration and
 319 oxidation of peridotite in closed-system conditions with respect to the atmosphere (Ta-
 320 ble 3; Barnes et al., 1967; Barnes & O’Neil, 1969; Neal & Stanger, 1985; Bruni et al.,
 321 2002; Cipolli et al., 2004; P. B. Kelemen et al., 2011; A. N. Paukert et al., 2012)). Ca^{2+} -
 322 OH^- waters had higher conductivities ($930 \mu\text{S}\cdot\text{cm}^{-1}$ to $3350 \mu\text{S}\cdot\text{cm}^{-1}$) than Mg^{2+} -
 323 HCO_3^- waters ($498 \mu\text{S}\cdot\text{cm}^{-1}$ to $1183 \mu\text{S}\cdot\text{cm}^{-1}$) (Table 1). The increase in conductiv-
 324 ity from Mg^{2+} - HCO_3^- waters to Ca^{2+} - OH^- waters is driven by enrichments in Ca^{2+}
 325 derived from dissolution of primary silicate minerals in addition to Na^+ and Cl^- derived
 326 from mineral dissolution, sea spray, and/or leaching of sea salts introduced during sub-
 327 seafloor alteration and/or ophiolite emplacement (Neal & Stanger, 1985; Stanger, 1986;
 328 Murad & Krishnamurthy, 2004; A. N. Paukert et al., 2012; Rempfert et al., 2017). The
 329 increase in pH from Mg^{2+} - HCO_3^- waters (pH 8.66 to 9.62) to Ca^{2+} - OH^- waters
 330 (10.51 to 11.39) was accompanied by a shift to lower f_{O_2} and Eh ($\sim 10^{-51}$ bar and -174 mV
 331 to -253 mV, respectively, in most Ca^{2+} - OH^- waters) (Table 1), indicating reduced
 332 conditions in Ca^{2+} - OH^- waters.

333 Concentrations of $\sum \text{CO}_2$ were relatively high in Mg^{2+} - HCO_3^- waters and gab-
 334 bro waters (up to $3490 \mu\text{mol}\cdot\text{L}^{-1}$), but below the limit of quantitation ($< 12 \mu\text{mol}\cdot$
 335 L^{-1}) in most Ca^{2+} - OH^- waters (Table 3). This is consistent with water-harzburgite
 336 reaction path modeling that terminates at chrysotile-brucite-diopside-calcite equilibrium,
 337 corresponding to a $c_{\sum \text{CO}_2}$ of $8 \mu\text{mol}\cdot\text{L}^{-1}$ at 25°C and 1 bar (Leong & Shock, 2020).

Table 3. Chemical and isotopic composition of water samples.

Well	δD_{H_2O}	$\delta^{18}O_{H_2O}$	$\sum CO_2$	$\delta^{13}C_{\sum CO_2}$	$\sum Na$	$\sum Ca$	$\sum Mg$	$\sum Fe$	$\sum Si$	NO_3^-	SO_4^{2-}	Cl^-	Br^-
<i>gabbro-hosted groundwaters</i>													
WAB103	-0.5	0.34	$2.67 \cdot 10^3$	-13.54	$1.18 \cdot 10^3$	$2.58 \cdot 10^2$	$1.87 \cdot 10^3$	7.35	$4.63 \cdot 10^2$	$4.72 \cdot 10^2$	$1.57 \cdot 10^3$	$6.25 \cdot 10^3$	$1.39 \cdot 10^2$
WAB188	-2.1	-0.71	$3.48 \cdot 10^3$	-13.52	$4.06 \cdot 10^3$	$1.41 \cdot 10^3$	$1.82 \cdot 10^3$	$2.90 \cdot 10^1$	$4.77 \cdot 10^2$	$3.21 \cdot 10^2$	$1.41 \cdot 10^3$	$4.22 \cdot 10^3$	$6.78 \cdot 10^1$
<i>Mg²⁺ - HCO₃⁻ groundwaters</i>													
WAB104	-0.5	-0.53	$3.62 \cdot 10^3$	-13.88	$7.53 \cdot 10^2$	$1.96 \cdot 10^2$	$2.30 \cdot 10^3$	3.88	$4.15 \cdot 10^2$	$3.14 \cdot 10^2$	$3.80 \cdot 10^2$	$7.76 \cdot 10^2$	3.55
WAB105	0.4	0.50	$3.32 \cdot 10^3$	-10.88	$1.18 \cdot 10^3$	$2.58 \cdot 10^2$	$1.87 \cdot 10^3$	4.83	$2.83 \cdot 10^2$	$3.02 \cdot 10^2$	$2.92 \cdot 10^2$	$8.54 \cdot 10^2$	8.60
WAB55	2.2	0.26	$2.40 \cdot 10^3$	-12.63	$4.44 \cdot 10^3$	$5.06 \cdot 10^1$	$3.34 \cdot 10^3$	2.52	$3.58 \cdot 10^1$	$3.02 \cdot 10^2$	$8.03 \cdot 10^2$	$6.54 \cdot 10^3$	$1.12 \cdot 10^2$
<i>Ca²⁺ - OH⁻ groundwaters</i>													
WAB56	n.d.	n.d.	$1.3 \cdot 10^{2a}$	n.d.	$3.56 \cdot 10^{3a}$	$5.43 \cdot 10^{2a}$	1.00^a	n.d.	$2.22 \cdot 10^2$	3.00^a	6.00^a	$1.33 \cdot 10^{1a}$	$1.79 \cdot 10^{-1a}$
NSHQ04	-15 ^a	-3.0 ^a	$1.8 \cdot 10^1$	-29.7	$1.04 \cdot 10^{4a}$	$7.79 \cdot 10^{3a}$	$1.80 \cdot 10^{1a}$	$8.20 \cdot 10^{-1a}$	$3.60 \cdot 10^{1a}$	3.00^a	$6.83 \cdot 10^{2a}$	$1.82 \cdot 10^{4a}$	1.25^a
WAB71	-3.0	-0.40	$< 1.2 \cdot 10^1$	n.d.	$6.25 \cdot 10^3$	$4.14 \cdot 10^3$	$< 2.06 \cdot 10^{-1}$	$8.48 \cdot 10^1$	$2.35 \cdot 10^1$	$1.84 \cdot 10^2$	$6.08 \cdot 10^1$	$1.17 \cdot 10^4$	$1.50 \cdot 10^2$
CM2A	1.7	0.67	$< 1.2 \cdot 10^1$	n.d.	$2.07 \cdot 10^4$	$1.75 \cdot 10^3$	9.49	$4.03 \cdot 10^1$	$2.81 \cdot 10^1$	$1.64 \cdot 10^2$	$5.56 \cdot 10^2$	$1.85 \cdot 10^4$	$2.48 \cdot 10^2$
NSHQ14	0.2	0.43	$< 1.2 \cdot 10^1$	n.d.	$1.03 \cdot 10^4$	$3.60 \cdot 10^3$	6.23	$8.48 \cdot 10^1$	$1.03 \cdot 10^1$	$3.60 \cdot 10^2$	$1.57 \cdot 10^2$	$1.36 \cdot 10^4$	$1.67 \cdot 10^2$

Concentrations reported in $\mu\text{mol} \cdot \text{L}^{-1}$. \sum indicates the sum of all dissolved species of the element. All δ values reported in ‰ units. $\delta^{18}O$ and δD reported relative to VSMOW. $\delta^{13}C$ reported relative to VPDB. Samples obtained in February-March 2018, unless noted. *Abbreviations:* n.d., not determined.

^aNot determined during 2018 sampling, so most recent prior data is reported (2015 to 2017; (Rempfert et al., 2017; Fones et al., 2019)).

338 Literature values for $c_{\sum \text{CO}_2}$ in ophiolitic $\text{Ca}^{2+}-\text{OH}^-$ waters are often higher than those
 339 predicted by reaction path modeling, but the lower range of reported values approaches
 340 $1 \mu\text{mol} \cdot \text{L}^{-1}$ (Barnes et al., 1967; Barnes & O’Neil, 1969; Barnes et al., 1978; Neal &
 341 Stanger, 1985; Bruni et al., 2002; Cipolli et al., 2004; A. N. Paukert et al., 2012; Falk et
 342 al., 2016; Brazelton et al., 2017; Canovas III et al., 2017; Crespo-Medina et al., 2017; Rempfert
 343 et al., 2017; Fones et al., 2019; Paukert Vankeuren et al., 2019). This spread in the data
 344 could reflect groundwater mixing, atmospheric contamination during sampling, differ-
 345 ences in reaction temperature and progress, and/or kinetic inhibitions to carbonate min-
 346 eral precipitation. In $\text{Mg}^{2+}-\text{HCO}_3^-$ waters and waters from gabbroic aquifers, $\delta^{13}\text{C}_{\sum \text{CO}_2}$
 347 ranged from -13.54‰ VPDB to -10.88‰ VPDB (Table 3), which is comparable to $\delta^{13}\text{C}_{\sum \text{CO}_2}$
 348 of $\text{Mg}^{2+}-\text{HCO}_3^-$ waters elsewhere in the ophiolite (-15.56‰ VPDB to -13.60‰ VPDB ;
 349 (Matter et al., 2006; D. Nothaft et al., 2021)).

350 Variable concentrations of H_2 and CH_4 across wells suggest spatial heterogeneities
 351 in sources and sinks of these gases in the ophiolite. In some $\text{Ca}^{2+}-\text{OH}^-$ waters, c_{H_2}
 352 was high (up to $253 \mu\text{mol} \cdot \text{L}^{-1}$), but c_{H_2} was below limits of quantitation in other $\text{Ca}^{2+}-$
 353 OH^- waters (Figure 2; Table 4). In $\text{Mg}^{2+}-\text{HCO}_3^-$ waters and waters from gabbroic
 354 aquifers, c_{H_2} was generally below limits of quantitation. However, up to $0.992 \mu\text{mol} \cdot \text{L}^{-1}$
 355 H_2 was measured in well WAB188, which is in gabbro near a faulted contact with peri-
 356 dotites that contain $\text{Ca}^{2+}-\text{OH}^-$ waters (Figure 1; Table 1). This suggests production
 357 of H_2 within the gabbro host rock or migration of H_2 from peridotites into gabbros sur-
 358 rounding WAB188. In most $\text{Ca}^{2+}-\text{OH}^-$ waters, c_{CH_4} was high (up to $483 \mu\text{mol} \cdot \text{L}^{-1}$;
 359 Figure 2, Table 4). However, wells with high c_{CH_4} did not always have high c_{H_2} (Fig-
 360 ure 2; Table 4). In $\text{Mg}^{2+}-\text{HCO}_3^-$ waters and gabbro waters, c_{CH_4} was typically lower
 361 ($\leq 0.1 \mu\text{mol} \cdot \text{L}^{-1}$), although c_{CH_4} reached $1.83 \mu\text{mol} \cdot \text{L}^{-1}$ in well WAB188, where c_{H_2}
 was also quantifiable.

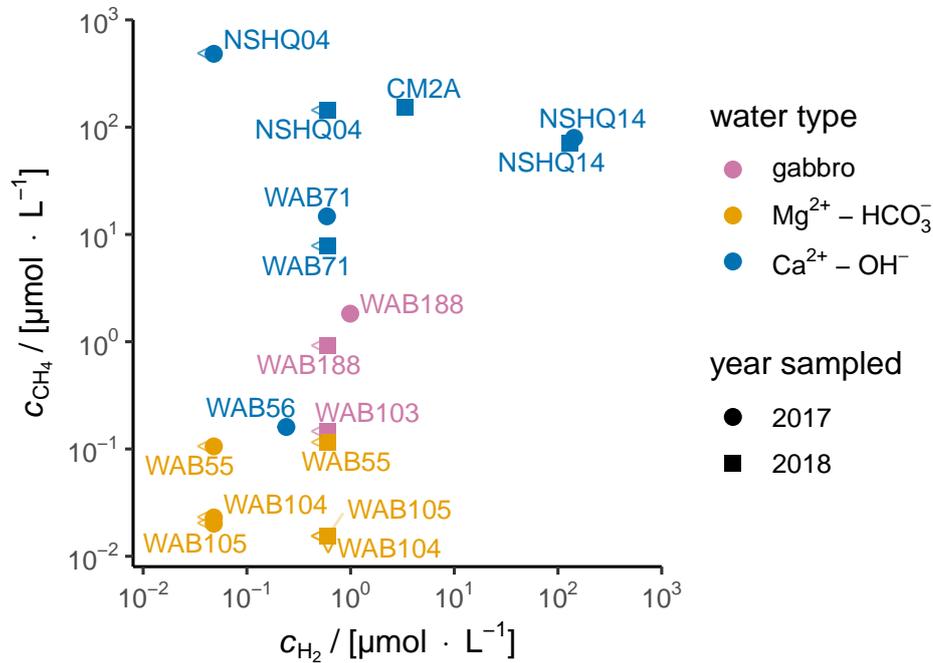


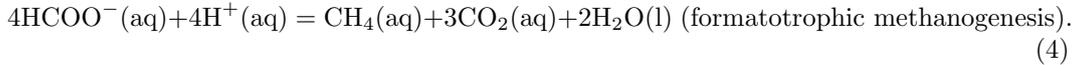
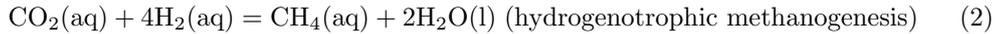
Figure 2. Aqueous concentrations of CH_4 and H_2 in Oman groundwater samples from 2017 and 2018. Left and down carrots denote “below limit of quantitation” for CH_4 and H_2 , respectively, with the adjacent point plotted at the limit of quantitation for that gas and year of analysis.

4.2 Origin of CH₄ and co-occurring short-chain alkanes in the Samail Ophiolite

We begin our examination of CH₄ origin in the Samail Ophiolite by calculating Gibbs free energies (ΔG_r) of potential CH₄-forming reactions under relevant environmental conditions and discussing these results in light of recent microbiological studies on methanogenesis in the study area. Subsequent discussion focuses on fluid and particulate samples from a subset of wells (NSHQ14, NSHQ04, and WAB188) that yielded particularly rich data sets from which we infer key CH₄ cycle processes. Discussion of three additional wells (WAB71, WAB56, and CM2A) in Supporting Information Text S1 illustrates that the processes outlined below occur throughout the broader study area with some variation due to local hydrogeologic factors.

4.2.1 Assessing which CH₄-forming reactions might occur using thermodynamic and microbiological data

To assess which CH₄-forming aqueous reactions might occur within the Samail Ophiolite, ΔG_r 's were calculated for the following reactions:



Gas-phase, abiotic reactions are also possible (Etiopie & Ionescu, 2015; Etiopie et al., 2018), but measurements of partial pressures of relevant gases in unsaturated zones of the subsurface in the study area are absent. Thus, ΔG_r 's of gas-phase reactions were not calculated. In addition to the common hydrogenotrophic and acetoclastic modes of methanogenesis, formatotrophic methanogenesis (Equation 4) was considered because formate can be produced abiotically in serpentinizing settings (McCollom & Seewald, 2003; McDermott et al., 2015; Miller, Mayhew, et al., 2017) and has been suggested as an important substrate for microbial metabolism in these settings (Lang et al., 2018), including for methanogenesis (Fones et al., 2020).

Rather than calculate ΔG_r 's of the above reactions for each individual groundwater chemical analysis, we investigate a range of generalized cases to highlight the most important factors controlling ΔG_r 's and to assess energetic states of the system that lay beyond our analytical limits. For instance, $\sum \text{CO}_2$ was below the limit of quantitation for the majority of the Ca²⁺ – OH[–] groundwaters sampled in 2018 (< 12 $\mu\text{mol} \cdot \text{L}^{-1}$; Table 3). H₂ was also below the limit of quantitation for several Ca²⁺ – OH[–] and Mg²⁺ – HCO₃[–] groundwaters (< 0.048 nmol · L^{–1} in 2017 and < 0.598 nmol · L^{–1} in 2018; Table 4). Further, formate and acetate were not measured explicitly for this study, but were measured on groundwaters from the studied wells sampled in 2015 (Rempfert et al., 2017). Thus, while robust constraints on the above parameters are available for the study area, complete sets of these parameters were generally not directly or simultaneously measured.

