JGR Biogeosciences

RESEARCH ARTICLE
10.1029/2020JG006025

Special Section:
Ophiolites and Oceanic Lithosphere, with a focus on the Samail ophiolite in Oman

Geochemical, Biological, and Clumped Isotopologue Evidence for Substantial Microbial Methane Production Under Carbon Limitation in Serpentinites of the Samail Ophiolite, Oman

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Abstract In hyperalkaline (pH >10) fluids that have participated in low-temperature (<150°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 ($^{13}CH_3D$ and $^{12}CH_2D_2$) relative abundances less than equilibrium, consistent with substantial microbial CH_4 production. Furthermore, we observed an inverse relationship between dissolved inorganic C concentrations and $\delta^{13}C_{CH_4}$ 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 $\delta^{13}C$ 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.

Key Points:

- 16S rRNA gene sequences affiliated with methanogens and CH_4 clumped isotopologue compositions suggest substantial microbial CH_4 production
- A second CH_4 source, release of CH_4 from fluid inclusions, is indicated by ^{13}C-enriched ethane and propane
- C availability may influence the apparent C isotope effect of microbial methanogenesis

Supporting Information:

Supporting Information may be found in the online version of this article.

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Citation:

Nothaft, D. B., Templeton, A. S., Rhim, J. H., Wang, D. T., Labidi, J., Miller, H. M., et al. (2021). Geochemical, biological, and clumped isotopologue evidence for substantial microbial methane production under carbon limitation in serpentinites of the Samail Ophiolite, Oman. *Journal of Geophysical Research: Biogeosciences*, *126*, e2020JG006025. https://doi.org/10.1029/2020JG006025

Received 22 AUG 2020
Accepted 6 AUG 2021

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1. Introduction

At temperatures and pressures near the Earth's surface (<400°C, <100MPa), 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; Klein & Bach, 2009; Klein et al., 2009, 2019; McCollom & Bach, 2009). 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°C), there is disagreement regarding the origin of CH_4. Three key potential CH_4 sources have been identified in these environments. One potential source is the abiotic reduction of CO_2 to CH_4 at warmer-than-present temperatures in fluid inclusions within crystals that can store CH_4 and subsequently release it. Another potential source is the abiotic, mineral-catalyzed reduction of CO_2 to CH_4 at the low temperatures that prevail in the present-day weathering environment. A third potential source is microbial methanogenesis.

Storage of CH_4 produced at temperatures of 270°C to 800°C in fluid inclusions in minerals such as olivine and the release of this CH_4 through subsequent chemical/physical alteration are the dominant processes contributing to CH_4 fluxes from sediment-poor seafloor hydrothermal vents (Kelley, 1996; Kelley & Früh-Green, 1999; Labidi et al., 2020; McDermott et al., 2015; Wang et al., 2018). In continental, low-temperature serpentinizing settings, however, debate continues as to whether fluid inclusions can sustain observed CH_4 fluxes (Etiope & Whiticar, 2019; Grozeva et al., 2020).

Abiotic reduction of CO_2 to CH_4 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 CO_2 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 CH_4 production can also be mediated by microbes called "methanogens." Microbial CH_4 has traditionally been viewed as a minor/negligible source of CH_4 in serpentinizing settings. This is due in large part to the relatively ^{13}C-enriched composition of CH_4 in serpentinizing settings ($\delta^{13}C$ commonly – 20‰VPDB to 5‰VPDB), which contrasts with the more ^{13}C-depleted composition of CH_4 in sedimentary settings dominated by microbial methanogenesis ($\delta^{13}C$ commonly – 90‰VPDB to – 50‰VPDB; Etiope, 2017; Etiope & Whiticar, 2019; Milkov & Etiope, 2018). However, cultures of methanogens can produce CH_4 with minimal C isotope fractionation in H_2-rich, CO_2-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 CO_2 to CH_4 when CO_2 was the limiting substrate. Such results illustrate that ^{13}C-enriched CH_4 in natural serpentinizing settings does not necessarily derive from non-microbial sources. Still, the quantity and isotopic composition of microbial CH_4 in serpentinizing settings remains uncertain.

In this study, we assessed sources and sinks of CH_4 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 CH_4 sampled in 2014 have been previously reported (Fones et al., 2019, 2020; Kraus et al., 2021; Miller et al., 2016; Rempfert et al., 2017) 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 CH_4, ethane (C_2H_6), and propane (C_3H_8) from samples obtained from 2015 through 2018. Further, we report analyses of multiply-substituted "clumped" isotopologues of CH_4, $^{13}CH_3D$ and $^{12}CH_2D_2$, for the first time on samples from this ophiolite. Leveraging one of the largest longitudinal data sets on CH_4 biogeochemistry in an ophiolite, we have identified robust trends across years

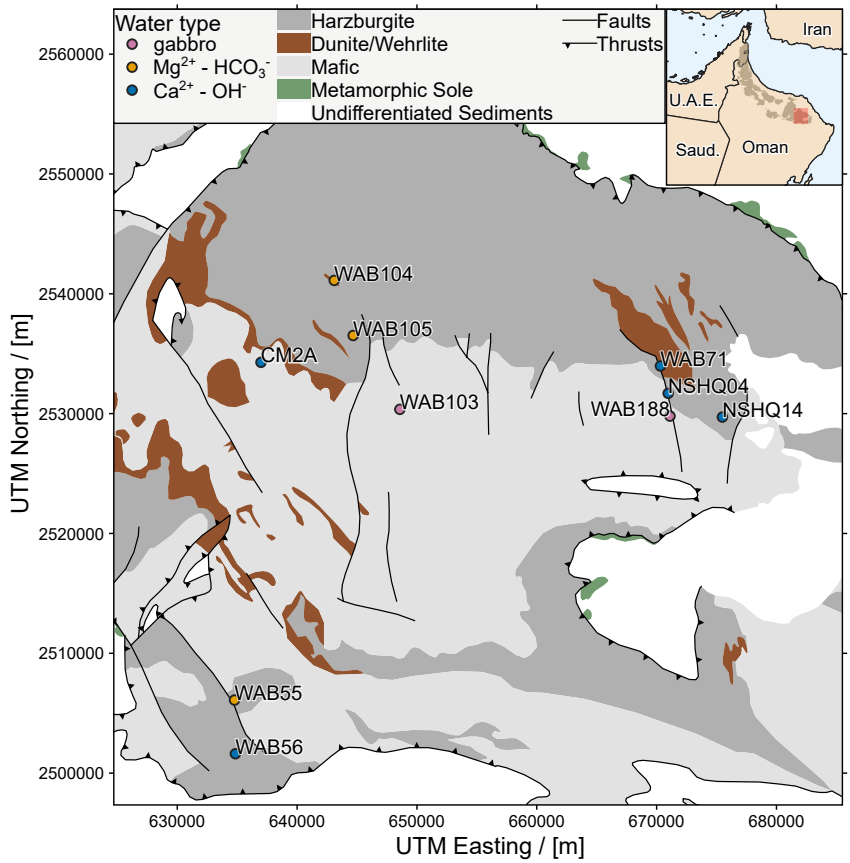

Figure 1. Study area in Samail Ophiolite, Sultanate of Oman. Geologic map data from Nicolas et al. (2000) were georeferenced and provided in GIS-compatible format in Nothaft (2021). Inset: overview of Samail Ophiolite (shaded in brown) with study area (larger map) indicated by the red shaded box. A topographic map of the study area is provided in Figure S1.

and hydrogeologic settings. We observed a wide range of C isotopic compositions of CH_4 and short-chain alkanes, intramolecular isotopologue disequilibrium in CH_4, and widespread occurrence of gene sequences affiliated with methanogens, which collectively indicate that substantial quantities of microbial CH_4 are produced and mix with abiotic CH_4 released from fluid inclusions in the Samail Ophiolite. Our finding that microbial methanogenesis proceeds even in hyperalkaline fluids lends insight into the functioning of microbial ecosystems that leverage reactions between water and ultramafic rocks to power metabolic processes on Earth and perhaps on other rocky bodies of the Solar System (Glein & Zolotov, 2020; Ménez, 2020).

2. Geologic Setting

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

The mantle section of the ophiolite is mainly composed of highly depleted, residual mantle harzburgites, together with 5% to 15% dunite, which both contain a few percent chromian spinel (Boudier & Coleman, 1981;

Table 1
Well Data and Field Measurements

Well	UTM coordinates (WGS-84) Easting	Northing	Geologic description	Well depth/ [mbgl]	Screen interval/ [mbct]	Water level/ [mbct]	Pump depth/ [mbct]	Conductivity/ [$\mu S \cdot cm^{-1}$]	Temperature/ [°C]	pH	*Eh*/ [mV]	f_{O_2}/[bar][a]
WAB103	648577	2530362	Gabbro	101	90 – 98	15	70.	1410	34.9	8.51	167[b]	$2.99 \cdot 10^{-36}$
WAB188	671123	2529798	Gabbro, near contact with harzburgite	78	34.5 – 51	9.5	50.	1120	35.6	8.16	214[b]	$2.01 \cdot 10^{-34}$
WAB104	643099	2541124	Harzburgite	120.4	100.8 – 104	40.	85	548	33.7	8.79	133	$1.23 \cdot 10^{-37}$
WAB105	644678	2536524	Harzburgite	120.5	110 – 117	16.5	60.	498	33.7	8.66	162	$2.99 \cdot 10^{-36}$
WAB55	634777	2506101	Harzburgite with abundant carbonate veins, near contact with gabbro	102	8 – 97	7.5[b]	50.[b]	1183[b]	36.2[b]	9.62[b]	269[b]	$7.17 \cdot 10^{-25}$
WAB56	634851	2501617	Harzburgite	106	7 – 27	7.62[b]	30.[b]	930.[b]	35.6[b]	10.61[b]	20.2[b]	$2.81 \cdot 10^{-37}$
NSHQ04	670971	2531699	Harzburgite, near fault with gabbro	304	open > 5.8	4.7	8	3350	33.4	10.51[b]	−174	$5.14 \cdot 10^{-51}$
WAB71	670322	2533981	Dunite, near fault with harzburgite	136.5	128 – 131	8.3	70.	1970	34.9	11.22	−229	$2.52 \cdot 10^{-51}$
CM2A	636988	2534284	Mostly dunite with occasional gabbro and harzburgite	400.	open > 23.7	13.4	75	2860	33.6	11.32	n.d.	n.d.
NSHQ14	675495	2529716	Harzburgite	304	open > 5.8	9.2	85	2670	36.7	11.39	−253[b]	$1.19 \cdot 10^{-51}$

Note. Measurements refer to sampling February–March, 2018, unless noted. Well elevations are given in Figure S1. Casings extend ~1m above ground level.
[a]Calculated from temperature, pH, and *Eh*. Where one or more of these parameters were obtained during different sampling years, f_{O_2} should be considered a representative estimate. [b]Not determined during 2018 sampling, so most recent prior data is reported (2015–2017; Fones et al., 2019; Rempfert et al., 2017).

Collier, 2012; Godard et al., 2000; Hanghøj et al., 2010). The extent of serpentinization is typically 30% to 60%, reaching 100% in some cases (Boudier et al., 2009; Dewandel et al., 2003; Kelemen et al., 2020; Miller et al., 2016). Chromitites are most often found in association with dunites near the crust-mantle transition, possibly representing bases of cumulate piles, but are also found dispersed throughout the mantle section (Rollinson, 2005).

Geologic reservoirs of C underlying the ophiolite include Mid Permian to Late Cretaceous shallow marine carbonates, which host oil and gas fields in parts of northern Oman and the United Arab Emirates (Alsharhan, 1989; Etiope et al., 2015; Terken, 1999). Maastrictian to Eocene limestones that partially overly the ophiolite have been shown to transfer inorganic C to peridotites where they are in contact (de Obeso & Kelemen, 2018). C is also stored within the ophiolite, primarily in the form of carbonate minerals (Kelemen et al., 2011; Kelemen & Matter, 2008; Neal & Stanger, 1985; Noël et al., 2018). Hydration and carbonation of >20 000km^3 of peridotite continue today in the Samail Ophiolite, largely at <60°C (Chavagnac, Ceuleneer, et al., 2013; Chavagnac, Monnin, et al., 2013; Falk et al., 2016; Kelemen et al., 2011; Kelemen & Matter, 2008; Mervine et al., 2014; Miller et al., 2016; Neal & Stanger, 1983, 1985; Streit et al., 2012; Vankeuren et al., 2012, 2019).

3. Methods

3.1. Fluid Sampling and Field Measurements

Wells were drilled into the Samail Ophiolite by the Ministry of Regional Municipalities and Water Resources of the Sultanate of Oman prior to 2006 ("WAB" and "NSHQ" wells in this study) and by the Oman Drilling Project in 2016 through 2018 ("CM"; Kelemen et al., 2013; Parsons International & Co LLC, 2005). Infor-

Table 2
Isotopic Compositions of CH_4, C_2H_6, and C_3H_8

Well	Sample year	Pump depth/ [mbct]	Laboratory	$\delta^{13}C_{CH_4}$	δD_{CH_4}	$\Delta^{13}CH_3D$	$\Delta^{12}CH_2D_2$	$\delta^{13}C_{C_2H_6}$	$\delta^{13}C_{C_3H_8}$
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.

Note. All isotopic values reported in ‰ units. $\delta^{13}C$ and δD reported in the VPDB and VSMOW reference frames, respectively. Data from 2014 previously reported by Miller et al. (2016).

mation on well location, construction, and water level are given in Table 1. In sampling campaigns in 2014 and 2015, a 12V submersible Typhoon® pump (Proactive Environmental Products, Bradenton, FL, USA) with typical flow rates of $5L \cdot min^{-1}$ was used. This pump was used in all years of sampling at well NSHQ04 due to partial obstruction of this well. In all other sampling from 2016 onwards, a larger submersible pump (Grundfos SQ 2–85) with typical flow rates of $20L \cdot min^{-1}$ was used. The pumping depths are reported in Tables 1 and 2. For fluids sampled in 2018, temperature, conductivity, and pH were measured using a ColeParmer PC100 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, $0.01\mu S \cdot cm^{-1}$, and 1mV, respectively. Each well was pumped for ≥20min prior to sampling. Sampling commenced once fluid pH and conductivity measurements stabilized.

3.2. Chemical and Isotopic Analyses of Fluids

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 $\sum$Na, $\sum$Ca, $\sum$Mg, $\sum$Al, $\sum$Fe, and $\sum$Si were measured by inductively coupled plasma 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 ion chromatograph with an AS9-HC IonPac column, with the exception of NO_3^-, which was measured on a Dionex 4500I ion chromatograph with an IonPac AS14 column using EPA method 300.0 (analytical uncertainty of 2%).

The concentration and $\delta^{13}C$ of dissolved inorganic C ($\sum CO_2$) were measured by acidification of water samples and transfer of resultant $CO_2(g)$ via a Thermo Fisher GasBench II to a Thermo Delta V Plus isotope ratio mass spectrometer. We optimized the methods of Assayag et al. (2006) for the wide range of $c_{\sum CO_2}$ observed in ophiolite groundwaters. Complete methodological details are available in Nothaft (2019b). Sample $\delta^{13}C$ values were converted to the VPDB reference frame using measured $\delta^{13}C$ values of international reference materials (Harding Iceland Spar and LSVEC). Isotopic reference frame calculations were performed using the Isoverse suite of packages (Kopf et al., 2021) for the statistical programing language, R (R Core Team, 2019).