In light of this, we considered a representative Mg²⁺ – HCO₃[–] groundwater and a representative Ca²⁺ – OH[–] groundwater, made informed assumptions when direct con-

Table 4. Aqueous gas concentrations, reported in $\mu\text{mol} \cdot \text{L}^{-1}$.

Well	Sample year	H ₂	CO	CH ₄	C ₂ H ₆	C ₃ H ₈	<i>i</i> -C ₄ H ₁₀	<i>n</i> -C ₄ H ₁₀	<i>i</i> -C ₅ H ₁₂	<i>n</i> -C ₅ H ₁₂	C ₆ H ₁₄ ^a
WAB103	2018	$< 5.98 \cdot 10^{-1}$	$< 1.32 \cdot 10^{-1}$	$1.45 \cdot 10^{-1}$	$< 9.88 \cdot 10^{-4}$	$< 7.60 \cdot 10^{-4}$	$< 4.61 \cdot 10^{-4}$	$6.05 \cdot 10^{-3}$	$< 3.43 \cdot 10^{-4}$	$8.73 \cdot 10^{-4}$	$< 2.81 \cdot 10^{-4}$
WAB188	2018	$< 5.98 \cdot 10^{-1}$	$< 1.32 \cdot 10^{-1}$	$9.17 \cdot 10^{-1}$	$< 9.88 \cdot 10^{-4}$	$< 7.60 \cdot 10^{-4}$	$< 4.61 \cdot 10^{-4}$	$< 5.78 \cdot 10^{-4}$	$< 3.43 \cdot 10^{-4}$	$< 3.81 \cdot 10^{-4}$	$< 2.81 \cdot 10^{-4}$
	2017	$9.92 \cdot 10^{-1}$	$< 2.79 \cdot 10^{-1}$	1.83	$< 1.01 \cdot 10^{-3}$	$< 7.79 \cdot 10^{-4}$	$< 4.72 \cdot 10^{-4}$	$< 6.01 \cdot 10^{-4}$	$< 3.50 \cdot 10^{-4}$	$< 3.91 \cdot 10^{-4}$	$< 2.88 \cdot 10^{-4}$
WAB104	2018	$< 5.98 \cdot 10^{-1}$	$< 1.32 \cdot 10^{-1}$	$< 1.53 \cdot 10^{-2}$	$< 9.88 \cdot 10^{-4}$	$< 7.60 \cdot 10^{-4}$	$4.82 \cdot 10^{-4}$	$< 5.78 \cdot 10^{-4}$	$7.56 \cdot 10^{-4}$	$< 3.81 \cdot 10^{-4}$	$< 2.81 \cdot 10^{-4}$
	2017	$< 4.80 \cdot 10^{-2}$	$< 2.79 \cdot 10^{-1}$	$2.30 \cdot 10^{-2}$	$< 1.01 \cdot 10^{-3}$	$< 7.79 \cdot 10^{-4}$	$< 4.72 \cdot 10^{-4}$	$< 6.01 \cdot 10^{-4}$	$< 3.50 \cdot 10^{-4}$	$< 3.91 \cdot 10^{-4}$	$< 2.88 \cdot 10^{-4}$
WAB105	2018	$< 5.98 \cdot 10^{-1}$	$< 1.32 \cdot 10^{-1}$	$< 1.53 \cdot 10^{-2}$	$< 9.88 \cdot 10^{-4}$	$< 7.60 \cdot 10^{-4}$	$3.70 \cdot 10^{-2}$	$< 5.78 \cdot 10^{-4}$	$< 3.43 \cdot 10^{-4}$	$< 3.81 \cdot 10^{-4}$	$< 2.81 \cdot 10^{-4}$
	2017	$< 4.80 \cdot 10^{-2}$	$< 2.79 \cdot 10^{-1}$	$2.01 \cdot 10^{-2}$	$< 1.01 \cdot 10^{-3}$	$< 7.79 \cdot 10^{-4}$	$< 4.72 \cdot 10^{-4}$	$< 6.01 \cdot 10^{-4}$	$< 3.50 \cdot 10^{-4}$	$< 3.91 \cdot 10^{-4}$	$< 2.88 \cdot 10^{-4}$
WAB55	2018	$< 5.98 \cdot 10^{-1}$	$< 1.32 \cdot 10^{-1}$	$1.15 \cdot 10^{-1}$	$1.55 \cdot 10^{-3}$	$< 7.60 \cdot 10^{-4}$	$2.25 \cdot 10^{-3}$	$7.91 \cdot 10^{-4}$	$1.60 \cdot 10^{-3}$	$< 3.81 \cdot 10^{-4}$	$5.52 \cdot 10^{-3}$
	2017	$< 4.80 \cdot 10^{-2}$	$< 2.79 \cdot 10^{-1}$	$1.06 \cdot 10^{-1}$	$< 1.01 \cdot 10^{-3}$	$< 7.79 \cdot 10^{-4}$	$< 4.72 \cdot 10^{-4}$	$< 6.01 \cdot 10^{-4}$	$< 3.50 \cdot 10^{-4}$	$< 3.91 \cdot 10^{-4}$	$< 2.88 \cdot 10^{-4}$
WAB56	2017	$2.40 \cdot 10^{-1}$	$< 2.79 \cdot 10^{-1}$	$1.60 \cdot 10^{-1}$	$< 1.01 \cdot 10^{-3}$	$< 7.79 \cdot 10^{-4}$	$< 4.72 \cdot 10^{-4}$	$< 6.01 \cdot 10^{-4}$	$< 3.50 \cdot 10^{-4}$	$< 3.91 \cdot 10^{-4}$	$< 2.88 \cdot 10^{-4}$
NSHQ04	2018	$< 5.98 \cdot 10^{-1}$	$< 1.32 \cdot 10^{-1}$	$1.44 \cdot 10^2$	$2.45 \cdot 10^{-2}$	$2.22 \cdot 10^{-3}$	$< 4.61 \cdot 10^{-4}$	$< 5.78 \cdot 10^{-4}$	$< 3.43 \cdot 10^{-4}$	$< 3.81 \cdot 10^{-4}$	$< 2.81 \cdot 10^{-4}$
	2017	$< 4.80 \cdot 10^{-2}$	$< 2.79 \cdot 10^{-1}$	$4.83 \cdot 10^2$	$< 1.01 \cdot 10^{-3}$ ^b	$1.03 \cdot 10^{-3}$	$< 4.72 \cdot 10^{-4}$	$< 6.01 \cdot 10^{-4}$	$< 3.50 \cdot 10^{-4}$	$< 3.91 \cdot 10^{-4}$	$< 2.88 \cdot 10^{-4}$
WAB71	2018	$< 5.98 \cdot 10^{-1}$	$< 1.32 \cdot 10^{-1}$	7.76	$1.00 \cdot 10^{-3}$	$< 7.60 \cdot 10^{-4}$	$< 4.61 \cdot 10^{-4}$	$< 5.78 \cdot 10^{-4}$	$< 3.43 \cdot 10^{-4}$	$< 3.81 \cdot 10^{-4}$	$< 2.81 \cdot 10^{-4}$
	2017	$5.92 \cdot 10^{-1}$	$< 2.79 \cdot 10^{-1}$	$1.48 \cdot 10^1$	$< 1.01 \cdot 10^{-3}$	$< 7.79 \cdot 10^{-4}$	$< 4.72 \cdot 10^{-4}$	$1.94 \cdot 10^{-2}$	$< 3.50 \cdot 10^{-4}$	$4.79 \cdot 10^{-4}$	$< 2.88 \cdot 10^{-4}$
CM2A	2018	3.38	$< 1.32 \cdot 10^{-1}$	$1.52 \cdot 10^2$	$4.11 \cdot 10^{-2}$	$1.75 \cdot 10^{-3}$	$< 4.61 \cdot 10^{-4}$	$6.48 \cdot 10^{-3}$	$< 3.43 \cdot 10^{-4}$	$< 3.81 \cdot 10^{-4}$	$< 2.81 \cdot 10^{-4}$
NSHQ14	2018	$1.31 \cdot 10^2$	$< 1.32 \cdot 10^{-1}$	$7.12 \cdot 10^1$	$7.32 \cdot 10^{-2}$	$7.64 \cdot 10^{-3}$	$2.26 \cdot 10^{-3}$	$2.88 \cdot 10^{-3}$	$1.27 \cdot 10^{-3}$	$2.23 \cdot 10^{-3}$	$1.12 \cdot 10^{-3}$
	2017	$2.53 \cdot 10^2$	$< 2.79 \cdot 10^{-1}$	$1.06 \cdot 10^2$	$7.98 \cdot 10^{-2}$	$9.00 \cdot 10^{-3}$	$1.53 \cdot 10^{-3}$	$4.77 \cdot 10^{-3}$	$< 3.50 \cdot 10^{-4}$	$< 3.91 \cdot 10^{-4}$	$9.70 \cdot 10^{-4}$

^aHexane isomers not chromatographically resolved.^bHigh C₁/(C₂ + C₃) at NSHQ04 resulted in CH₄ tailing into and preventing quantitation of the C₂H₆ peak in 2017. Chromatographic improvements were made between analyses of 2017 and 2018 samples.

Table 5. Gibbs free energies of potential CH₄-forming reactions and log activities of relevant species. *Abbreviations:* H, hydrogenotrophic (Equation 2); A, acetoclastic (Equation 3); F, formatotrophic methanogenesis (Equation 4).

water type	H ⁺	CO ₂ (aq)	log (activity)			H ₂ (aq)	$\Delta G_r / [\text{kJ} \cdot \text{mol}^{-1}]$		
			HCOO ⁻	CH ₃ COO ⁻	CH ₄ (aq)		H	A	F
Ca ²⁺ – OH ⁻	-11.1	-11.6	-6.1	-6.1	-4.0	-9.0	64	-115	-90
						-6.0	-6	-115	-90
						-3.0	-77	-115	-90
Mg ²⁺ – HCO ₃ ⁻	-8.7	-4.9	-6.0	-6.0	-7.0	-9.0	8	-107	-47
						-6.0	-63	-107	-47

399 concentration measurements were lacking, and evaluated ΔG_r 's for a range of H₂ concen-
400 trations. Measurements of major inorganic dissolved constituents, pH, and *Eh* from wells
401 WAB105 and NSHQ14 were used for the model Mg²⁺ – HCO₃⁻ and Ca²⁺ – OH⁻ flu-
402 ids, respectively (Tables 1 and 3). Since measured $c_{\sum \text{CO}_2}$ was below the limit of quan-
403 titation in the water sample from NSHQ14, 8 $\mu\text{mol} \cdot \text{kg}^{-1}$ was taken as the $c_{\sum \text{CO}_2}$ of
404 the representative Ca²⁺ – OH⁻ water, corresponding to the value at chrysotile-brucite-
405 diopside-calcite equilibrium at 25 °C and 1 bar obtained from water-harzburgite reaction
406 path modeling (Leong & Shock, 2020). Concentrations of formate and acetate were both
407 assumed to be 1 $\mu\text{mol} \cdot \text{kg}^{-1}$, which is consistent with their concentrations in earlier sam-
408 ples from wells in Samail Ophiolite (Rempfert et al., 2017). Concentrations of CH₄ were
409 assumed to be 100 $\mu\text{mol} \cdot \text{kg}^{-1}$ and 0.1 $\mu\text{mol} \cdot \text{kg}^{-1}$ for the representative Ca²⁺ – OH⁻
410 and Mg²⁺ – HCO₃⁻ waters, respectively, reflecting typical concentrations for these flu-
411 ids (Table 4, Figure 2). H₂ concentrations vary widely between and within fluid types
412 (Table 4, Figure 2), so calculations were performed for multiple H₂ concentrations (1 $\text{mmol} \cdot \text{kg}^{-1}$,
413 1 $\mu\text{mol} \cdot \text{kg}^{-1}$, and 1 $\text{nmol} \cdot \text{kg}^{-1}$) encompassing the range of concentrations observed in Ca²⁺ –
414 OH⁻ fluids. The 1 $\text{mmol} \cdot \text{kg}^{-1}$ H₂ case was omitted for the Mg²⁺ – HCO₃⁻ fluid, where
415 such high H₂ concentrations are not observed. The log activities (*a*) of all relevant species
416 are tabulated in Table 5.

417 The calculated ΔG_r 's (Table 5) indicate that all of the CH₄-forming reactions con-
418 sidered here can have sufficient chemical potential to sustain microbial life in certain states
419 of the system. That is, $\Delta G_r > \Delta G_{\text{min}}$, where ΔG_{min} (also known as the Biological En-
420 ergy Quantum) is the minimum free energy that must be available to sustain life in a
421 given environment (thought to be around $-9 \text{kJ} \cdot \text{mol}^{-1}$ to $-20 \text{kJ} \cdot \text{mol}^{-1}$; (Schink, 1997;
422 Hoehler, 2004; Schink & Stams, 2006)). Acetoclastic methanogenesis had the most nega-
423 tive ΔG_r in all conditions tested. formatotrophic methanogenesis had more negative
424 ΔG_r than hydrogenotrophic methanogenesis in all Ca²⁺ – OH⁻ conditions tested, but
425 formatotrophic methanogenesis had less negative ΔG_r than hydrogenotrophic methano-
426 genesis in the Mg²⁺ – HCO₃⁻ case at 1 $\mu\text{mol} \cdot \text{kg}^{-1}$ H₂. Hydrogenotrophic methanogen-
427 esis had sufficient chemical potential to sustain microbial life only when a_{H_2} was high
428 enough, with the threshold a_{H_2} being higher in Ca²⁺ – OH⁻ waters, where $a_{\text{CO}_2(\text{aq})}$ is
429 lower. These calculations are generally consistent with those of Canovas III et al. (2017),
430 who found that hydrogenotrophic methanogenesis had modest potential energy yields
431 in waters from surface seeps in the Samail Ophiolite at pH ranging from 8 to 12.