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

Gases dissolved in pumped groundwaters were sampled by injecting water into N_2 purged vials for headspace gas analysis using methods described by Miller et al. (2016) in field campaigns occurring from 2014 to 2017. In addition, the bubble strip method (modified from (Kampbell et al., 1998)) was used from 2016 to 2018. Details on bubble strip gas sampling are available in Nothaft (2019a). The gas concentrations reported in this study were determined from bubble strip samples. These concentrations were measured on an SRI 8610C gas chromatograph (GC) with N_2 as the carrier gas. H_2, CO, CH_4, and CO_2 were separated with a 2mm by 1mm ID micropacked ShinCarbon ST column, whereas alkanes of 2 to 6 C atoms ("$C_2 - C_6$ short-chain alkanes") were separated with a PORAPAK Q 6ft by 0.085in ID column. Peak intensities were measured concurrently using a thermal conductivity detector (TCD) and a flame ionization detector (FID) and calibrated with standard gas mixes (Supelco Analytical, Bellefonte, PA, USA; accuracy of ± 2% of reported concentration). Measurement repeatability expressed as relative standard deviation was 5% over most of the calibrated range. The limit of quantitation was defined as the signal at which the relative standard deviation increased to 20%. In 2018, H_2 and CO were analyzed on a Peak Performer 1 gas chromatograph equipped with a reducing compound photometer (RCP). Due to the high sensitivity of the RCP, the signal at limit of quantitation (S_{LQ}) for these analyses was defined as $S_{LQ} = S_b + 10 \cdot \sigma_b$, where S_b is the mean signal of blanks prepared in field and σ_b is the population standard deviation of these blanks, in accordance with American Chemical Society guidelines (MacDougall & Crummett., 1980). Gaseous concentrations were converted to aqueous concentrations using gas solubilities (Sander, 2015) and corrected for temperature and volume changes between sampling and analysis.

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

From 2017 onwards, bulk stable isotope analyses of CH_4 and co-occurring alkane gases were conducted at the University of Colorado - Boulder (CUB) by GC/C/Pyr/IRMS using a Trace 1310 GC equipped with an Agilent J & W GS-CarbonPLOT column (30m length, 0.32mm ID, 3.0 μm film) coupled to a Thermo Scientific MAT253 IRMS. CH_4 isotope standards purchased from Airgas (uncertainties of ± 0.3‰ for $\delta^{13}C$ and ± 5‰ for δD) were used for calibration. Over the range of peak amplitudes of analyses reported here, the repeatability expressed as 1s on analyses of standards is ± 0.6‰ for $\delta^{13}C$ and ± 7‰ for δD. The analytical

uncertainty (accuracy) expressed as 1 standard error on a 3-point calibration was <0.3 ‰ for $\delta^{13}C$ and <9 ‰ for δD (Supporting Information Section S1).

The relative abundances of CH_4 isotopologues, including the doubly substituted isotopologue, $^{13}CH_3D$, were measured at the Massachusetts Institute of Technology (MIT) by tunable infrared laser direct absorption spectroscopy following the methods described by Ono et al. (2014). Abundances of CH_4 isotopologues, including both $^{13}CH_3D$ and $^{12}CH_2D_2$, were measured at the University of California, Los Angeles (UCLA) by high-mass-resolution gas-source isotope ratio mass spectrometry following the procedure of Young et al. (2016). The abundance of $^{13}CH_3D$ relative to a random (stochastic) distribution of isotopes among the isotopologues in a CH_4 sample is described by its $\Delta^{13}CH_3D$ value, which is defined as: $\Delta^{13}CH_3D = \ln Q$, where Q is the reaction quotient of the isotope exchange reaction:

$$^{13}CH_4 + {}^{12}CH_3D \rightleftharpoons {}^{12}CH_4 + {}^{13}CH_3D. \quad (1)$$

Analogous expressions can be written for doubly deuterated CH_4, $^{12}CH_2D_2$.

3.3. 16S rRNA Gene Sequencing and Analysis

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

Sequences were demultiplexed with idemp (Wu, 2017). The resultant fastq files were quality filtered using Figaro v1.1.1 (Weinstein, 2019) and the DADA2 v1.16 R package (Callahan et al., 2016). Amplicon sequence variants were assigned taxonomy to the genus level using the RDP classifier (Wang et al., 2007) trained on the Silva SSU 138 reference database (Quast et al., 2012) using the DADA2 assignTaxonomy function. Species level assignments were based on exact matching between amplicon sequence variants and sequenced reference strains using the DADA2 addSpecies function. Sequences assigned to mitochondria, chloroplast, and Eukaryota, or not assigned at the domain level (collectively <1% of sequences), were removed. After all of the above filtering, 24000 to 40000 reads per sample remained for the samples presented here obtained in 2018. In addition, 16S rRNA gene sequencing data from previous Oman sampling campaigns (2014–2017; Kraus et al., 2021; Miller et al., 2016; Rempfert et al., 2017) were reprocessed in accordance with the methods outlined here to facilitate comparisons across the data sets. The complete data processing pipeline for samples across all years, from raw data provided by the sequencing facility through to taxonomic assignment, are available in Nothaft, Rempfert, and Kraus (2021). Additional analyses and plotting can be found in Nothaft, Templeton, Rhim, et al. (2021). For samples presented in this study, demultiplexed fastq files (without additional processing) are also accessible on the NCBI Short Read Archive under accession PRJNA655565.

3.4. Thermodynamic Calculations

Oxidation-reduction potential, pH, and concentrations of major ions and $\sum CO_2$ were used as inputs for the modeling software PHREEQC (Charlton & Parkhurst, 2011; Parkhurst & Appelo, 2013), with which fluids were speciated using the LLNL database. Activities of formate and acetate were separately calculated

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^-
Gabbro-hosted groundwaters													
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	$4.06 \cdot 10^3$	$1.41 \cdot 10^3$	$1.82 \cdot 10^3$	$2.90 \cdot 10^1$	$4.77 \cdot 10^2$	$3.21 \cdot 10^2$	$1.41 \cdot 10^3$	$4.22 \cdot 10^3$	$6.78 \cdot 10^1$
$Mg^{2+} - HCO_3^-$ *groundwaters*													
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	$3.58 \cdot 10^1$	$3.02 \cdot 10^2$	$8.03 \cdot 10^2$	$6.54 \cdot 10^3$	$1.12 \cdot 10^2$
$Ca^{2+} - OH^-$ *groundwaters*													
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	$8.48 \cdot 10^1$	$1.03 \cdot 10^1$	$3.60 \cdot 10^2$	$1.57 \cdot 10^2$	$1.36 \cdot 10^4$	$1.67 \cdot 10^2$

Note. Concentrations reported in $\mu mol \cdot L^{-1}$. $\sum$ 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.

[a]Not determined during 2018 sampling, so most recent prior data is reported (2015–2017; Fones et al., 2019; Rempfert et al., 2017).

according to the Debye-Hückel equation. Activities of the aqueous gases were assumed equivalent to their concentrations, which is reasonable for neutral species in low ionic strength solutions. Standard Gibbs free energies (ΔG_r°) of the CH_4-forming reactions were calculated using the program SUPCRTBL (Johnson et al., 1992; Zimmer et al., 2016) using conditions of 1bar and 35°C to approximate *in situ* conditions. Gibbs free energies were then calculated as $\Delta G_r = \Delta G_r^\circ + RT \ln Q_r$, where R is the universal gas constant, T is temperature, and Q_r is the reaction quotient. All of the above calculations and software inputs and outputs can be found in Nothaft, Templeton, Rhim, et al. (2021).

4. Results and Discussion

4.1. Controls on Groundwater Chemistry

To assess the source and reaction histories of Samail Ophiolite groundwaters, we measured their stable isotopic compositions and solute concentrations. Groundwater δD and $\delta^{18}O$ plotted near local and global meteoric water lines (Terzer et al., 2013; Weyhenmeyer et al., 2002), indicating that the groundwaters derive from rain (Table 3; Figure S2; Matter et al., 2006; Miller et al., 2016; Vankeuren et al., 2019). The sampled groundwaters included oxidized and moderately alkaline $Mg^{2+} - HCO_3^-$ waters, typical of reaction with peridotite in communication with the atmosphere, and reduced and hyperalkaline $Ca^{2+} - OH^-$ waters, typical of extensive hydration and oxidation of peridotite in closed-system conditions with respect to the atmosphere (Table 3; Barnes et al., 1967; Barnes & O'Neil, 1969; Bruni et al., 2002; Cipolli et al., 2004; Kelemen et al., 2011; Neal & Stanger, 1985; Vankeuren et al., 2012). $Ca^{2+} - OH^-$ waters had higher conductivities ($930 \mu S \cdot cm^{-1}$ to $3{,}350 \mu S \cdot cm^{-1}$) than $Mg^{2+} - HCO_3^-$ waters ($498 \mu S \cdot cm^{-1}$ to $1{,}183 \mu S \cdot cm^{-1}$; Table 1). The increase in conductivity from $Mg^{2+} - HCO_3^-$ waters to $Ca^{2+} - OH^-$ waters is driven by enrichments in Ca^{2+} derived from dissolution of primary silicate minerals in addition to Na^+ and Cl^- derived from mineral dissolution, sea spray, and/or leaching of sea salts introduced during subseafloor alteration and/or ophiolite emplacement (Neal & Stanger, 1985; Rempfert et al., 2017; Stanger, 1986; Vankeuren et al., 2012). The increase in pH from $Mg^{2+} - HCO_3^-$ waters (pH 8.66 to 9.62) to $Ca^{2+} - OH^-$ waters (10.51–11.39) was accompanied by a shift to lower f_{O_2} and Eh ($\sim 10^{-51}$bar and −174mV to −253mV, respectively, in most $Ca^{2+} - OH^-$ waters; Table 1), indicating reduced conditions in $Ca^{2+} - OH^-$ waters.

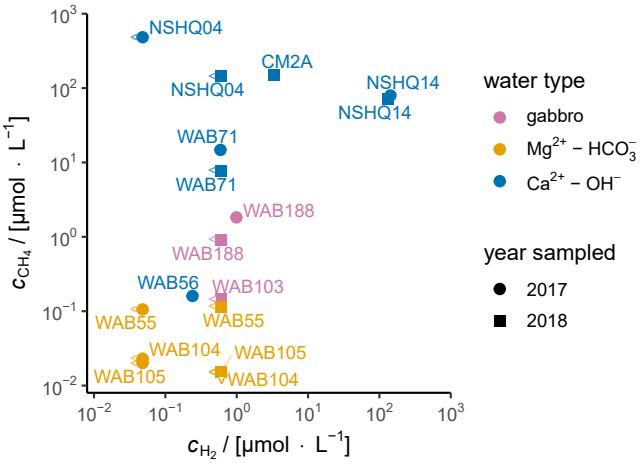

Figure 2. Aqueous concentrations of CH_4 and H_2 in Oman groundwater samples from 2017 and 2018. Left and down carrots denote "below limit of quantitation" for CH_4 and H_2, respectively, with the adjacent point plotted at the limit of quantitation for that gas and year of analysis.

Concentrations of $\sum CO_2$ were relatively high in $Mg^{2+} - HCO_3^-$ waters and gabbro waters (up to 3490 μmol · L^{-1}), but below the limit of quantitation (<12 μmol · L^{-1}) in most $Ca^{2+} - OH^-$ waters (Table 3). This is consistent with water-harzburgite reaction path modeling that terminates at chrysotile-brucite-diopside-calcite equilibrium, corresponding to a $c_{\sum CO_2}$ of 8 μmol · L^{-1} at 25°C and 1bar (Leong & Shock, 2020). Literature values for $c_{\sum CO_2}$ in ophiolitic $Ca^{2+} - OH^-$ waters are often higher than those predicted by reaction path modeling, but the lower range of reported values approaches 1 μmol · L^{-1} (Barnes et al., 1967, 1978; Barnes & O'Neil, 1969; Brazelton et al., 2017; Bruni et al., 2002; Canovas et al., 2017; Cipolli et al., 2004; Crespo-Medina et al., 2017; Falk et al., 2016; Fones et al., 2019; Neal & Stanger, 1985; Rempfert et al., 2017; Vankeuren et al., 2012, 2019). This spread in the data could reflect groundwater mixing, atmospheric contamination during sampling, differences in reaction temperature and progress, and/or kinetic inhibitions to carbonate mineral precipitation. In $Mg^{2+} - HCO_3^-$ waters and waters from gabbroic aquifers, $\delta^{13}C_{\sum CO_2}$ ranged from −13.54‰VPDB to −10.88‰VPDB (Table 3), which is comparable to $\delta^{13}C_{\sum CO_2}$ of $Mg^{2+} - HCO_3^-$ waters elsewhere in the ophiolite (−15.56‰VPDB to − 13.60‰VPDB (Matter et al., 2006; Nothaft, Templeton, Boyd, et al., 2021).

Variable concentrations of H_2 and CH_4 across wells suggest spatial heterogeneities in sources and sinks of these gases in the ophiolite. In some $Ca^{2+} - OH^-$ waters, c_{H_2} was high (up to 253 μmol · L^{-1}), but c_{H_2} was below limits of quantitation in other $Ca^{2+} - OH^-$ waters (Figure 2 and Table 4). In $Mg^{2+} - HCO_3^-$

Table 4
Aqueous Gas Concentrations, Reported in μmol · L^{-1}

Well	Sample year	H_2	CO	CH_4	C_2H_6	C_3H_8	i-C_4H_{10}	n-C_4H_{10}	i-C_5H_{12}	n-C_5H_{12}	C_6H_{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 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	$2.53 \cdot 10^{2}$	$<2.79 \cdot 10^{-1}$	$1.06 \cdot 10^{2}$	$7.98 \cdot 10^{-2}$	$9.00 \cdot 10^{-3}$	$1.53 \cdot 10^{-3}$	$4.77 \cdot 10^{-3}$	$<3.50 \cdot 10^{-4}$	$<3.91 \cdot 10^{-4}$	$9.70 \cdot 10^{-4}$

[a]Hexane isomers not chromatographically resolved. [b]High $C_1/(C_2 + C_3)$ at NSHQ04 resulted in CH_4 elution tailing into and preventing quantitation of the C_2H_6 peak in 2017. Chromatographic improvements were made between analyses of 2017 and 2018 samples.

waters and waters from gabbroic aquifers, c_{H_2} was generally below limits of quantitation. However, up to $0.992\,\mu mol \cdot L^{-1}$ H_2 was measured in well WAB188, which is in gabbro near a faulted contact with peridotites that contain $Ca^{2+} - OH^-$ waters (Figure 1 and Table 1). This suggests production of H_2 within the gabbro host rock or migration of H_2 from peridotites into gabbros surrounding WAB188. In most $Ca^{2+} - OH^-$ waters, c_{CH_4} was high (up to $483\,\mu mol \cdot L^{-1}$; Figure 2 and Table 4). However, wells with high c_{CH_4} did not always have high c_{H_2} (Figure 2 and Table 4). In $Mg^{2+} - HCO_3^-$ waters and gabbro waters, c_{CH_4} was typically lower ($\leq 0.1\,\mu mol \cdot L^{-1}$), although c_{CH_4} reached $1.83\,\mu mol \cdot L^{-1}$ in well WAB188, where c_{H_2} was also quantitatable.