432 Several additional factors should be considered when interpreting the ΔG_r results.
433 First, reactions proceeding in environmental systems are often drawn towards equilib-
434 rium, and thus a large negative ΔG_r of a given reaction may indicate that that reaction
435 is not actively occurring, but only has the potential to occur. Second, substrate trans-
436 port into the cell is not addressed in our calculations. A more complete model would ac-
437 count for rates of CO₂ diffusion across the cell membrane and/or energy expended to

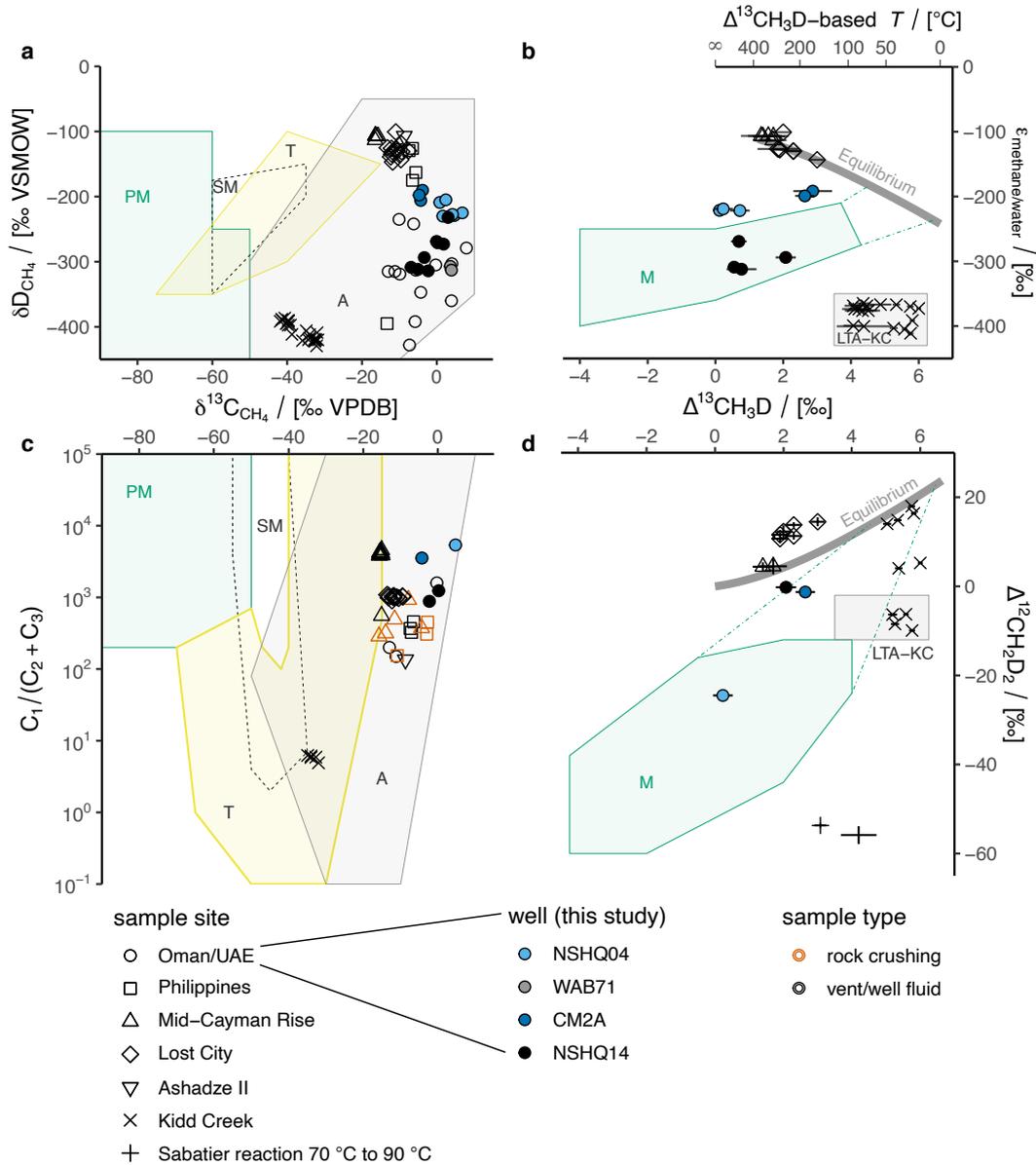
438 transport charged species such as formate and acetate into the cell (Hoehler, 2004). Third,
 439 mixing is not explicitly accounted for in our calculations. Mixing has been suggested as
 440 a key factor controlling energetic favorability of various reactions in the Samail Ophi-
 441 olite. This is especially pertinent to hydrogenotrophic methanogenesis because c_{CO_2} is
 442 so much lower in endmember hyperalkaline fluids than in near-surface, atmosphere-influenced
 443 fluids (Canovas III et al., 2017; Leong & Shock, 2020). The c_{CO_2} used for the example
 444 $\text{Ca}^{2+}-\text{OH}^-$ fluid in our calculations is representative of a minimum value for the sys-
 445 tem (Leong & Shock, 2020). Mixing would tend to inject CO_2 into the fluids and increase
 446 the energetic favorability of hydrogenotrophic methanogenesis.

447 In addition to energetic considerations, microbiological approaches can help elu-
 448 cidate which CH_4 -forming reactions occur. Kraus et al. (2021) found higher transcript
 449 abundances of carbonic anhydrase and formate dehydrogenase relative to acetate kinase
 450 and phosphate acetyltransferase in hyperalkaline groundwaters from wells in the Samail
 451 Ophiolite, suggesting that $\text{CO}_2/\text{HCO}_3^-$ and formate are more actively used substrates
 452 for methanogenesis than acetate in these conditions. Further, Fones et al. (2020) iden-
 453 tified two lineages of *Methanobacterium* in Samail Ophiolite groundwaters that were shown
 454 by genomic and microcosm-based radiotracer approaches to use different methanogenic
 455 pathways. *Methanobacterium* Type I lineage predominated in circumneutral waters and
 456 is capable of using either CO_2 or formate for methanogenesis. *Methanobacterium* Type
 457 II lineage, which was more abundant in hyperalkaline waters and which branched from
 458 the Type I lineage, was exclusively capable of formatotrophic methanogenesis. It was pos-
 459 tulated that gene loss and acquisition in Type II lineage allowed it to be specially suited
 460 to the high-pH and low- $\sum \text{CO}_2$ conditions resulting from extensive serpentinization. Thus,
 461 microbiological data suggest that hydrogenotrophic or formatotrophic methanogenesis
 462 are the most likely pathways for methanogenesis in the Samail Ophiolite and that the
 463 relative contributions of each of these pathways to microbial CH_4 production at a given
 464 site may depend on local geochemical factors such as $a_{\text{CO}_2(\text{aq})}$. This notion is generally
 465 supported by our calculations in that formatotrophic methanogenesis had more nega-
 466 tive ΔG_r than hydrogenotrophic methanogenesis in all $\text{Ca}^{2+}-\text{OH}^-$ conditions tested,
 467 whereas the reverse was true for the $\text{Mg}^{2+}-\text{HCO}_3^-$ case at $1 \mu\text{mol} \cdot \text{kg}^{-1} \text{H}_2$.

468 Remarkably, although acetoclastic methanogenesis had the most negative ΔG_r of
 469 the investigated CH_4 -forming reactions (Table 5), it has the least microbiological evi-
 470 dence of being a major methanogenic pathway in the Samail Ophiolite. Conversion of
 471 isotopically labeled acetate ($^{13}\text{CH}_3\text{OO}^-$) to $^{13}\text{CH}_4$, has, however, been documented in
 472 cultures from serpentinite springs in the Voltri Massif, Italy (Brazelton et al., 2017), in-
 473 dicated that acetoclastic methanogenesis can operate in some serpentinizing settings.
 474 In the aquifers sampled via wells in the Samail Ophiolite, methanogens may be out-competed
 475 for acquisition of acetate by other groups of microbes, such as sulfate reducers. Indeed,
 476 geochemical evidence of microbial acetate oxidation coupled to sulfate reduction has been
 477 reported in alkaline, H_2 -rich, crystalline rock aquifers inhabited by microbial communi-
 478 ties dominated by sulfate reducing bacteria and methanogens (Moser et al., 2005).

479 **4.2.2 Abiotic, ^{13}C -enriched CH_4 , C_2H_6 , and C_3H_8 mixed with micro-** 480 **bial CH_4 produced under C-limited conditions in the $\text{Ca}^{2+}-\text{OH}^-$** 481 **waters of well NSHQ14**

482 Well NSHQ14 is situated in a catchment dominated by partially serpentinized harzbur-
 483 gite with meter-scale partially serpentinized dunite bands (Figure 1; Supporting Infor-
 484 mation Figure S1; Table 1). The well is cased to 5.8 meters below ground level (mbgl)
 485 and drilled to 304 mbgl (Table 1). Groundwaters accessed via NSHQ14 had the highest
 486 pH (11.39), and lowest Eh (-253 mV) and f_{O_2} ($1.19 \cdot 10^{-51} \text{ bar}$) among the wells in-
 487 vestigated (Table 1), indicating that fluids sampled from NSHQ14 have extensively par-
 488 ticipated in serpentinization. This is also reflected in the c_{H_2} of groundwaters sampled
 489 at NSHQ14, which was the highest among the studied wells ($253 \mu\text{mol} \cdot \text{L}^{-1}$ and $131 \mu\text{mol} \cdot$



490 L^{-1} in 2017 and 2018, respectively; Table 4; Figure 2). NSHQ14 waters also had high
 491 c_{CH_4} ($106 \mu\text{mol} \cdot L^{-1}$ and $71.2 \mu\text{mol} \cdot L^{-1}$ in 2017 and 2018, respectively).

492 CH_4 has ranged in $\delta^{13}C$ from $-6.89‰$ VPDB to $+3.7‰$ VPDB in fluid samples
 493 from NSHQ14, with a mean weighted by sample year of $-0.8‰$ VPDB (Figure 3a; Ta-
 494 ble 2). These $\delta^{13}C$ values are generally higher than those of CH_4 emanating from sediment-
 495 poor seafloor hydrothermal vents, where a dominantly abiotic origin has been proposed
 496 ((Welhan & Craig, 1983; Merlivat et al., 1987; J. L. Charlou et al., 1996; J. Charlou et
 497 al., 2000, 2002; Proskurowski et al., 2008; Kumagai et al., 2008; McDermott et al., 2015;
 498 D. T. Wang et al., 2018); represented by Mid-Cayman Rise, Lost City, and Ashadze II
 499 in Figure 3a), higher than typical mantle values (Deines, 2002), and similar to marine
 500 carbonate (Schidlowski, 2001). CH_4 $\delta^{13}C$ at NSHQ14 is generally higher than $\delta^{13}C$ of
 501 carbonate veins in NSHQ14 ($-7.05‰$ VPDB to $-4.69‰$ VPDB; (Miller et al., 2016)),
 502 which is opposite to that which would be expected at equilibrium (Bottinga, 1969), in-
 503 dicated that CH_4 is not in isotopic equilibrium with co-existing carbonate minerals.

Figure 3. Molecular and isotopic compositions of natural gases. (a) Plot of δD_{CH_4} vs. $\delta^{13}C_{CH_4}$. Shaded fields of typical gas origin after Milkov and Etiope (2018). *Abbreviations:* PM, primary microbial; SM, secondary microbial; T, thermogenic; A, abiotic. (c) Plot of ratio of methane (C_1) to the sum of ethane (C_2) and propane (C_3) vs. $\delta^{13}C_{CH_4}$. Only analyses for which C_2 was above limit of quantitation are plotted. If C_3 was below limit of quantitation, its contribution to $C_1/(C_2 + C_3)$ was assumed to be negligible, and therefore C_1/C_2 is plotted. Fields and abbreviations same as in (a). In (a) and (c), uncertainties are smaller than plotted symbols. (b) Plot of $\epsilon_{methane/water}$ vs. $\Delta^{13}CH_3D$. X and Y axes are swapped with respect to original publication of this type of plot (D. T. Wang et al., 2015) so that (b) is comparable against (d). The data from (b) are plotted in the D. T. Wang et al. (2015) orientation in Supporting Information Figure S4. Equilibrium line from Horibe and Craig (1995) and E. Young et al. (2017). *Abbreviations:* LTA-KC, low-temperature abiotic (Kidd Creek-type); M, microbial. Green dot-dashed lines in (b) and (d) indicate a range of CH_4 isotopic compositions that have been attributed to either low cell-specific rates of methanogenesis or anaerobic oxidation of methane; that is, they start at isotopic compositions produced by methanogen cultures and end at isotopic equilibrium between 5 °C and 70 °C, which is the range of temperatures over which anaerobic oxidation of methane has been documented (D. T. Wang et al., 2015; D. Stolper et al., 2015; E. Young et al., 2017; Ash & Egger, 2019; Giunta et al., 2019). (d) Plot of $\Delta^{13}CH_3D$ vs. $\Delta^{12}CH_2D_2$, after E. Young et al. (2017). Fields, abbreviations, and temperature axis same as in (b). In (b) and (d), error bars represent 95 % confidence interval for analyses performed at MIT, and 1 standard error for analyses performed at UCLA. Contextual data from ophiolites: Oman/UAE (Fritz et al., 1992; Etiope et al., 2015; Boulart et al., 2013; Miller et al., 2016; Vacquand et al., 2018), the Philippines (Abrajano et al., 1990; Grozeva et al., 2020); sediment-poor seafloor hydrothermal vents: Mid-Cayman Rise (McDermott et al., 2015; D. T. Wang et al., 2018; Grozeva et al., 2020), Lost City (Proskurowski et al., 2008; D. T. Wang et al., 2018; Labidi et al., 2020), Ashadze II (J. L. Charlou et al., 2010); Precambrian Shield: Kidd Creek, Canada (Sherwood Lollar et al., 2008; E. Young et al., 2017); and laboratory Sabatier reaction catalyzed by Ru (E. Young et al., 2017).

504 CH_4 is accompanied by C_2 – C_6 alkanes in fluids from NSHQ14 (Table 4). These
 505 alkanes had $C_1/(C_2 + C_3)$ ratios of 1240 in 2017 and 881 in 2018, which are similar to
 506 fluid samples and rock crushings from other ophiolites and sediment-poor seafloor hy-
 507 drothermal vents (Abrajano et al., 1990; J. L. Charlou et al., 2010; McDermott et al.,
 508 2015; Grozeva et al., 2020), but 10^2 times higher than those of Kidd Creek mine, Canada,
 509 for which a low-temperature, abiotic origin of alkanes has been proposed (Sherwood Lol-
 510 lar et al., 2002, 2008; E. Young et al., 2017) (Figure 3c). Thus, $C_1/(C_2 + C_3)$ ratios could
 511 reflect differences in alkane formation mechanisms or extents of reaction in Precambrian
 512 shield sites like Kidd Creek versus ophiolites and sediment-poor seafloor hydrothermal
 513 vents.

514 C_2H_6 and C_3H_8 at NSHQ14 are strongly ^{13}C -enriched ($\delta^{13}C$ of -6.0% VPDB and
 515 $+3.3\%$ VPDB, respectively; Table 2; Figure 4). The observed $\delta^{13}C$ values are $\sim 15\%$
 516 higher than those in the most mature (and therefore most ^{13}C -enriched) thermogenic
 517 C_2H_6 and C_3H_8 samples from confined systems (Milkov & Etiope, 2018; Fiebig et al.,
 518 2019). Increases in $\delta^{13}C_{C_3}$ of $\sim 15\%$ have been attributed to microbial oxidation of short-
 519 chain alkanes, which enriches the residual in ^{13}C (Martini et al., 2003). However, short-
 520 chain alkane oxidizing microbial species (Shennan, 2006; Singh et al., 2017; Laso-Pérez
 521 et al., 2019) were not detected in 16S rRNA gene sequences of DNA obtained from NSHQ14.
 522 Thus, there is not strong evidence to suggest that $\delta^{13}C_{C_2}$ and $\delta^{13}C_{C_3}$ at NSHQ14 re-

523 sult from post-genetic microbial alteration. Rather, $\delta^{13}\text{C}_{\text{C}_2}$ and $\delta^{13}\text{C}_{\text{C}_3}$ should reflect
524 formation conditions and C source(s).

525 C_2H_6 and C_3H_8 at NSHQ14 are not likely to derive from nearby organic matter.
526 Hydrocarbon-rich sedimentary formations in northern Oman not only lack a clear struc-
527 tural connection to the ophiolite aquifer, but also yield oils with $\delta^{13}\text{C}$ values (Terken,
528 1999) at least 20 ‰ lower than those of C_2H_6 and C_3H_8 at NSHQ14. Furthermore, to-
529 tal organic C in peridotites exposed to alteration at the seafloor, a proxy for organic C
530 endogenous to the Samail Ophiolite, is also relatively ^{13}C -depleted (approximately $-25 \pm$
531 5 ‰ VPDB; (Alt et al., 2013; Alt, Garrido, et al., 2012; Alt, Shanks, et al., 2012; Dela-
532 cour et al., 2008)). Closed-system thermal cracking of these organic matter sources is
533 unlikely to have produced the comparatively ^{13}C -enriched C_2H_6 and C_3H_8 at NSHQ14
534 and previously reported elsewhere in the ophiolite (Figure 4; (Fritz et al., 1992)).