4.2. Origin of CH_4 and Co-Occurring Short-Chain Alkanes in the Samail Ophiolite

We begin our examination of CH_4 origin in the Samail Ophiolite by calculating Gibbs free energies (ΔG_r) of potential CH_4-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 CH_4 cycle processes. Discussion of three additional wells (WAB71, WAB56, and CM2A) in 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 CH_4-Forming Reactions Might Occur Using Thermodynamic and Microbiological Data

To assess which CH_4-forming aqueous reactions might occur within the Samail Ophiolite, ΔG_r's were calculated for the following reactions:

$$CO_2(aq) + 4H_2(aq) = CH_4(aq) + 2H_2O(l) \left(\text{hydrogenotrophic methanogenesis}\right) \quad (2)$$

$$CH_3COO^-(aq) + H^+(aq) = CH_4(aq) + CO_2(aq) \left(\text{acetoclastic methanogenesis}\right) \quad (3)$$

$$4HCOO^-(aq) + 4H^+(aq) = CH_4(aq) + 3CO_2(aq) + 2H_2O(l) \left(\text{formatotrophic methanogenesis}\right). \quad (4)$$

Gas-phase, abiotic reactions are also possible (Etiope et al., 2018; Etiope & Ionescu, 2015), 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, $\sum CO_2$ was below the limit of quantitation for the majority of the $Ca^{2+} - OH^-$ groundwaters sampled in 2018 ($<12\,\mu mol \cdot L^{-1}$; Table 3). H_2 was also below the limit of quantitation for several $Ca^{2+} - OH^-$ and $Mg^{2+} - HCO_3^-$ groundwaters ($<0.048 nmol \cdot L^{-1}$ in 2017 and $<0.598 nmol \cdot L^{-1}$ in 2018; Table 4). Furthermore, 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 $Mg^{2+} - HCO_3^-$ groundwater and a representative $Ca^{2+} - OH^-$ groundwater, made informed assumptions when direct concentration measurements were lacking, and evaluated ΔG_r's for a range of H_2 concentrations. Measurements of major inorganic dissolved constituents, pH, and *Eh* from wells WAB105 and NSHQ14 were used for the model $Mg^{2+} - HCO_3^-$ and $Ca^{2+} - OH^-$ fluids, respectively (Tables 1 and 3). Since measured $c_{\sum CO_2}$ was below the limit of quantitation in the water sample from NSHQ14, $8\,\mu mol \cdot kg^{-1}$ was taken as the $c_{\sum CO_2}$ of the representative $Ca^{2+} - OH^-$ water, corresponding to the value at chrysotile-brucite-diopside-calcite equilibrium at 25°C and 1bar obtained from water-harzburgite reaction path modeling (Leong & Shock, 2020). Concentrations of formate and acetate were both assumed to be $1\,\mu mol \cdot kg^{-1}$, which is consistent with their concentrations in earlier samples from wells in Samail Ophiolite (Rempfert et al., 2017). Concentrations of CH_4 were assumed to be $100\,\mu mol \cdot kg^{-1}$

Table 5
Gibbs Free Energies of Potential CH_4-Forming Reactions and Log Activities of Relevant Species

	log(activity)						ΔG_r / [kJ · mol^{-1}]		
water type	H^+	CO_2(aq)	$HCOO^-$	CH_3COO^-	CH_4(aq)	H_2(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

and 0.1μmol · kg^{-1} for the representative Ca^{2+} – OH^- and Mg^{2+} – HCO_3^- waters, respectively, reflecting typical concentrations for these fluids (Table 4, Figure 2). H_2 concentrations vary widely between and within fluid types (Table 4, Figure 2), so calculations were performed for multiple H_2 concentrations (1mmol · kg^{-1}, 1μmol · kg^{-1}, and 1nmol · kg^{-1}) encompassing the range of concentrations observed in Ca^{2+} – OH^- fluids. The 1mmol · kg^{-1} H_2 case was omitted for the Mg^{2+} – HCO_3^- fluid, where such high H_2 concentrations are not observed. The log activities (a) of all relevant species are tabulated in Table 5.

The calculated ΔG_r's (Table 5) indicate that all of the CH_4-forming reactions considered here can have sufficient chemical potential to sustain microbial life in certain states of the system. That is, $\Delta G_r > \Delta G_{min}$, where ΔG_{min} (also known as the Biological Energy Quantum) is the minimum free energy that must be available to sustain life in a given environment (thought to be around 9 kJ · mol^{-1} to 20 kJ · mol^{-1} (Hoehler, 2004; Schink, 1997; Schink & Stams, 2006)). Acetoclastic methanogenesis had the most negative ΔG_r in all conditions tested. Formatotrophic methanogenesis had more negative ΔG_r than hydrogenotrophic methanogenesis in all Ca^{2+} – OH^- conditions tested, but formatotrophic methanogenesis had less negative ΔG_r than hydrogenotrophic methanogenesis in the Mg^{2+} – HCO_3^- case at 1μmol · kg^{-1} H_2. Hydrogenotrophic methanogenesis had sufficient chemical potential to sustain microbial life only when a_{H_2} was high enough, with the threshold a_{H_2} being higher in Ca^{2+} – OH^- waters, where $a_{CO_{2(aq)}}$ is lower. These calculations are generally consistent with those of Canovas et al. (2017), who found that hydrogenotrophic methanogenesis had modest potential energy yields in waters from surface seeps in the Samail Ophiolite at pH ranging from 8 to 12.

Several additional factors should be considered when interpreting the ΔG_r results. First, reactions proceeding in environmental systems are often drawn toward equilibrium, and thus a large negative ΔG_r of a given reaction may indicate that that reaction is not actively occuring, but only has the potential to occur. Second, substrate transport into the cell is not addressed in our calculations. A more complete model would account for rates of CO_2 diffusion across the cell membrane and/or energy expended to transport charged species such as formate and acetate into the cell (Hoehler, 2004). Third, mixing is not explicitly accounted for in our calculations. Mixing has been suggested as a key factor controlling energetic favorability of various reactions in the Samail Ophiolite. This is especially pertinent to hydrogenotrophic methanogenesis because c_{CO_2} is so much lower in endmember hyperalkaline fluids than in near-surface, atmosphere-influenced fluids (Canovas et al., 2017; Leong & Shock, 2020). The c_{CO_2} used for the example Ca^{2+} – OH^- fluid in our calculations is representative of a minimum value for the system (Leong & Shock, 2020). Mixing would tend to inject CO_2 into the fluids and increase the energetic favorability of hydrogenotrophic methanogenesis.

In addition to energetic considerations, microbiological approaches can help elucidate which CH_4-forming reactions occur. Kraus et al. (2021) found higher transcript abundances of carbonic anhydrase and formate dehydrogenase relative to acetate kinase and phosphate acetyltransferase in hyperalkaline groundwaters from wells in the Samail Ophiolite, suggesting that CO_2/HCO_3^- and formate are more actively used substrates for methanogenesis than acetate in these conditions. Further, Fones et al. (2020) identified two lineages of *Methanobacterium* in Samail Ophiolite groundwaters that were shown by genomic and microcosm-based radiotracer approaches to use different methanogenic pathways. *Methanobacterium* Type I lineage predominated in circumneutral waters and is capable of using either CO_2 or formate for methano-

genesis. *Methanobacterium* Type II lineage, which was more abundant in hyperalkaline waters and which branched from the Type I lineage, was exclusively capable of formatotrophic methanogenesis. It was postulated that gene loss and acquisition in Type II lineage allowed it to be specially suited to the high-pH and low-$\sum CO_2$ conditions resulting from extensive serpentinization. Thus, microbiological data suggest that hydrogenotrophic or formatotrophic methanogenesis are the most likely pathways for methanogenesis in the Samail Ophiolite and that the relative contributions of each of these pathways to microbial CH_4 production at a given site may depend on local geochemical factors such as $a_{CO_2(aq)}$. This notion is generally supported by our calculations in that formatotrophic methanogenesis had more negative ΔG_r than hydrogenotrophic methanogenesis in all Ca^{2+} – OH^- conditions tested, whereas the reverse was true for the Mg^{2+} – HCO_3^- case at $1 \mu mol \cdot kg^{-1}$ H_2.

Remarkably, although acetoclastic methanogenesis had the most negative ΔG_r of the investigated CH_4-forming reactions (Table 5), it has the least microbiological evidence of being a major methanogenic pathway in the Samail Ophiolite. Conversion of isotopically labeled acetate ($^{13}CH_3OO^-$) to $^{13}CH_4$, has, however, been documented in cultures from serpentinite springs in the Voltri Massif, Italy (Brazelton et al., 2017), indicating that acetoclastic methanogenesis can operate in some serpentinizing settings. In the aquifers sampled via wells in the Samail Ophiolite, methanogens may be out-competed for acquisition of acetate by other groups of microbes, such as sulfate reducers. Indeed, geochemical evidence of microbial acetate oxidation coupled to sulfate reduction has been reported in alkaline, H_2-rich, crystalline rock aquifers inhabited by microbial communities dominated by sulfate reducing bacteria and methanogens (Moser et al., 2005).

4.2.2. Abiotic, ^{13}C-Enriched CH_4, C_2H_6, and C_3H_8 Mixed With Microbial CH_4 Produced Under C-Limited Conditions in the Ca^{2+} — OH^- Waters of Well NSHQ14

Well NSHQ14 is situated in a catchment dominated by partially serpentinized harzburgite with meter-scale partially serpentinized dunite bands (Figure 1 and Table 1; Figure S1). The well is cased to 5.8 meters below ground level (mbgl) and drilled to 304mbgl (Table 1). Groundwaters accessed via NSHQ14 had the highest pH (11.39), and lowest *Eh* (– 253mV) and f_{O_2} ($1.19 \cdot 10^{-51}$bar) among the wells investigated (Table 1), indicating that fluids sampled from NSHQ14 have extensively participated in serpentinization. This is also reflected in the c_{H_2} of groundwaters sampled at NSHQ14, which was the highest among the studied wells ($253 \mu mol \cdot L^{-1}$ and $131 \mu mol \cdot L^{-1}$ in 2017 and 2018, respectively, Figure 2 and Table 4). NSHQ14 waters also had high c_{CH_4} ($106 \mu mol \cdot L^{-1}$ and $71.2 \mu mol \cdot L^{-1}$ in 2017 and 2018, respectively).

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

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

C_2H_6 and C_3H_8 at NSHQ14 are strongly ^{13}C-enriched ($\delta^{13}C$ of –6.0‰VPDB and + 3.3‰VPDB, respectively; Table 2 and Figure 4). The observed $\delta^{13}C$ values are ~15‰ higher than those in the most mature (and therefore most ^{13}C-enriched) thermogenic C_2H_6 and C_3H_8 samples from confined systems (Fiebig et al., 2019; Milkov & Etiope, 2018). Increases in $\delta^{13}C_{C_3}$ of ~15‰ have been attributed to microbial oxidation of short-

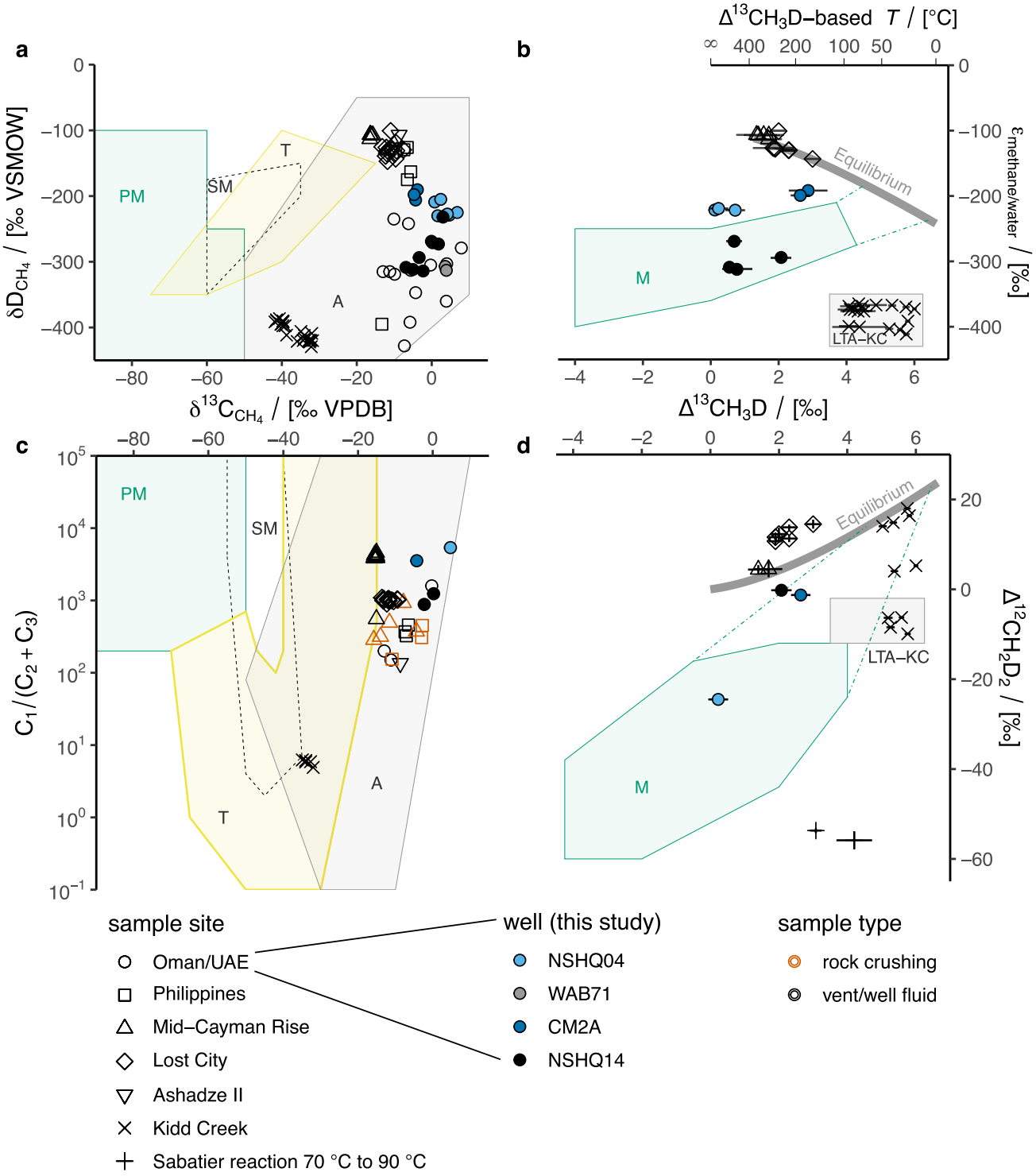

Figure 3.

chain alkanes, which enriches the residual in ^{13}C (Martini et al., 2003). However, short-chain alkane oxidizing microbial species (Laso-Pérez et al., 2019; Shennan, 2006; Singh et al., 2017) were not detected in 16S rRNA gene sequences of DNA obtained from NSHQ14. Thus, there is not strong evidence to suggest that $\delta^{13}C_{C_2}$ and $\delta^{13}C_{C_3}$ at NSHQ14 result from post-genetic microbial alteration. Rather, $\delta^{13}C_{C_2}$ and $\delta^{13}C_{C_3}$ should reflect formation conditions and C source(s).