535 Thermal cracking of organic matter and open-system degassing can enrich late-produced
536 short-chain alkanes in ^{13}C due to kinetic isotope effects associated with the cleavage of
537 precursor sites in the parent organic matter and the resultant Rayleigh distillation of these
538 sites (Rooney et al., 1995; Fiebig et al., 2019). Thermogenic gas production can proceed
539 slowly at temperatures as low as 60 °C, but substantial thermogenic gas production typ-
540 ically occurs at reservoir temperatures above 120 °C (Burnham, 1989; Hunt, 1996; D. A. Stolper
541 et al., 2018; Cumming et al., 2019; Fiebig et al., 2019). These temperatures are higher
542 than temperatures along groundwater flow paths intersecting the wells in this study. Mea-
543 sured groundwater temperatures in the study area are ~ 35 °C (Table 1), and H_2 – H_2O
544 isotope thermometry and C–O clumped isotope thermometry on carbonate veins with
545 significant ^{14}C contents in Samail Ophiolite peridotites both indicate equilibrium ≤ 60 °C
546 (P. B. Kelemen & Matter, 2008; P. B. Kelemen et al., 2011; Mervine et al., 2014; Miller
547 et al., 2016). Geothermal gradients derived from geophysical logs of NSHQ14 are 25 °C·
548 km^{-1} (A. Paukert, 2014; Matter et al., 2017), which is typical of near-surface, continen-
549 tal settings (Lowell et al., 2014). At the low temperatures and ordinary geothermal gra-
550 dients within the active alteration zone of the Samail Ophiolite, thermal cracking of or-
551 ganic matter is unlikely to proceed at sufficient rates to attain the high extents of reac-
552 tion progress necessary to explain the observed ^{13}C enrichments in short-chain alkanes
553 at NSHQ14 over relevant timescales.

554 Alternatively, short-chain alkanes in NSHQ14 fluids may have an abiotic source.
555 Several studies have demonstrated storage of large quantities of CH_4 and associated short-
556 chain alkanes in fluid inclusions in ophiolites (Sachan et al., 2007; Klein et al., 2019; Grozeva
557 et al., 2020). However, the findings of these studies disagree with those of Etiope et al.
558 (2018), who measured relatively low concentrations of CH_4 stored in serpentinized peri-
559 dotites from Greek ophiolites. Since the rocks analyzed by Etiope et al. (2018) were sam-
560 pled from outcrops, it is possible that chemical or physical processes associated with sur-
561 face exposure resulted in loss of CH_4 once stored in peridotite-hosted fluid inclusions prior
562 to analysis. Although further study of the quantity and spatial distribution of CH_4 stor-
563 age in ophiolitic rocks is warranted, the presence of $\text{CH}_4 + \text{H}_2$ inclusions in olivine and
564 $\text{CH}_4 \pm$ graphite inclusions in orthopyroxene in Samail Ophiolite harzburgites (Miura et
565 al., 2011) requires that fluid inclusions be considered as a potential source for abiotic CH_4
566 and associated short-chain alkanes at NSHQ14 and elsewhere in the ophiolite.

567 A fluid inclusion source of CH_4 and short-chain alkanes is compatible with C sta-
568 ble isotopic compositions of these compounds in groundwaters pumped from NSHQ14.
569 CH_4 , C_2H_6 , and C_3H_8 $\delta^{13}\text{C}$ values at NSHQ14 (-6.89 ‰ VPDB to $+3.7$ ‰ VPDB; Ta-
570 ble 2) overlap with CH_4 and C_2H_6 $\delta^{13}\text{C}$ values measured by Grozeva et al. (2020) in rock
571 crushing experiments on CH_4 -rich fluid inclusion-bearing peridotites and dunites sam-
572 pled from the Zambales ophiolite in the Philippines (-12.4 ‰ VPDB to -0.9 ‰ VPDB;
573 Figure 4), which, in turn, overlap with $\delta^{13}\text{C}$ values of CH_4 from nearby gas seeps at Los
574 Fuegos Eternos and Nagsasa in the Philippines (-7.4 ‰ VPDB to -5.6 ‰ VPDB; Fig-
575 ure 3a; (Abrajano et al., 1990; Vacquand et al., 2018)). Grozeva et al. (2020) also crushed

576 CH₄-rich fluid inclusion-bearing rocks from the Mid-Cayman Rise. Of the Mid-Cayman
 577 Rise samples that yielded sufficient CH₄ and C₂H₆ for precise C isotopic analysis, which
 578 were all mafic intrusive rocks, $\delta^{13}\text{C}$ values ranged from -14.0‰ VPDB to $+0.7\text{‰}$ VPDB.
 579 The lower end of Mid-Cayman Rise rock crushing short-chain alkane $\delta^{13}\text{C}$ values are sim-
 580 ilar to those measured in Mid-Cayman Rise hydrothermal vent fluids (-15.8‰ VPDB
 581 to -9.7‰ VPDB; (McDermott et al., 2015)), whereas the higher end are similar to those
 582 of NSHQ14 (Figure 4). Furthermore, C₂H₆ and C₃H₈ $\delta^{13}\text{C}$ values of NSHQ14 fluids re-
 583 semble those of fluids discharging from the sediment-poor hydrothermal vents at Ashadze
 584 II, Mid-Atlantic Ridge (Figure 4; (J. L. Charlou et al., 2010)). The similarities in short-
 585 chain alkane $\delta^{13}\text{C}$ values between circulating fluids and rock-hosted fluid inclusions in
 586 ophiolites and present-day oceanic lithospheric sites suggest that circulating fluids in both
 587 environments derive much of their CH₄ and short-chain alkanes from fluid inclusions.

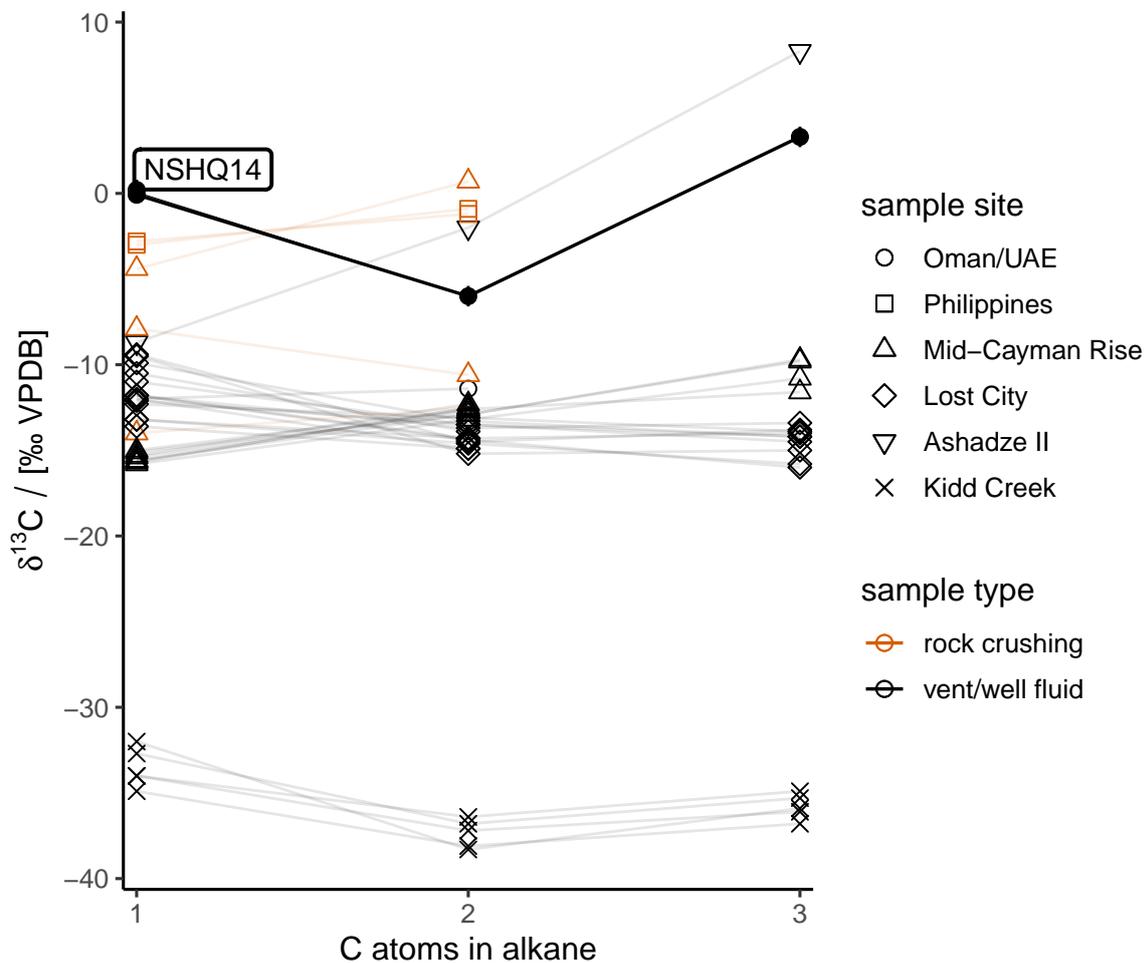


Figure 4. Plot of $\delta^{13}\text{C}$ of CH₄ and co-occurring *n*-alkanes vs. the number of C atoms per molecule. Error bars represent uncertainties on $\delta^{13}\text{C}$ analyses performed at CUB. Only samples for which $\delta^{13}\text{C}_{\text{C}_2}$ was determined are plotted. Contextual data from ophiolites: Oman/UAE (Fritz et al., 1992), the Philippines (Grozeva et al., 2020); sediment-poor seafloor hydrothermal vents: Mid-Cayman Rise (McDermott et al., 2015; Grozeva et al., 2020), Lost City (Proskurowski et al., 2008), Ashadze II (J. L. Charlou et al., 2010); and Precambrian Shield: Kidd Creek, Canada (Sherwood Lollar et al., 2008).

588 Sources of CH₄ can also be assessed by measuring H isotopic compositions and clumped
 589 isotopologue relative abundances of CH₄ and comparing these isotopic compositions to
 590 temperature-dependent equilibria. These isotopic equilibria are represented by thick gray
 591 lines in Figure 3b and d. Intra-CH₄ equilibrium is governed by the increasing relative
 592 stability of bonds between two heavy isotopes (more “clumping”) at lower temperatures,
 593 which is reflected in higher $\Delta^{13}\text{CH}_3\text{D}$ and $\Delta^{12}\text{CH}_2\text{D}_2$ values. However, isotopic equi-
 594 librium will only be expressed if kinetics allow it. In the first study to publish clumped
 595 isotopologue ($\Delta^{13}\text{CH}_3\text{D}$) data on CH₄- and H₂- rich gases from sediment-poor seafloor
 596 hydrothermal vents, D. T. Wang et al. (2018) found that these gases yielded apparent
 597 CH₄–H₂O H isotopic and $\Delta^{13}\text{CH}_3\text{D}$ equilibrium temperatures of 270 °C to 360 °C, de-
 598 spite having a range of effluent fluid temperatures from 96 °C to 370 °C. This was in-
 599 terpreted as evidence for a closure temperature of 270 °C for H isotope exchange in the
 600 CH₄–H₂O and CH₄–H₂ systems in seafloor hydrothermal settings (e.g. Mid-Cayman
 601 Rise in Figure 3b and d). However, in a subsequent study that re-analyzed some of the
 602 same samples, plus a greater number of samples from low-temperature vents at Lost City
 603 (96 °C to 64 °C), and contributed the first $\Delta^{12}\text{CH}_2\text{D}_2$ values from these settings, Labidi
 604 et al. (2020) found evidence for re-equilibration of clumped isotopologue and CH₄–H₂O
 605 H isotopic systems at lower temperatures. Of these isotopic systems, that of $^{12}\text{CH}_2\text{D}_2$
 606 had the fastest apparent re-equilibration kinetics (approximately twice as fast as $^{13}\text{CH}_3\text{D}$),
 607 which was explained by differences in symmetry numbers among the isotopologues. The
 608 $^{12}\text{CH}_2\text{D}_2$ -based temperatures of the Lost City samples, which were as low as 69_{-4}^{+4} °C,
 609 closely matched their end member vent fluid temperatures. As a result of the apparent
 610 faster re-equilibration of $^{12}\text{CH}_2\text{D}_2$, the Lost City data plot above the $^{13}\text{CH}_3\text{D}$ – $^{12}\text{CH}_2\text{D}_2$
 611 equilibrium line (towards higher $\Delta^{12}\text{CH}_2\text{D}_2$) in Figure 3d. Therefore, isotopic compo-
 612 sitions of CH₄ formed in fluid inclusions in the oceanic lithosphere and stored for mil-
 613 lions of years at low temperatures may be expected to fall somewhere along a contin-
 614 uum from $\Delta^{13}\text{CH}_3\text{D}$, $\Delta^{12}\text{CH}_2\text{D}_2$, and CH₄–H₂O isotopic equilibrium at ~ 330 °C to
 615 compositions approaching lower temperature (~ 70 °C or perhaps even lower) equilib-
 616 rium, with $^{12}\text{CH}_3\text{D}$, $^{13}\text{CH}_3\text{D}$, CH₄–H₂O isotopic re-equilibration proceeding at vary-
 617 ing rates. This is not the case for Samail Ophiolite samples, as detailed below.

618 Across five years of samples from NSHQ14, $\delta\text{D}_{\text{CH}_4}$ has ranged from -232 ‰ VSMOW
 619 to -311.73 ‰ VSMOW, with a mean weighted by sample year of -275 ‰ VSMOW (Fig-
 620 ure 3a; Table 2). This CH₄ is D-enriched with respect to coexisting H₂ ($\delta\text{D}_{\text{H}_2} = -685$ ‰ VSMOW;
 621 (Miller et al., 2016)) and D-depleted with respect to coexisting water ($\delta\text{D}_{\text{H}_2\text{O}} = +0.2$ ‰ VSMOW
 622 in 2018; Table 3). Although H₂ and water reflect H isotopic equilibrium at ~ 50 °C (Miller
 623 et al., 2016), both H₂ and water are in H isotopic disequilibrium with CH₄ (Figure 3b).
 624 Moreover, NSHQ14 fluids exhibit intra-CH₄ disequilibrium, as indicated by $\Delta^{13}\text{CH}_3\text{D}$
 625 and $\Delta^{12}\text{CH}_2\text{D}_2$ values (Table 2) plotting below the equilibrium line in Figure 3d. These
 626 non-equilibrium isotopic compositions indicate that post-genetic alteration of CH₄ must
 627 have occurred or that fluid inclusions are not the only source of CH₄ at NSHQ14.