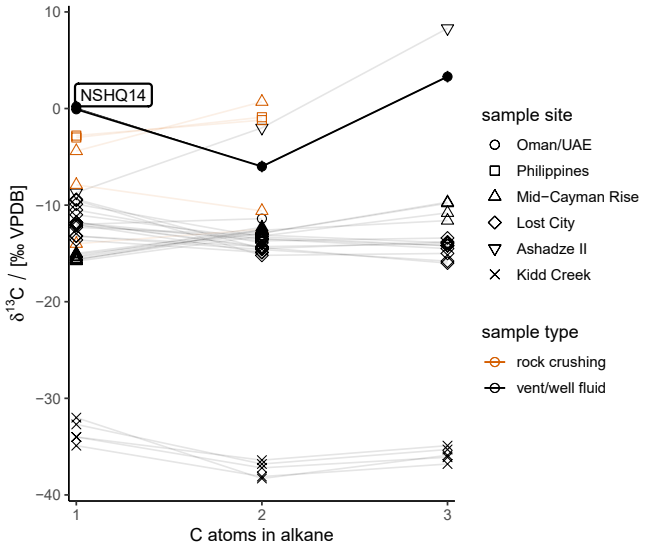

Figure 4. Plot of $\delta^{13}C$ of CH_4 and co-occurring *n*-alkanes versus the number of C atoms per molecule. Error bars represent uncertainties on $\delta^{13}C$ analyses performed at CUB. Only samples for which $\delta^{13}C_{C_2}$ was determined are plotted. Contextual data from ophiolites: Oman/UAE (Fritz et al., 1992), the Philippines (Grozeva et al., 2020); sediment-poor seafloor hydrothermal vents: Mid-Cayman Rise (Grozeva et al., 2020; McDermott et al., 2015), Lost City (Proskurowski et al., 2008), Ashadze II (Charlou et al., 2010), and Precambrian Shield: Kidd Creek, Canada (Sherwood Lollar et al., 2008).

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

Thermal cracking of organic matter and open-system degassing can enrich late-produced short-chain alkanes in ^{13}C due to kinetic isotope effects associated with the cleavage of precursor sites in the parent organic matter and the resultant Rayleigh distillation of these sites (Fiebig et al., 2019; Rooney et al., 1995). Thermogenic gas production can proceed slowly at temperatures as low as 60°C, but substantial thermogenic gas production typically occurs at reservoir temperatures above 120°C (Burnham, 1989; Cumming et al., 2019; Fiebig et al., 2019; Hunt, 1996; Stolper et al., 2018). These temperatures are higher than temperatures along groundwater flow paths intersecting the wells in this study. Measured groundwater temperatures in the study area are ~35°C (Table 1), and $H_2 - H_2O$ isotope thermometry and $C - O$ clumped isotope thermometry on carbonate veins with significant ^{14}C contents in Samail Ophiolite peridotites both indicate equilibrium ≤60°C (Kelemen et al., 2011; Kelemen & Matter, 2008; Mervine et al., 2014; Miller et al., 2016). Geothermal gradients derived from geophysical logs of NSHQ14 are $25°C \cdot km^{-1}$ (Matter et al., 2017; Paukert, 2014), which is typical of near-surface, continental settings (Lowell et al., 2014). At the low temperatures and ordinary geothermal gradients within the active alteration zone of the Samail Ophiolite, thermal cracking of organic matter is unlikely to proceed at sufficient rates to attain the high extents of reaction progress necessary to explain the observed ^{13}C enrichments in short-chain alkanes at NSHQ14 over relevant timescales.

Alternatively, short-chain alkanes in NSHQ14 fluids may have an abiotic source. Several studies have demonstrated storage of large quantities of CH_4 and associated short-chain alkanes in fluid inclusions in ophiolites (Grozeva et al., 2020; Klein et al., 2019; Sachan et al., 2007). However, the findings of these studies disagree with those of Etiope et al. (2018), who measured relatively low concentrations of CH_4 stored in serpentinized peridotites from Greek ophiolites. Since the rocks analyzed by Etiope et al. (2018) were sampled

Figure 3. Molecular and isotopic compositions of natural gases. (a) Plot of δD_{CH_4} versus $\delta^{13}C_{CH_4}$. 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) versus $\delta^{13}C_{CH_4}$. 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 $\varepsilon_{methane/water}$ versus $\Delta^{13}CH_3D$. *X* and *Y* axes are swapped with respect to original publication of this type of plot (Wang et al., 2015) so that (b) is comparable against (d). The data from (b) are plotted in the Wang et al. (2015) orientation in Figure S4. Equilibrium line from Horibe and Craig (1995) and 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 (Ash & Egger, 2019; Giunta et al., 2019; Stolper et al., 2015; Wang et al., 2015; Young et al., 2017). (d) Plot of $\Delta^{13}CH_3D$ versus $\Delta^{12}CH_2D_2$, after 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 (Boulart et al., 2013; Etiope et al., 2015; Fritz et al., 1992; 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 (Grozeva et al., 2020; McDermott et al., 2015; Wang et al., 2018), Lost City (Labidi et al., 2020; Proskurowski et al., 2008; Wang et al., 2018), Ashadze II (Charlou et al., 2010); Precambrian Shield: Kidd Creek, Canada (Sherwood Lollar et al., 2008; Young et al., 2017); and laboratory Sabatier reaction catalyzed by Ru (Young et al., 2017).

from outcrops, it is possible that chemical or physical processes associated with surface exposure resulted in loss of CH_4 once stored in peridotite-hosted fluid inclusions prior to analysis. Although further study of the quantity and spatial distribution of CH_4 storage in ophiolitic rocks is warranted, the presence of $CH_4 + H_2$ inclusions in olivine and $CH_4 \pm$ graphite inclusions in orthopyroxene in Samail Ophiolite harzburgites (Miura et al., 2011) requires that fluid inclusions be considered as a potential source for abiotic CH_4 and associated short-chain alkanes at NSHQ14 and elsewhere in the ophiolite.

A fluid inclusion source of CH_4 and short-chain alkanes is compatible with C stable isotopic compositions of these compounds in groundwaters pumped from NSHQ14. CH_4, C_2H_6, and C_3H_8 $\delta^{13}C$ values at NSHQ14 (−6.89‰VPDB to +3.7‰VPDB; Table 2) overlap with CH_4 and C_2H_6 $\delta^{13}C$ values measured by Grozeva et al. (2020) in rock crushing experiments on CH_4-rich fluid inclusion-bearing peridotites and dunites sampled from the Zambales ophiolite in the Philippines (−12.4‰VPDB to −0.9‰VPDB; Figure 4), which, in turn, overlap with $\delta^{13}C$ values of CH_4 from nearby gas seeps at Los Fuegos Eternos and Nagsasa in the Philippines (−7.4‰VPDB to −5.6‰VPDB; Figure 3a; Abrajano et al., 1990; Vacquand et al., 2018). Grozeva et al. (2020) also crushed CH_4-rich fluid inclusion-bearing rocks from the Mid-Cayman Rise. Of the Mid-Cayman Rise samples that yielded sufficient CH_4 and C_2H_6 for precise C isotopic analysis, which were all mafic intrusive rocks, $\delta^{13}C$ values ranged from −14.0‰VPDB to +0.7‰VPDB. The lower end of Mid-Cayman Rise rock crushing short-chain alkane $\delta^{13}C$ values are similar to those measured in Mid-Cayman Rise hydrothermal vent fluids (−15.8‰VPDB to −9.7‰VPDB; McDermott et al., 2015), whereas the higher end are similar to those of NSHQ14 (Figure 4). Furthermore, C_2H_6 and C_3H_8 $\delta^{13}C$ values of NSHQ14 fluids resemble those of fluids discharging from the sediment-poor hydrothermal vents at Ashadze II, Mid-Atlantic Ridge (Figure 4; Charlou et al., 2010). The similarities in short-chain alkane $\delta^{13}C$ values between circulating fluids and rock-hosted fluid inclusions in ophiolites and present-day oceanic lithospheric sites suggest that circulating fluids in both environments derive much of their CH_4 and short-chain alkanes from fluid inclusions.