628 One potential post-genetic alteration mechanism is diffusion. However, CH₄ at NSHQ14
 629 cannot be the diffusion residual of CH₄ that was originally at intramolecular equilibrium
 630 (or with $\Delta^{12}\text{CH}_2\text{D}_2$ above the apparent $\Delta^{13}\text{CH}_3\text{D}$ equilibrium temperature) because the
 631 diffusion slope (change in $\Delta^{12}\text{CH}_2\text{D}_2$ over change in $\Delta^{13}\text{CH}_3\text{D}$) is shallower than the equi-
 632 librium line slope over the relevant temperature range (E. Young et al., 2017). Another
 633 potential alteration mechanism is microbial CH₄ oxidation. Two types of microbial CH₄
 634 oxidation have been studied for their effects on CH₄ clumped isotopologue relative abun-
 635 dances: anaerobic methane oxidation of the ANME type and aerobic CH₄ oxidation. ANME-
 636 type anaerobic methane oxidation is suggested to be a highly reversible metabolic path-
 637 way (Knittel & Boetius, 2009; Timmers et al., 2017). This reversibility has been proposed
 638 to bring $\Delta^{13}\text{CH}_3\text{D}$ and $\Delta^{12}\text{CH}_2\text{D}_2$ towards equilibrium at low temperatures (70 °C to
 639 30 °C) through continuous breaking and reforming of bonds in the CH₄ molecule (E. Young
 640 et al., 2017; Ash & Egger, 2019; Giunta et al., 2019). Thus, the comparatively low $\Delta^{13}\text{CH}_3\text{D}$
 641 and $\Delta^{12}\text{CH}_2\text{D}_2$ values observed in samples from NSHQ14 and other wells in this study

(Figure 3b and d) do not support a major role for anaerobic methane oxidation in the study area. Aerobic CH₄ oxidation is less reversible than ANME-type anaerobic methane oxidation due to differences in the enzymes and electron acceptors used for those respective processes. For this reason, aerobic CH₄ oxidation does not bring CH₄ into isotopic equilibrium, but rather imparts a normal, classical kinetic isotope effect during CH₄ consumption. In a study of the effect of aerobic CH₄ oxidation on $\Delta^{13}\text{CH}_3\text{D}$, D. T. Wang et al. (2016) found that the fractionation factor for $^{13}\text{CH}_3\text{D}$ was closely approximated by the product of the fractionation factors for $^{13}\text{CH}_4$ and $^{12}\text{CH}_3\text{D}$. Although it has not yet been demonstrated experimentally, it is hypothesized that the fractionation factor for $^{12}\text{CH}_2\text{D}_2$ during aerobic CH₄ oxidation may likewise be approximated by the square of the fractionation factor for $^{12}\text{CH}_3\text{D}$ (E. D. Young, 2020). This “product rule” for isotopic fractionation during aerobic CH₄ oxidation results in decreases in $\Delta^{13}\text{CH}_3\text{D}$ and $\Delta^{12}\text{CH}_2\text{D}_2$ with concomitant increases in $\delta^{13}\text{C}$ and δD in residual CH₄ (D. T. Wang et al., 2016; E. D. Young, 2020). Thus, aerobic CH₄ oxidation could draw $\Delta^{13}\text{CH}_3\text{D}$ and $\Delta^{12}\text{CH}_2\text{D}_2$ values originally near equilibrium down below the equilibrium line in Figure 3d. However, if CH₄ samples from NSHQ14 were originally near H isotope equilibrium with water of SMOW-like isotopic composition, aerobic methane oxidation would push the residual CH₄ towards higher δD (and $\epsilon_{\text{methane/water}}$) values (above the equilibrium line in Figure 3b), which is inconsistent with the comparatively low $\delta\text{D}_{\text{CH}_4}$ observed at NSHQ14.

For the reasons outlined above, post-genetic alteration of CH₄ near CH₄ – H₂O and intramolecular isotopic equilibrium does not explain the observed isotopic compositions of CH₄ sampled from NSHQ14. Therefore, the release of CH₄ stored in fluid inclusions cannot account for all of the CH₄ at NSHQ14. Alternative processes that do produce CH₄ with $\Delta^{13}\text{CH}_3\text{D}$ and $\Delta^{12}\text{CH}_2\text{D}_2$ values lower than equilibrium include microbial methanogenesis and low-temperature ($\leq 90^\circ\text{C}$) abiotic reduction of CO₂ or CO through Sabatier or Fischer-Tropsch-type reactions. In Figure 3b and d, microbial methanogenesis is represented by samples from cultures (green shaded areas; (D. T. Wang et al., 2015; D. Stolper et al., 2015; E. Young et al., 2017; Gruen et al., 2018; E. D. Young, 2020)), and low-temperature Sabatier or Fischer-Tropsch-type reactions are represented by field samples from Kidd Creek (gray shaded areas; (E. Young et al., 2017; Sherwood Lollar et al., 2002, 2008)) and laboratory experiments with synthetic Ru catalysts (E. Young et al., 2017; Etiope & Ionescu, 2015).

To independently assess the potential influences of microbial processes on CH₄ concentration and isotopic composition, DNA was extracted from biomass in pumped groundwaters and subjected to amplification and sequencing of 16S rRNA genes. 16S rRNA gene sequences of biomass collected in 2018 were searched for matches to known CH₄-cycling taxa, as compiled previously by Crespo-Medina et al. (2017). Sequences closely affiliated with both methanogenic and methanotrophic taxa were found to be widespread in the aquifer (Figure 5). Based on phylogenetic inference, the dominant methanogenic taxon was related to the genus *Methanobacterium*, whose members can produce CH₄ from H₂ and CO₂, CO, or formate (Balch et al., 1979). *Methanobacterium* comprised a high proportion (24%) of 16S rRNA gene sequences at NSHQ14 in 2018. Relative abundances of *Methanobacterium* 16S rRNA gene reads were similarly high in 2017 (12%) and 2016 (28%), but lower (< 1%) in 2015 and 2014 (Miller et al., 2016; Rempfert et al., 2017; Kraus et al., 2021). The increase in the relative abundance of 16S rRNA genes affiliated with *Methanobacterium* in samples collected in 2016 and onwards versus those collected in 2014 and 2015 coincided with a change in sampling methods from smaller, lower-flow pumps (maximum depth 20 m) prior to 2016 to larger, higher-flow pumps (maximum depth 90 m). The obligate anaerobic nature of this methanogen genus (Boone, 2015) is consistent with its higher relative gene abundances in fluids sampled from greater depths, which presumably receive less input of atmospheric O₂ than do shallower fluids.

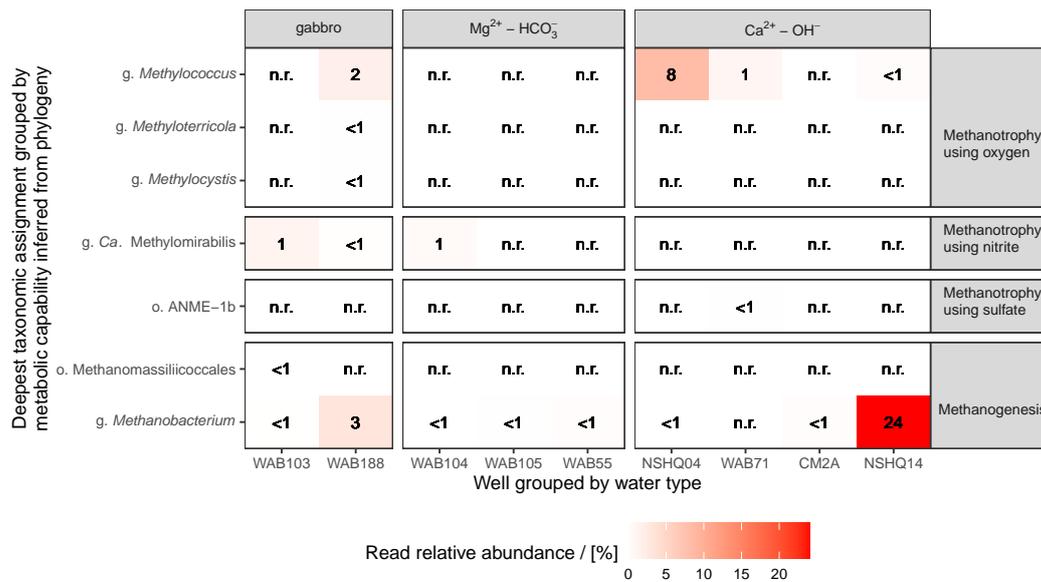


Figure 5. 16S rRNA gene read relative abundances of DNA extracted from Samail Ophiolite groundwaters sampled in 2018 affiliated with CH₄-cycling taxa. Read relative abundances are reported as percentages rounded to the ones place. Cases when a taxon was detected in a sample and was < 1% read relative abundance after rounding are labeled “< 1”. Cases when no reads of a taxon were detected in a sample are labeled “n.r.” Data shown are from unique field samples. Previous 16S rRNA gene sequencing studies that obtained field samples in triplicate from Samail Ophiolite groundwaters through similar methods to those used here have found typical standard deviations of relative abundances less than or equal to 25% of the mean relative abundance (Kraus et al., 2021).

694 Consortia capable of anaerobic oxidation of CH₄ coupled to SO₄²⁻ reduction, in-
 695 cluding ANME, were not detected by 16S rRNA gene sequencing of samples obtained
 696 from NSHQ14 in 2018 (Figure 5), 2016, or 2014 (Miller et al., 2016; Rempfert et al., 2017),
 697 although sequences affiliated with order ANME-1b were detected in low abundance (<
 698 1% of reads) in samples obtained from NSHQ14 in 2017 and 2015 (Rempfert et al., 2017;
 699 Kraus et al., 2021). This scarcity of ANME may result from metabolic inhibition by high
 700 c_{H₂} in groundwaters at NSHQ14 and elsewhere in the Samail Ophiolite. It has been pro-
 701 posed that the thermodynamics of “reverse methanogenesis” require low c_{H₂} (e.g. ≤ 1 nM
 702 in a marine cold seep environment (Boetius et al., 2000)). Indeed, the bioenergetics of
 703 SO₄²⁻-driven oxidation of CH₄ are less favorable than SO₄²⁻-driven oxidation of H₂ or
 704 non-CH₄ organics, or other metabolisms such as methanogenesis or acetogenesis in the
 705 Samail Ophiolite (Canovas III et al., 2017) and in deep continental settings where ra-
 706 diolytic H₂ accumulates (Kieft et al., 2005; Moser et al., 2005; Kieft, 2016).

707 While 16S rRNA gene sequences affiliated with anaerobic CH₄ oxidizing microbes
 708 have only occasionally been detected at NSHQ14, 16S rRNA gene sequences affiliated
 709 with the genus *Methylococcus*, which contains aerobic methanotrophs (Hanson & Han-
 710 son, 1996), have been detected in all samples from NSHQ14, ranging from 1% to < 1%
 711 of reads in samples obtained from 2014 to 2018 (Figure 5; (Miller et al., 2016; Rempfert
 712 et al., 2017; Kraus et al., 2021)). Since the aerobic lifestyle of *Methylococcus* is at odds
 713 with that of the obligate anaerobe, *Methanobacterium*, it seems most likely that these
 714 two taxa are spatially separated in the aquifer, and that waters containing each of them

were mixed during open borehole pumping. Still, the > 10 times higher abundances of *Methanobacterium*-related 16S rRNA genes relative to those of *Methylococcus* at NSHQ14 in samples from 2016 to 2018 suggest that the microbial CH₄ cycle at this well is dominated by CH₄ production, rather than consumption.

16S rRNA gene sequencing of subsurface biomass from NSHQ14 is complemented by other observations that suggest that methanogens are not only prevalent, but active. Genes involved in methanogenesis are enriched (Fones et al., 2019) and actively transcribed in waters sampled from NSHQ14 (Kraus et al., 2021). Transformation of both ¹⁴C-labeled HCO₃⁻ and ¹⁴C-labeled formate to CH₄ have been shown to occur in water samples from NSHQ14 at significantly higher rates than in killed controls, with formatotrophic methanogenesis greatly outpacing hydrogenotrophic methanogenesis (Fones et al., 2019, 2020). Taken together with a cell abundance of 1.15 · 10⁵ cells · mL⁻¹ in groundwater at NSHQ14 (Fones et al., 2019), these data suggest that aquifer regions accessed by NSHQ14 host abundant active methanogenic cells (thousands per mL, assuming ~ 24 % of cells are methanogens based on 16S rRNA gene data). These active cells could influence CH₄ concentration and isotopic composition.

The genomic and cultivation data of Fones et al. (2020) indicate that formate is the dominant substrate for methanogenesis at NSHQ14. Formate concentrations are 1 μmol · L⁻¹ to 2 μmol · L⁻¹ in the studied wells (Rempfert et al., 2017), which are roughly two orders of magnitude lower than formate concentrations at unsedimented seafloor hydrothermal vents impacted by serpentinization at warmer conditions than present in the Samail Ophiolite (McDermott et al., 2015; Lang et al., 2018). These relatively low formate concentrations in the ophiolite suggest that formate might be the primary limiting substrates for methanogenesis in Ca²⁺-OH⁻ waters, such as at NSHQ14. Coexisting hydrogenotrophic methanogens may produce CH₄ through direct uptake of ∑ CO₂ in H₂-rich Ca²⁺-OH⁻ water, where kinetic inhibitions to abiotic ∑ CO₂ reduction to CH₄ allow for a modest energy yield for hydrogenotrophic methanogens (Section 5; (Leong & Shock, 2020)). Methanogens using ∑ CO₂ could benefit from greater chemical disequilibrium if they inhabit zones where deeply-sourced, H₂-rich Ca²⁺-OH⁻ water mixes with shallow, Mg²⁺-HCO₃⁻ water (Zwicker et al., 2018; Leong & Shock, 2020). In addition to direct uptake of ∑ CO₂, carbonate minerals may serve as a C source for methanogenesis in carbonated peridotites (Miller et al., 2018). Another potential C source is carbon monoxide (CO). CO has always been below limits of quantitation in Oman wells (< 132 nmol · L⁻¹ in 2018; Table 4), but it is unclear whether this indicates minimal CO production or rapid CO turnover.

The microbiological data from NSHQ14 fluids are compatible with δD_{CH₄}, Δ¹³CH₃D, and Δ¹²CH₂D₂ values that collectively indicate a substantial addition of microbial CH₄ to an otherwise abiotic pool of CH₄. Although the data presented here do not enable us to precisely determine the mole fractions and isotopic compositions of the microbial and abiotic components of CH₄ at NSHQ14, the δD_{CH₄} data alone suggest that perhaps the majority of CH₄ at NSHQ14 formed through non-equilibrium processes, which include microbial methanogenesis. Thus, the high δ¹³C of CH₄ at NSHQ14 suggests that the microbial component is more ¹³C-enriched than microbial CH₄ formed in sedimentary environments, which typically ranges from -90 ‰ VPDB to -50 ‰ VPDB ((Milkov & Etiope, 2018); Figure 3a). In cultures of a hydrogenotrophic strain of *Methanobacterium* provided CaCO₃ (s) as a C source at pH ~ 9, Miller et al. (2018) observed suppressed apparent isotope effects during methanogenesis (α_{CO₂/CH₄} = 1.028). The authors attributed this to the slow kinetics of carbonate dissolution at high pH and the near-total conversion of the resultant CO₂ (aq) to CH₄ by *Methanobacterium*. If the primary mode of methanogenesis at NSHQ14 is in fact formatotrophic methanogenesis and abiotic formate production is the rate-limiting step in the overall process through which ∑ CO₂ is converted to CH₄, similar isotopic bottlenecks could apply. Cellular formate uptake and enzymatic conversion processes whose isotope effects remain unknown could be important drivers of the isotopic composition of CH₄ in hyperalkaline, serpentinizing set-

768 tings. In such settings, the suppression of C isotope fractionation during methanogen-
 769 esis is supported by observations of high $\delta^{13}\text{C}$ values (up to +14 ‰ VPDB) of lipid biomark-
 770 ers thought to be produced by methanogens at Chimaera, Turkey (Zwicker et al., 2018)
 771 and at Lost City (Bradley et al., 2009). Evaluation of these hypotheses will require fur-
 772 ther physiological studies of methanogens aimed at understanding substrate selection and
 773 limitation systematics in hyperalkaline, low-C conditions and the isotopic implications
 774 of these factors.

775 While the data support substantial microbial CH_4 and abiotic, fluid inclusion-derived
 776 CH_4 in NSHQ14 fluids, we find less evidence for abiotic CH_4 production at the low tem-
 777 peratures that pervade the modern weathering horizon in the ophiolite. Below 100 °C,
 778 access of gas-phase H_2 and CO_2 or CO to the catalytic metals Ru or Rh is required for
 779 CH_4 to form at appreciable rates (Thampi et al., 1987; Jacquemin et al., 2010; Etiope
 780 & Ionescu, 2015; McCollom, 2016). It has been proposed that the spatial concentration
 781 of potentially-catalytic Ru-rich chromites in chromitites is important for catalysis of low-
 782 temperature CO_2 reduction to CH_4 in ophiolites (Etiope & Ionescu, 2015; Etiope et al.,
 783 2018). While peridotites in Oman ubiquitously contain a few percent distributed chromite
 784 (Hanghøj et al., 2010), massive chromitites were not reported in lithologic descriptions
 785 of cores or drill cuttings from NSHQ14 or any of the six additional wells ranging from
 786 300 m to 400 m depth that have been drilled in the same catchment by the Oman Drilling
 787 Project (P. Kelemen et al., 2020). Nor are chromitites notably abundant in outcrop within
 788 this catchment. Further, although some flow paths of meteoric water through the ophi-
 789 olite may result in saturation in H_2 and separation of a free gas phase (Canovas III et
 790 al., 2017), the depth to water is < 20 m in all wells in the catchment of NSHQ14, sug-
 791 gesting water-saturated conditions in the subsurface. Moreover, if free H_2 (g) were gen-
 792 erated at high extents of reaction progress, co-existing CO_2 (g) would be extremely scarce
 793 due to precipitation of carbonate minerals and high pH (Etiope & Ionescu, 2015; Leong
 794 & Shock, 2020). It has been proposed that CH_4 in ophiolites can form through reduc-
 795 tion of CO_2 (g) from non-atmospheric sources such as magma, the mantle, or sedimen-
 796 tary carbonate formations (Etiope & Ionescu, 2015). A magmatic/mantle CO_2 source
 797 is not supported at NSHQ14 because excess He above air saturation in groundwaters from
 798 this well has a dominantly radiogenic isotopic composition that is distinct from mantle-
 799 derived He (Paukert Vankeuren et al., 2019). Further, although sedimentary carbonates
 800 are present in the vicinity of NSHQ14 and elsewhere in the ophiolite (Boudier & Cole-
 801 man, 1981; de Obeso & Kelemen, 2018), there is no clear mechanism to liberate CO_2 (g)
 802 from mineral carbonates and transfer that CO_2 (g) to catalytic sites of reaction on chromites
 803 where H_2 (g) is also present. Thus, the apparent lack of massive chromites and free gaseous
 804 potential reactants suggest that the subsurface surrounding NSHQ14 is not conducive
 805 to low-temperature abiotic CH_4 production. While substantial low-temperature CH_4 pro-
 806 duction in the catchment of NSHQ14 seems unlikely, NSHQ14 groundwaters could be
 807 mere carriers of CH_4 that was produced elsewhere in the ophiolite under gaseous con-
 808 ditions and that has subsequently migrated into the aquifer. Some studies of CH_4 ori-
 809 gin in other peridotite bodies have favored such a hypothesis (Etiope et al., 2016; Mar-
 810 ques et al., 2018). However, it is not clear how this hypothesis could be tested in the case
 811 of the NSHQ14, nor how it addresses the issue of CO_2 source.

812 In summary, isotopic and microbiological data lead us to conclude that the high
 813 concentrations of CH_4 ($10^2 \mu\text{mol}\cdot\text{L}^{-1}$) in groundwaters accessed by NSHQ14 primarily
 814 result from microbial methanogenesis and the release of abiotic CH_4 from fluid inclusions.
 815 The known presence of CH_4 -bearing fluid inclusions in the Samail Ophiolite and our find-
 816 ing of high $\delta^{13}\text{C}$ values of CH_4 , C_2H_6 , and C_3H_8 that overlap with values reported from
 817 seafloor hydrothermal vents where CH_4 formed at > 270 °C in fluid inclusions predom-
 818 inates suggest a similar source in the ophiolite. However, deficits in $^{12}\text{CH}_3\text{D}$, $^{13}\text{CH}_3\text{D}$,
 819 and $^{12}\text{CH}_2\text{D}_2$ relative to equilibrium indicate the production of additional CH_4 at low
 820 temperatures. The $^{13}\text{CH}_3\text{D}$ deficit in particular is more compatible with a microbial ori-
 821 gin than a low-temperature abiotic origin. Moreover, genomic, transcriptomic, and phys-

822 iological data show that methanogens are abundant and active in aquifers accessed via
 823 NSHQ14. Organic geochemical and cultivation data from the literature suggest that C
 824 isotope effects of methanogenesis may be suppressed under C-limited conditions in ser-
 825 pentinizing settings. That genes associated with methanogens coexist with a smaller abun-
 826 dance of genes associated with methanotrophs (particularly aerobes) in NSHQ14 ground-
 827 waters suggests that some of the CH₄ has undergone microbial oxidation, which would
 828 further help explain the high δ¹³C values of CH₄ at this well.

829 **4.2.3 Abundant microbial CH₄ produced under C-limited conditions and** 830 **substantial microbial CH₄ oxidation in the Ca²⁺ – OH[–] waters** 831 **of well NSHQ04**

832 NSHQ04 is situated in partially serpentinized harzburgite 10 m away from a faulted
 833 contact with crustal gabbros (Figure 1; Supporting Information Figure S1). Surface rock
 834 exposures surrounding NSHQ04 are dominated by serpentinized harzburgites, with lesser
 835 dunites, gabbro lenses, and pyroxenite dikes. NSHQ04 is cased to 5.8 mbgl and drilled
 836 to 304 m depth (Table 1). As of 2017, the well is obstructed at 8 m below the casing top,
 837 precluding deeper sampling (Section 3.1; Table 1).

838 Primary differences in fluid composition between NSHQ04 and NSHQ14 include
 839 lower pH by ~ 1 and higher $c_{\sum Ca}$ and $c_{\sum Si}$ at NSHQ04 (Tables 1 and 3; (Miller et
 840 al., 2016; Rempfert et al., 2017; Paukert Vankeuren et al., 2019; Fones et al., 2019)). These
 841 differences could be related to the scarcity of fresh, near-surface olivine at NSHQ04, which
 842 may result in a greater influence of pyroxene serpentinization at NSHQ04 (Miller et al.,
 843 2016). Low-temperature pyroxene serpentinization generally continues after olivine is
 844 exhausted, and leads to higher $c_{\sum Si}$ and, depending on pyroxene chemical composition,
 845 can also lead to higher $c_{\sum Ca}$ and lower pH (Bach et al., 2006; Leong & Shock, 2020).
 846 The relatively low pH and high $c_{\sum Si}$ could also stem from mixing of Ca²⁺–OH[–] wa-
 847 ters with gabbro- or atmosphere-influenced fluids.

848 Compared to NSHQ14, NSHQ04 has generally had lower c_{H_2} (detected in 2014, but
 849 not in 2018, 2017, 2015, or 2012; Table 4; Figure 2; (Miller et al., 2016; Rempfert et al.,
 850 2017; Paukert Vankeuren et al., 2019)). The relatively low c_{H_2} measured in waters pumped
 851 from NSHQ04 is probably due at least in part to microbial H₂ oxidation. Although there
 852 are multiple enzymes with which which a diversity of microbes oxidize H₂ (Peters et al.,
 853 2015), aerobic H₂ oxidation by bacteria of the genus *Hydrogenophaga* has been identi-
 854 fied as a particularly prevalent process in serpentinizing settings, including the Samail
 855 Ophiolite (Suzuki et al., 2014; Rempfert et al., 2017; Marques et al., 2018). Sequences
 856 affiliated with *Hydrogenophaga* accounted for 20 % of 16S rRNA gene reads in DNA ex-
 857 tracted from biomass in waters pumped from NSHQ04 in 2018, which is similar to pre-
 858 vious years of sampling at NSHQ04 (6 % to 18 % in 2014, 2015, and 2017; inter-annual
 859 mean of 12 %) and higher than all other studied wells (Supporting Information Figure
 860 S3; (Rempfert et al., 2017; Miller et al., 2016; Kraus et al., 2021)).

861 While H₂ has only been transiently detected at NSHQ04, c_{CH_4} at this well has con-
 862 sistently been the highest among our sample sites (144 μmol·L⁻¹ in 2018 and 483 μmol·
 863 L⁻¹ in 2017. In comparison to NSHQ14, CH₄ at NSHQ04 is more ¹³C- and D-enriched
 864 (mean weighted by sample year δ¹³C = +3.3 ‰ VPDB, $s = 1.8 ‰$; δD = -220 ‰ VSMOW,
 865 $s = 11 ‰$; $n = 4$; Table 2; Figure 3a). Fluids sampled from NSHQ04 are in CH₄ –
 866 H₂O H isotopic disequilibrium and intra-CH₄ disequilibrium (Figure 3b and d), which
 867 is also true of fluids from NSHQ14. However, CH₄ sampled from NSHQ04 has distinctly
 868 negative Δ¹²CH₂D₂ (-24.502 ‰) and low Δ¹³CH₃D (mean weighted by sample year of
 869 0.36 ‰, $s = 0.32 ‰$, $n = 3$; Table 2). As such, CH₄ from NSHQ04 plots squarely among
 870 methanogen culture samples in Δ¹³CH₃D/Δ¹²CH₂D₂ space (Figure 3d), suggesting that
 871 CH₄ is dominantly microbial at NSHQ04. Moreover, alkane gases dissolved in waters pumped
 872 from NSHQ04 exhibited a C₁/(C₂ + C₃) ratio of 5.4·10³ in 2018, which is higher than

873 other wells in this study (Table 4; Figure 3c), further supporting a major component of
874 microbial CH₄ at NSHQ04.

875 Microbial CH₄ production at NSHQ04 is also indicated by microbiological data.
876 16S rRNA gene sequences affiliated with *Methanobacterium* have been detected in DNA
877 extracted from biomass filtered from waters pumped from NSHQ04, albeit in low relative
878 abundance (< 1 % of reads in 2018; Figure 5; also detected in < 1 % of reads in
879 2014, but not detected in 2015 and 2017; (Rempfert et al., 2017; Miller et al., 2016; Kraus
880 et al., 2021)). The apparent low relative abundance of *Methanobacterium* at NSHQ04
881 could have resulted from the relatively shallow depth from which samples were collected
882 at NSHQ04 due to well obstruction and the consequential sampling of groundwaters that
883 that may have experienced atmospheric O₂ infiltration. High relative read abundances of se-
884 quences affiliated with aerobes and transient H₂ across years of sampling NSHQ04 sug-
885 gest that zones of the aquifer that are not always anoxic were accessed. These conditions
886 may restrict methanogen abundance to greater depths than were sampled, but not con-
887 strain the upward diffusion of the product of their metabolism, CH₄. Nevertheless, flu-
888 ids obtained from NSHQ04 have yielded robust cultures of *Methanobacterium* (Miller
889 et al., 2018). In addition, high relative abundances of 16S rRNA gene reads of DNA ex-
890 tracted from biomass in waters sampled from NSHQ04 were related to an aerobic methan-
891 otroph of the genus *Methylococcus* (8 % of reads in 2018; inter-annual mean of 11 %; Fig-
892 ure 5; (Miller et al., 2016; Rempfert et al., 2017; Kraus et al., 2021)). Greater aerobic
893 methanotrophy at NSHQ04 relative to NSHQ14 may have contributed in part to the lower
894 $\Delta^{13}\text{CH}_3\text{D}$ and $\Delta^{12}\text{CH}_2\text{D}_2$ and higher $\delta^{13}\text{C}$ and δD of CH₄ sampled from NSHQ04.

895 Methanotrophic activity at NSHQ04 is consistent with the observed ¹³C-depletion
896 in $\sum \text{CO}_2$ at NSHQ04 (-29.7‰ VPDB $\delta^{13}\text{C}$; Table 2) relative to the other studied wells
897 because environments of active methanotrophy often have ¹³C-depleted $\sum \text{CO}_2$ (Barker
898 & Fritz, 1981; Michaelis et al., 2002). Indeed, $\delta^{13}\text{C}_{\sum \text{CO}_2}$ at NSHQ04 is compatible with
899 aerobic oxidation of CH₄ of $\sim 0\text{‰}$ VPDB $\delta^{13}\text{C}$ (Barker & Fritz, 1981; Feisthauer et al.,
900 2011). Alternatively, ¹³C-depletion in $\sum \text{CO}_2$ could be explained by kinetic isotope frac-
901 tionation during hydroxylation of atmospheric CO₂ upon contact with Ca²⁺–OH[–] wa-
902 ter, which has been interpreted as the cause of $\delta^{13}\text{C}$ as low as -27.21‰ VPDB in Ca-
903 rich carbonates from hyperalkaline seeps in the Samail Ophiolite (Clark et al., 1992; P. B. Kele-
904 men et al., 2011; Falk et al., 2016). Considering the relatively shallow sampling depth
905 at NSHQ04 in 2018 (Table 1), it is plausible that the sampled groundwaters continuously
906 interact with atmospheric CO₂. Although the relative influences of methanotrophy and
907 atmospheric CO₂ hydroxylation cannot be determined based on the available data, both
908 processes could affect $\delta^{13}\text{C}_{\sum \text{CO}_2}$ at NSHQ04.

909 In summary, low $\Delta^{13}\text{CH}_3\text{D}$ and $\Delta^{12}\text{CH}_2\text{D}_2$, high C₁/(C₂ + C₃), the presence of
910 *Methanobacterium* that were readily cultured, and high 16S rRNA gene relative abun-
911 dances of *Methylococcus* lead us to conclude that microbial production and consump-
912 tion of CH₄ are the dominant factors controlling CH₄ concentration and isotopic com-
913 position at NSHQ04.

914 **4.2.4 H₂-limited microbial methanogenesis with classic C isotope effect** 915 **expressed at well WAB188**

916 WAB188 is situated 2 km down-gradient from NSHQ04 and is set in gabbro on the
917 opposite side of a fault from NSHQ04 (Figure 1; Supporting Information Figure S1; Ta-
918 ble 1). Fluids pumped from WAB188 have had variable pH (8.72 to 5.75) and oxidation-
919 reduction potential (f_{O_2} of 10^{-61} bar to 10^{-34} bar and Eh of -220 mV to $+214$ mV) across
920 four years of sampling (Table 1; (Rempfert et al., 2017; Fones et al., 2019)). WAB188
921 has consistently had major ion compositions similar to the gabbro-hosted well WAB103,
922 except that WAB188 has had higher $c_{\sum \text{Ca}}$ (Table 3; (Rempfert et al., 2017; Fones et
923 al., 2019)). H₂ has occasionally been detected in fluids pumped from WAB188 (c_{H_2} =

0.992 $\mu\text{mol}\cdot\text{L}^{-1}$ in 2017), and CH_4 has consistently been detected at moderate concentrations ($c_{\text{CH}_4} = 1.83 \mu\text{mol}\cdot\text{L}^{-1}$ in 2017 and $0.917 \mu\text{mol}\cdot\text{L}^{-1}$ in 2018) (Table 4; (Rempfert et al., 2017; Fones et al., 2019)). The high $c_{\sum \text{Ca}}$ and moderate but variable pH, Eh , and c_{H_2} in fluids sampled from WAB188 suggest that fluid chemical composition at WAB188 is dominantly controlled by water-rock reaction with gabbro (McCollom, 1999; Hoehler, 2004), but may also be affected by inputs of fresh rainwater and/or H_2 -bearing $\text{Ca}^{2+} - \text{OH}^-$ water flowing from the peridotite aquifer into the gabbro aquifer across a fault at depth. Flows of water from higher-head, lower-permeability peridotite aquifers into gabbro aquifers in the Samail Ophiolite have been proposed on the basis of physical hydrologic data (Dewandel et al., 2005). Instead or in addition, serpentinization of olivine and pyroxene entirely within gabbro might have produced H_2 observed in water samples from WAB188.

Microbial methanogenesis at WAB188 is indicated by high relative abundances of 16S rRNA gene reads affiliated with methanogens in pumped groundwaters. Sequences affiliated with *Methanobacterium* accounted for 3% of 16S rRNA gene reads of DNA extracted from subsurface fluids sampled from WAB188 in 2018, which was second only to NSHQ14 among our sampling sites, and consistent with prior years of sampling at WAB188 (mean 2015 to 2018 of 4%; Figure 5; (Rempfert et al., 2017; Kraus et al., 2021)). There was also evidence for methanotrophy. 2% of 16S rRNA gene reads from WAB188 were affiliated with *Methylococcus* in 2018, which was second only to NSHQ04 among our sampling sites, and consistent with prior years of sampling (Figure 5; (Rempfert et al., 2017; Kraus et al., 2021)). Further, 16S rRNA gene sequences affiliated with genus *Candidatus* *Methylomirabilis*, which includes species that mediate anaerobic methane oxidation coupled to nitrite reduction (Ettwig et al., 2010; Luesken et al., 2012; Welte et al., 2016), were detected in samples from WAB188 in 2018 albeit at low relative gene abundance (< 1%). As a whole, the 16S rRNA gene sequencing data from WAB188 fluids are consistent with microbial production of CH_4 and, secondarily, methanotrophy using O_2 and/or NO_2^- . The 16S rRNA data are bolstered by genomic and cultivation data that demonstrate that *Methanobacterium* at WAB188 can produce CH_4 from CO_2 and/or formate (Fones et al., 2020) and that genes involved in methanogenesis are transcribed in groundwater samples obtained from WAB188 (Kraus et al., 2021).

While subsurface fluids sampled at WAB188, NSHQ14, and NSHQ04 all bear evidence of methanogenic activity, the conditions under which methanogenesis proceeds at WAB188 are fundamentally distinct. In contrast to the $\text{Ca}^{2+} - \text{OH}^-$ fluids from NSHQ14 and NSHQ04, the circumneutral fluids from WAB188 have $\sim 10^2$ to $\sim 10^3$ times higher $c_{\sum \text{CO}_2}$ (inter-annual mean of $2910 \mu\text{mol}\cdot\text{L}^{-1}$, $s = 620 \mu\text{mol}\cdot\text{L}^{-1}$, $n = 3$; Table 3) and $\sim 75\%$ lower $\delta^{13}\text{C}_{\text{CH}_4}$ (inter-annual mean $\delta^{13}\text{C} = -73\%$ VPDB, $s = 13\%$, $n = 3$; Table 2; Figure S5). Since WAB188 fluids contain relatively ^{13}C -depleted CH_4 that is not associated with substantial concentrations of $\text{C}_2 - \text{C}_6$ alkanes (Table 4), a standard interpretation (Bernard et al., 1977; Milkov & Etiope, 2018) would be that the source of CH_4 at WAB188 is dominantly microbial. Such an interpretation is largely based on data from sedimentary settings, where H_2 is typically more scarce than CO_2 . In this regard, conditions in sedimentary settings are analogous to those at WAB188. Evidence that considerable methanogenesis proceeds through a hydrogenotrophic pathway under H_2 -limited conditions at WAB188 include microbiological data confirming the capacity of *Methanobacterium* to perform hydrogenotrophic methanogenesis at WAB188 and thermodynamic calculations showing that hydrogenotrophic methanogenesis (with H_2 as limiting substrate) was more energetically favorable than formatotrophic methanogenesis for a fluid with $c_{\sum \text{CO}_2}$ and c_{H_2} similar to WAB188 in 2017 (Section 4.2.1; Table 5). Further, the apparent $\alpha_{\text{CO}_2/\text{CH}_4}$ at WAB188 (based on measured $\delta^{13}\text{C}_{\sum \text{CO}_2}$ of -13.52% VPDB; Table 3) is compatible with that of *Methanobacterium* cultures grown hydrogenotrophically with excess HCO_3^- (aq), which was greater than the $\alpha_{\text{CO}_2/\text{CH}_4}$ observed for parallel cultures under CO_2 -poor conditions (Miller et al., 2018). In sum, the conditions at

977 WAB188 contrast starkly with those that prevail in $\text{Ca}^{2+}-\text{OH}^-$ fluids, where C sub-
 978 strates for methanogenesis are often more scarce than H_2 . These differences may be re-
 979 flected in the inverse relationship between $c_{\sum \text{CO}_2}$ and $\delta^{13}\text{C}_{\text{CH}_4}$ across fluids from wells
 980 WAB188, NSHQ14, and NSHQ04 (Figure S5), which is consistent with an effect of C avail-
 981 ability on the apparent C isotope effect of microbial methanogenesis.

982 5 Conclusions

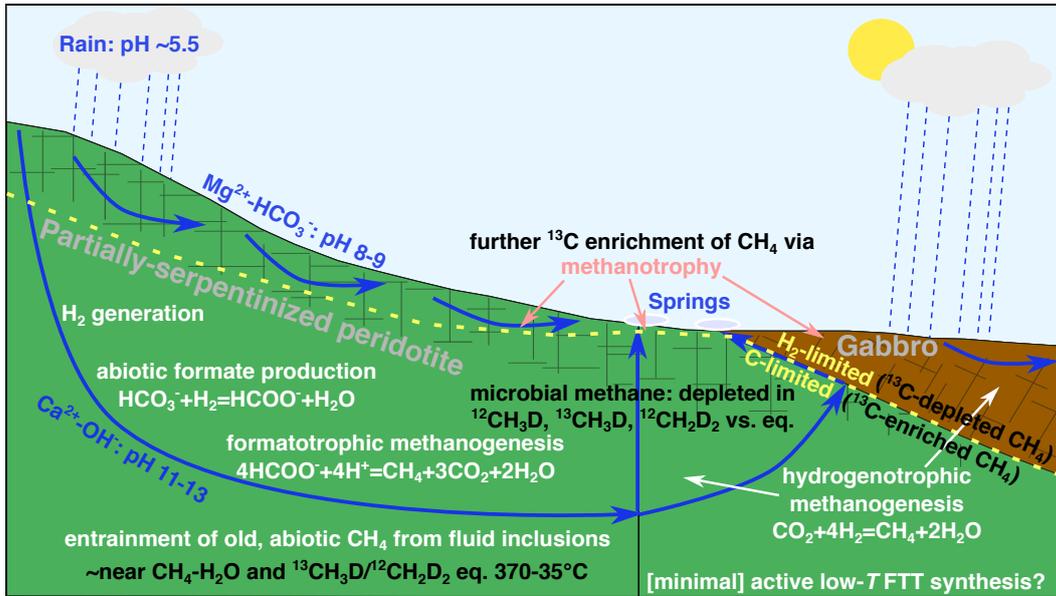


Figure 6. Conceptual model of CH_4 dynamics in Samail Ophiolite. Cross section after Neal and Stanger (1985), Dewandel et al. (2005), and Rempfert et al. (2017). Groundwater flow is depicted with blue arrows. Cross-hatching illustrates fissured zone of aquifer, extending to ~ 50 m depth. A deep tectonic fracture hosting upward groundwater flow is shown as a black line. Yellow dashed line indicates proposed transition between conditions where methanogenesis is limited by H_2 versus C availability. Isotopic systematics are written in black text. *Abbreviations:* eq., equilibrium; T , temperature; FTT, Fischer-Tropsch-type.

983 Through integration of isotopic, microbiological, and hydrogeochemical data, we
 984 conclude that substantial microbial CH_4 is produced under varying degrees of C or H_2
 985 limitation in subsurface waters of the Samail Ophiolite and mixes with abiotic CH_4 re-
 986 leased from fluid inclusions (Figure 6). Across subsurface fluids ranging in pH from cir-
 987 cumneutral to 11.39, microbial CH_4 production is evidenced by 16S rRNA gene sequenc-
 988 ing and other microbiological data indicating that methanogens are widespread and ac-
 989 tive in groundwaters in the ophiolite. We propose that CH_4 produced by these microbes
 990 constitutes a substantial portion of the total CH_4 pool, which is consistent with our find-
 991 ing of $^{13}\text{CH}_3\text{D}$ and $^{12}\text{CH}_2\text{D}_2$ relative abundances significantly less than equilibrium. Us-
 992 ing a simple thermodynamic model, we find that formatotrophic methanogenesis may
 993 become more energetically favorable than hydrogenotrophic methanogenesis as $\text{Mg}^{2+}-$
 994 HCO_3^- waters transition to $\text{Ca}^{2+}-\text{OH}^-$ waters where $\text{CO}_2(\text{aq})$ is extremely scarce, de-
 995 spite relatively low formate concentrations of $\sim 1 \mu\text{mol}\cdot\text{L}^{-1}$ across fluid types (Rempfert
 996 et al., 2017). This lends geochemical support to recent microbiological findings that in-
 997 dependently indicate that the activity of formatotrophic methanogens increases relative

998 to hydrogenotrophic methanogens as groundwater pH increases in the ophiolite (Fones
999 et al., 2020).

1000 In addition, an abiotic, fluid inclusion-derived source of CH₄, C₂H₆, and C₃H₈ is
1001 inferred from the widespread occurrence of CH₄ in fluid inclusions in peridotites, includ-
1002 ing those in Oman, and is supported by the relatively ¹³C-enriched compositions of CH₄,
1003 C₂H₆, and C₃H₈ measured in gases exsolved from peridotite-hosted groundwaters in this
1004 study. The measured $\delta^{13}\text{C}$ values overlap with those of CH₄, C₂H₆ and C₃H₈ from seafloor
1005 hydrothermal vents where fluid inclusions are the dominant source of these alkanes, sug-
1006 gesting similar CH₄ sources across these environments. In contrast, abiotic, low-temperature
1007 reduction of CO₂ to CH₄ appears less likely to contribute substantially to the CH₄ pool
1008 in the study area due to a scarcity of conditions favorable to catalysis, namely, access
1009 of gas-phase H₂ and CO₂/CO to Ru-bearing chromites.

1010 Further, we note an inverse relationship between $c_{\sum\text{CO}_2}$ and $\delta^{13}\text{C}_{\text{CH}_4}$ across ground-
1011 waters bearing microbiological evidence of methanogenic activity. This finding supports
1012 the hypothesis that the apparent C isotope fractionation between the C substrate used
1013 by methanogens and the CH₄ they produce is suppressed when the C substrate is lim-
1014 iting. Thus, our finding that $\delta^{13}\text{C}_{\text{CH}_4}$ varies by 90 ‰ in the Samail Ophiolite suggests
1015 that, in some settings, $\delta^{13}\text{C}_{\text{CH}_4}$ may be a powerful indicator of transitions from H₂-limited
1016 to C-limited conditions for microbial methanogenesis, rather than a discriminant between
1017 microbial versus abiotic CH₄. The 16S rRNA gene sequencing data also indicate the pres-
1018 ence of microbes capable of CH₄ oxidation, particularly those that can use O₂ as an ox-
1019 idant. This oxidation may also contribute in part to the ¹³C-enriched composition of CH₄
1020 in the ophiolite, which is considered unusual for CH₄ with a substantial microbial com-
1021 ponent.

1022 This study supports the premise that H₂ produced from water/rock reaction can
1023 fuel microbial life, even under challenging conditions of high pH and low oxidant avail-
1024 ability. By identifying where and how microbial methanogenesis can reasonably be ex-
1025 pected to occur in H₂-rich, subsurface environments, this work complements theoret-
1026 ical models in guiding the search for rock-hosted life, including extraterrestrial life. For
1027 example, our findings substantiate predictions that microbial methanogenesis could oc-
1028 cur in the reduced, alkaline ocean of Saturn’s moon, Enceladus (McKay et al., 2008; Glein
1029 et al., 2015; Waite et al., 2017) and in the Martian subsurface (Kral et al., 2014).

1030 Open Research

1031 Data (in Excel format) and source code (in R Markdown format) used to produce
1032 the figures, data tables and analyses for this paper (as well as additional data on ana-
1033 lytical uncertainties and trace element concentrations) are available online in D. B. Nothaft,
1034 Templeton, et al. (2021). Additional DNA sequence data processing codes are available
1035 in D. B. Nothaft, Rempfert, and Kraus (2021). The sequences are accessible on the NCBI
1036 Short Read Archive under accession PRJNA655565.

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Figure 1.

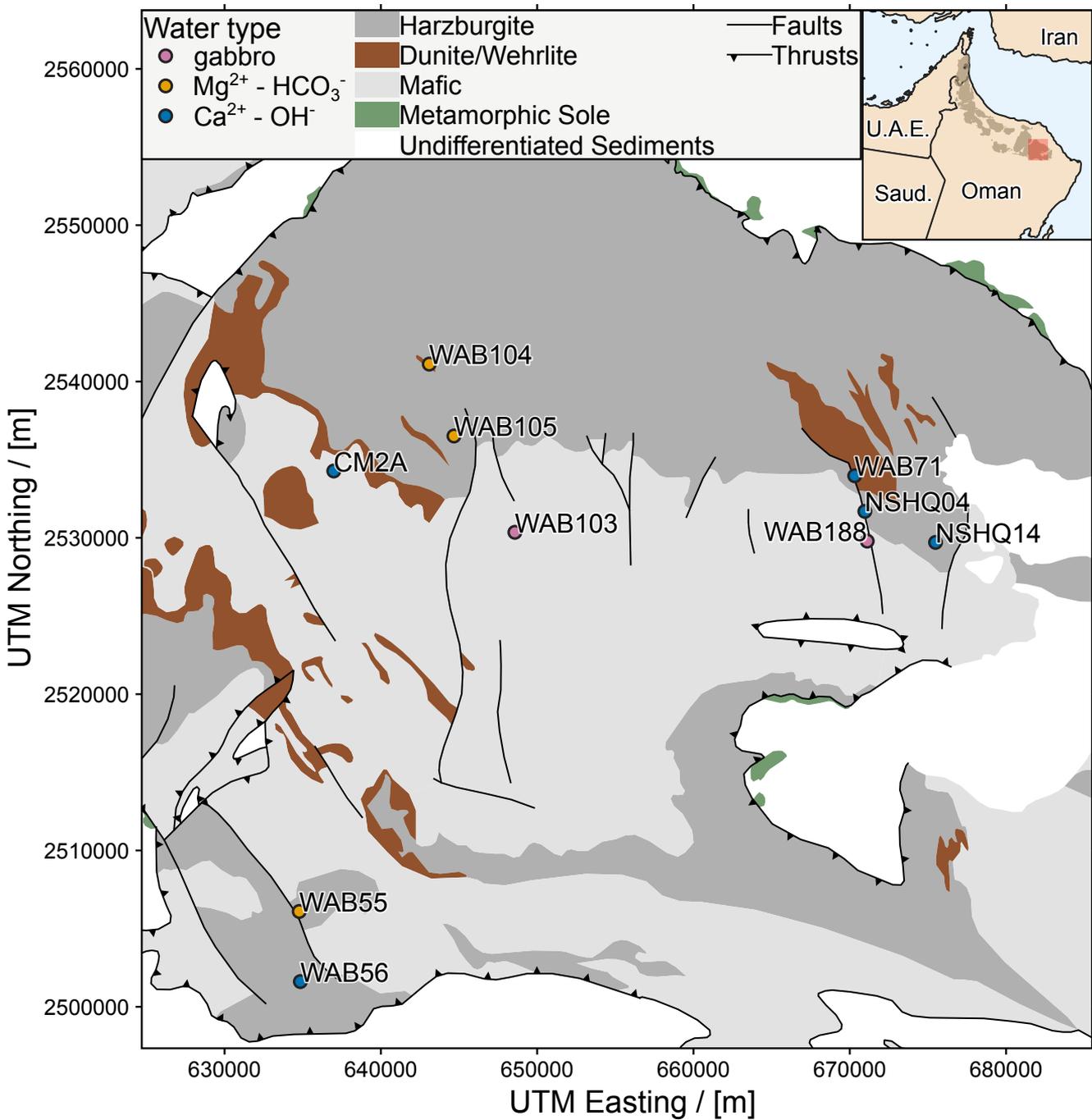


Figure 2.

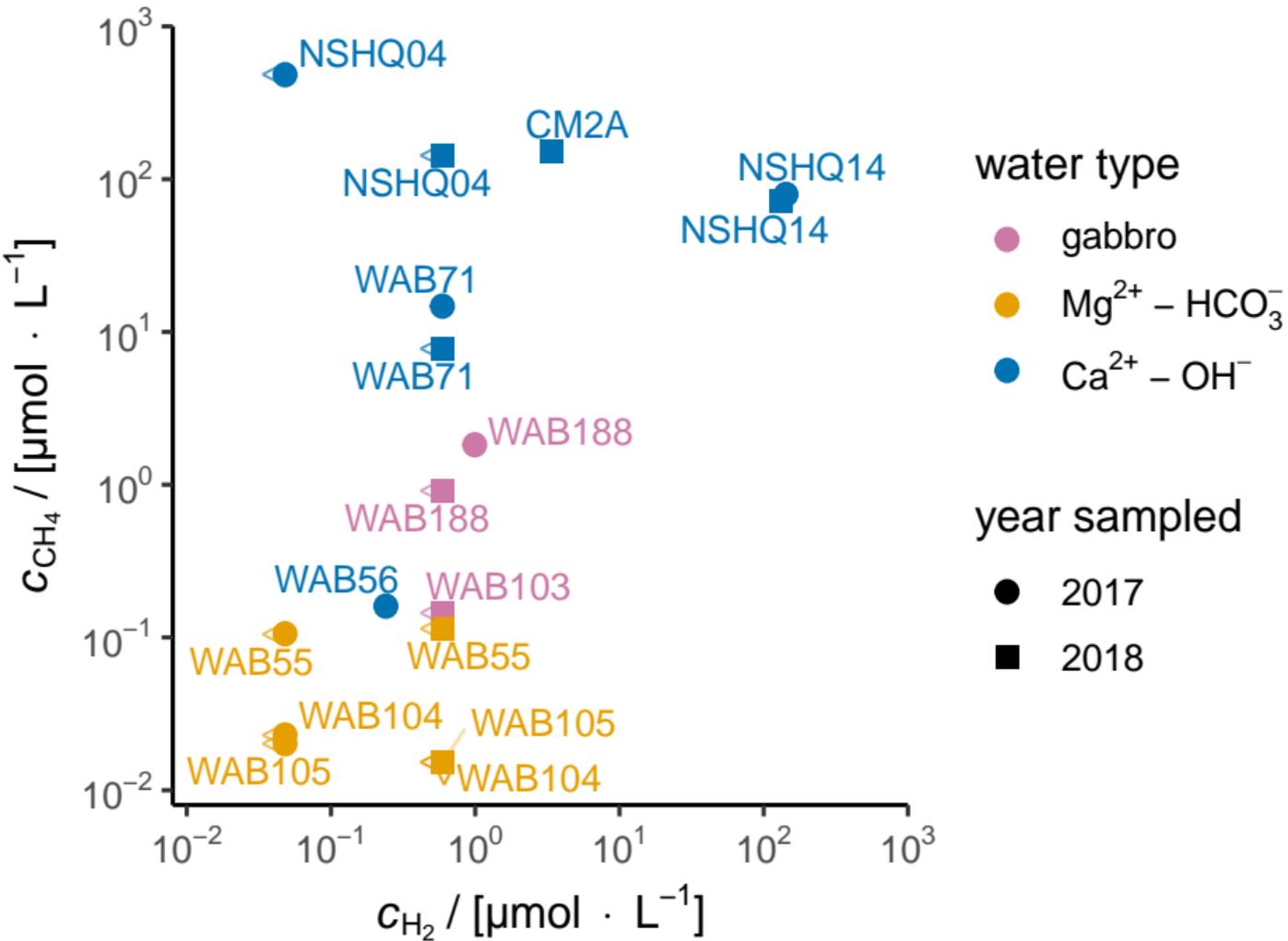


Figure 3.

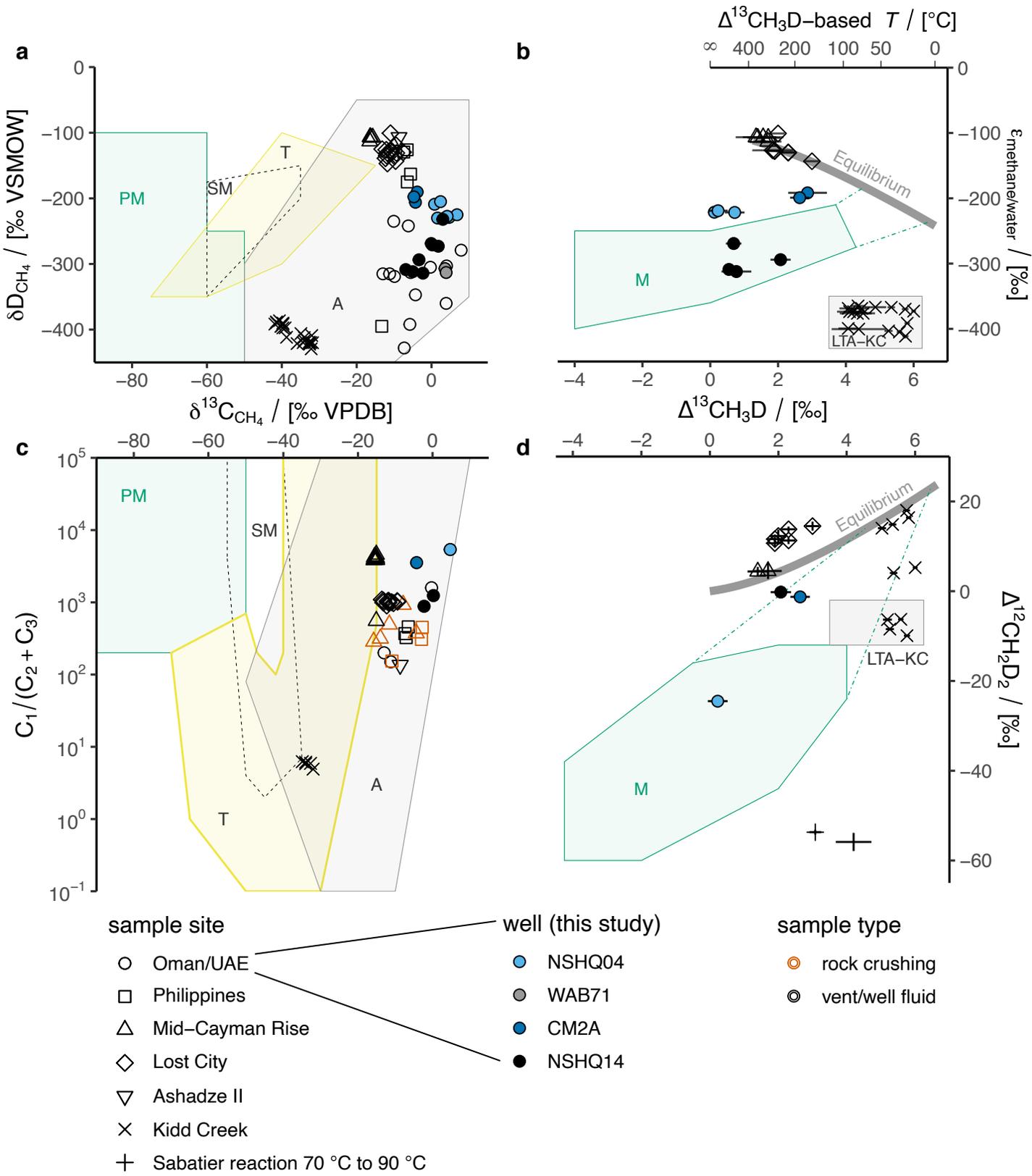
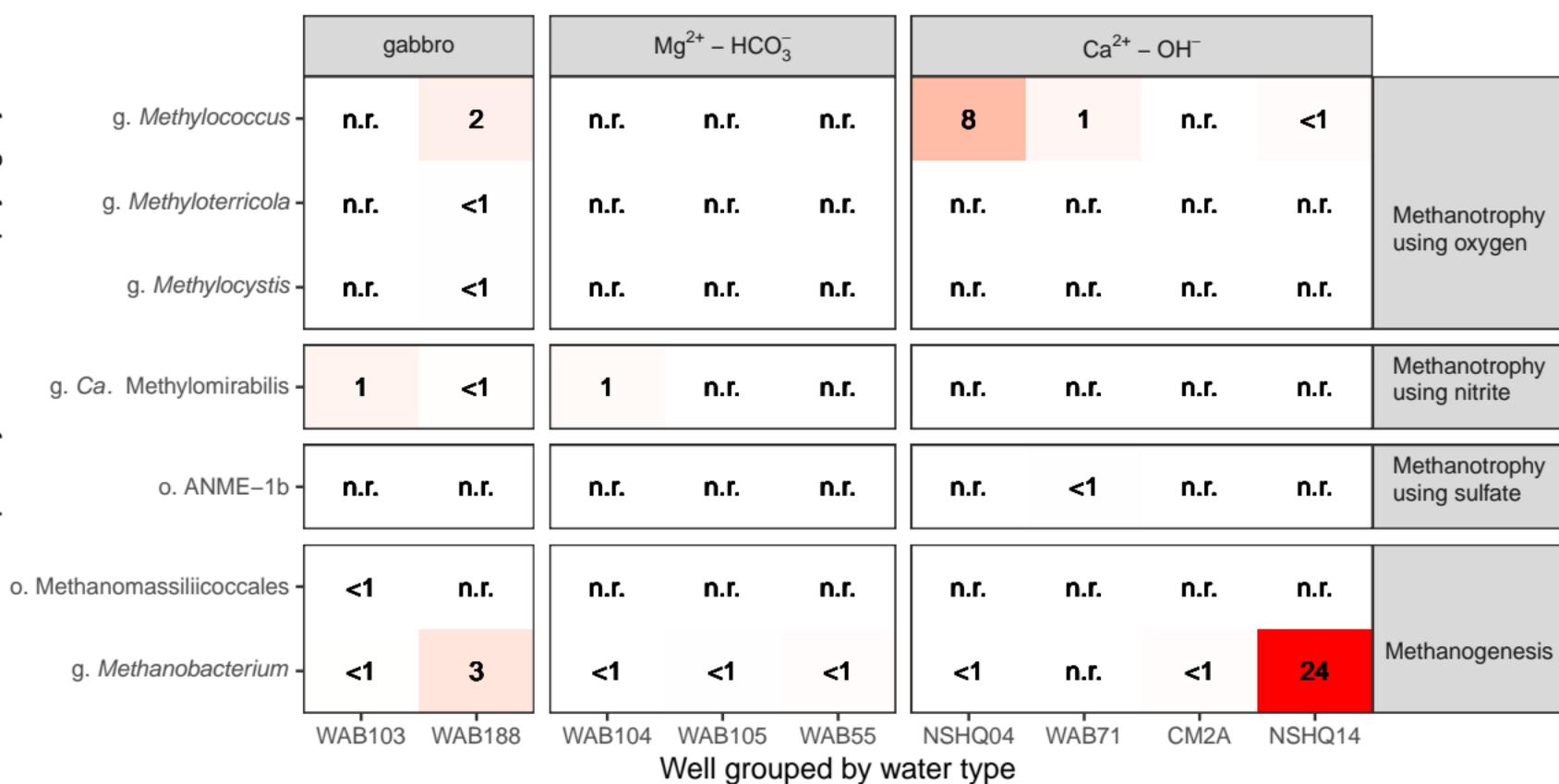


Figure 4.

Figure 5.

Deepest taxonomic assignment grouped by metabolic capability inferred from phylogeny



Well grouped by water type

Read relative abundance / [%]

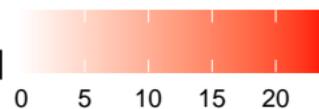
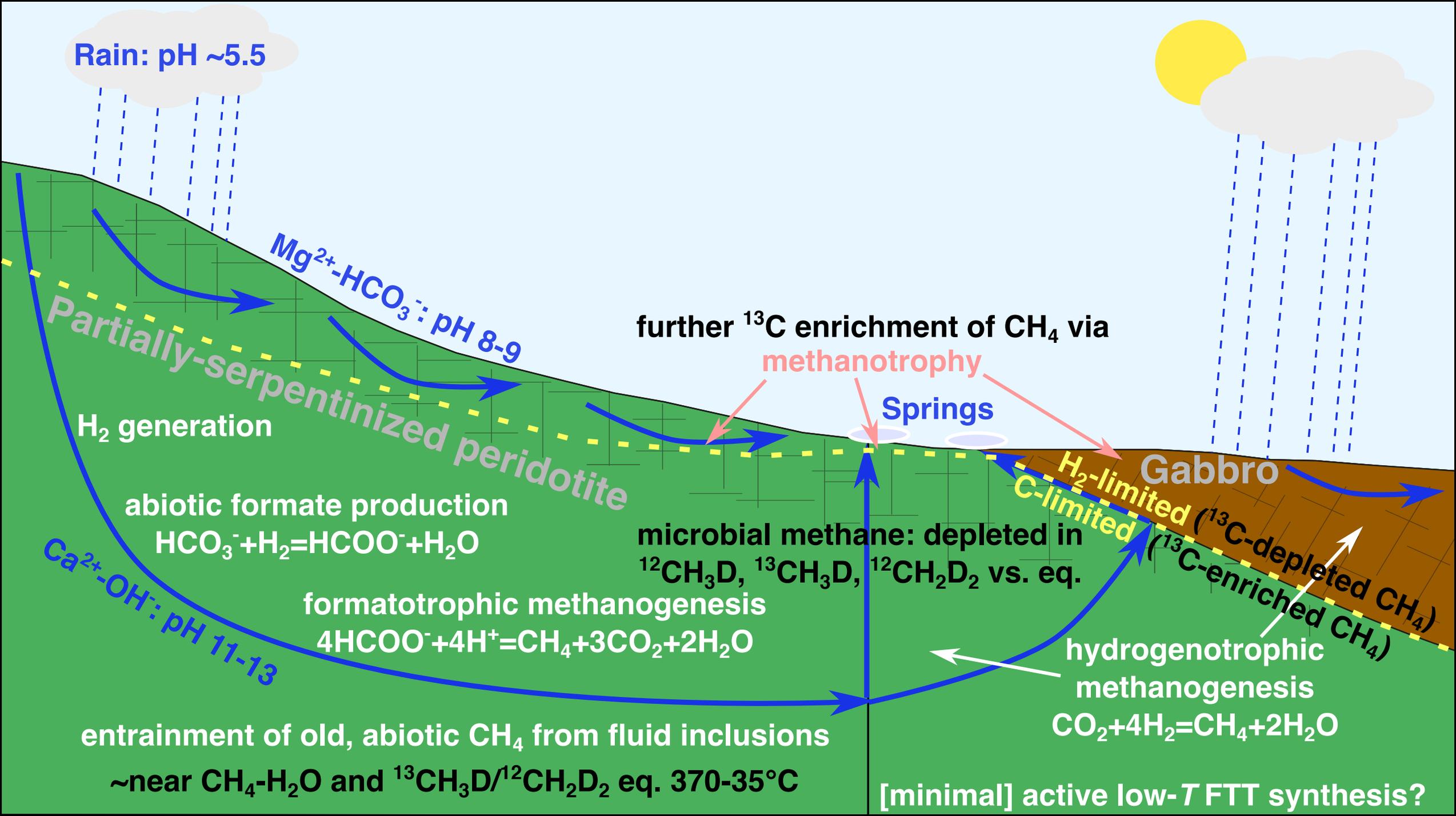


Figure 6.



Rain: pH ~5.5

$Mg^{2+}-HCO_3^-$: pH 8-9

Partially-serpentinized peridotite

H_2 generation

abiotic formate production
 $HCO_3^- + H_2 = HCOO^- + H_2O$

formatrophic methanogenesis
 $4HCOO^- + 4H^+ = CH_4 + 3CO_2 + 2H_2O$

$Ca^{2+}-OH^-$: pH 11-13

entrapment of old, abiotic CH_4 from fluid inclusions
 ~near CH_4-H_2O and $^{13}CH_3D/^{12}CH_2D_2$ eq. 370-35°C

further ^{13}C enrichment of CH_4 via
 methanotrophy

Springs

Gabbro

H_2 -limited (^{13}C -depleted CH_4)
 C-limited (^{13}C -enriched CH_4)

hydrogenotrophic methanogenesis
 $CO_2 + 4H_2 = CH_4 + 2H_2O$

[minimal] active low-T FTT synthesis?