Sources of CH_4 can also be assessed by measuring H isotopic compositions and clumped isotopologue relative abundances of CH_4 and comparing these isotopic compositions to temperature-dependent equilibria. These isotopic equilibria are represented by thick gray lines in Figures 3b and 3d. Intra-CH_4 equilibrium is governed by the increasing relative stability of bonds between two heavy isotopes (more "clumping") at lower temperatures, which is reflected in higher $\Delta^{13}CH_3D$ and $\Delta^{12}CH_2D_2$ values. However, isotopic equilibrium will only be expressed if kinetics allow it. In the first study to publish clumped isotopologue ($\Delta^{13}CH_3D$) data on CH_4- and H_2- rich gases from sediment-poor seafloor hydrothermal vents, Wang et al. (2018) found that these gases yielded apparent $CH_4 - H_2O$ H isotopic and $\Delta^{13}CH_3D$ equilibrium temperatures of 270°C to 360°C, despite having a range of effluent fluid temperatures from 96°C to 370°C. This was interpreted as evidence for a closure temperature of 270°C for H isotope exchange in the $CH_4 - H_2O$ and $CH_4 - H_2$ systems in seafloor hydrothermal settings (e.g., Mid-Cayman Rise in Figures 3b and 3d). However, in a subsequent study that re-analyzed some of the same samples, plus a greater number of samples from low-temperature vents at Lost City (96°C to 64°C), and contributed the first $\Delta^{12}CH_2D_2$ values from these settings, Labidi et al. (2020) found evidence for re-equilibration of clumped isotopologue and $CH_4 - H_2O$ H isotopic systems at lower temperatures. Of these isotopic systems, that of $^{12}CH_2D_2$ had the fastest apparent re-equilibration kinetics (approximately twice as fast as $^{13}CH_3D$), which was explained by differences in symmetry numbers among the isotopologues. The $^{12}CH_2D_2$-based temperatures of the Lost City samples, which were as low as 69^{+4}_{4}°C, closely matched their end member vent fluid temperatures. As a result of the apparent faster re-equilibration of $^{12}CH_2D_2$, the Lost City data plot above the $^{13}CH_3D - {}^{12}CH_2D_2$ equilibrium line (toward higher $\Delta^{12}CH_2D_2$) in Figure 3d. Therefore, isotopic compositions of CH_4 formed in fluid inclusions in the oceanic lithosphere and stored for millions of years at low temperatures may be expected to fall somewhere along a continuum from $\Delta^{13}CH_3D$, $\Delta^{12}CH_2D_2$, and $CH_4 - H_2O$ isotopic equilibrium at ~330°C to compositions approaching lower temperature (~70°C or perhaps even lower) equilibrium, with $^{12}CH_3D$, $^{13}CH_3D$, $CH_4 - H_2O$ isotopic re-equilibration proceeding at varying rates. This is not the case for Samail Ophiolite samples, as detailed below.

Across five years of samples from NSHQ14, δD_{CH_4} has ranged from −232‰VSMOW to −311.73‰VSMOW, with a mean weighted by sample year of −275‰VSMOW (Figure 3a and Table 2). This CH_4 is D-enriched with respect to coexisting H_2 (δD_{H_2} = −685‰VSMOW; Miller et al., 2016) and D-depleted with respect to coexisting water (δD_{H_2O} = +0.2‰VSMOW in 2018; Table 3). Although H_2 and water reflect H isotopic

equilibrium at ~50°C (Miller et al., 2016), both H_2 and water are in H isotopic disequilibrium with CH_4 (Figure 3b). Moreover, NSHQ14 fluids exhibit intra-CH_4 disequilibrium, as indicated by $\Delta^{13}CH_3D$ and $\Delta^{12}CH_2D_2$ values (Table 2) plotting below the equilibrium line in Figure 3d. These non-equilibrium isotopic compositions indicate that post-genetic alteration of CH_4 must have occurred or that fluid inclusions are not the only source of CH_4 at NSHQ14.

One potential post-genetic alteration mechanism is diffusion. However, CH_4 at NSHQ14 cannot be the diffusion residual of CH_4 that was originally at intramolecular equilibrium (or with $\Delta^{12}CH_2D_2$ above the apparent $\Delta^{13}CH_3D$ equilibrium temperature) because the diffusion slope (change in $\Delta^{12}CH_2D_2$ over change in $\Delta^{13}CH_3D$) is shallower than the equilibrium line slope over the relevant temperature range (Young et al., 2017). Another potential alteration mechanism is microbial CH_4 oxidation. Two types of microbial CH_4 oxidation have been studied for their effects on CH_4 clumped isotopologue relative abundances: anaerobic methane oxidation of the ANME type and aerobic CH_4 oxidation. ANME-type anaerobic methane oxidation is suggested to be a highly reversible metabolic pathway (Knittel & Boetius, 2009; Timmers et al., 2017). This reversibility has been proposed to bring $\Delta^{13}CH_3D$ and $\Delta^{12}CH_2D_2$ toward equilibrium at low temperatures (70°C to 30°C) through continuous breaking and reforming of bonds in the CH_4 molecule (Ash & Egger, 2019; Giunta et al., 2019; Young et al., 2017). Thus, the comparatively low $\Delta^{13}CH_3D$ and $\Delta^{12}CH_2D_2$ values observed in samples from NSHQ14 and other wells in this study (Figures 3b and 3d) do not support a major role for anaerobic methane oxidation in the study area. Aerobic CH_4 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 CH_4 oxidation does not bring CH_4 into isotopic equilibrium, but rather imparts a normal, classical kinetic isotope effect during CH_4 consumption. In a study of the effect of aerobic CH_4 oxidation on $\Delta^{13}CH_3D$, Wang et al. (2016) found that the fractionation factor for $^{13}CH_3D$ was closely approximated by the product of the fractionation factors for $^{13}CH_4$ and $^{12}CH_3D$. Although it has not yet been demonstrated experimentally, it is hypothesized that the fractionation factor for $^{12}CH_2D_2$ during aerobic CH_4 oxidation may likewise be approximated by the square of the fractionation factor for $^{12}CH_3D$ (Young, 2020). This "product rule" for isotopic fractionation during aerobic CH_4 oxidation results in decreases in $\Delta^{13}CH_3D$ and $\Delta^{12}CH_2D_2$ with concomitant increases in $\delta^{13}C$ and δD in residual CH_4 (Wang et al., 2016; Young, 2020). Thus, aerobic CH_4 oxidation could draw $\Delta^{13}CH_3D$ and $\Delta^{12}CH_2D_2$ values originally near equilibrium down below the equilibrium line in Figure 3d. However, if CH_4 samples from NSHQ14 were originally near H isotope equilibrium with water of SMOW-like isotopic composition, aerobic methane oxidation would push the residual CH_4 toward higher δD (and $\varepsilon_{methane/water}$) values (above the equilibrium line in Figure 3b), which is inconsistent with the comparatively low δD_{CH_4} observed at NSHQ14.

For the reasons outlined above, post-genetic alteration of CH_4 near $CH_4 - H_2O$ and intramolecular isotopic equilibrium does not explain the observed isotopic compositions of CH_4 sampled from NSHQ14. Therefore, the release of CH_4 stored in fluid inclusions cannot account for all of the CH_4 at NSHQ14. Alternative processes that do produce CH_4 with $\Delta^{13}CH_3D$ and $\Delta^{12}CH_2D_2$ values lower than equilibrium include microbial methanogenesis and low-temperature (≲90°C) abiotic reduction of CO_2 or CO through Sabatier or Fischer-Tropsch-type reactions. In Figures 3b and 3d, microbial methanogenesis is represented by samples from cultures (green shaded areas; Gruen et al., 2018; Stolper et al., 2015; Wang et al., 2015; Young, 2020; Young et al., 2017), and low-temperature Sabatier or Fischer-Tropsch-type reactions are represented by field samples from Kidd Creek (gray shaded areas; Sherwood Lollar et al., 2002, 2008; Young et al., 2017) and laboratory experiments with synthetic Ru catalysts (Etiope & Ionescu, 2015; Young et al., 2017).

To independently assess the potential influences of microbial processes on CH_4 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 CH_4-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 CH_4 from H_2 and CO_2, 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 (Kraus et al., 2021; Miller et al., 2016; Rempfert et al., 2017).

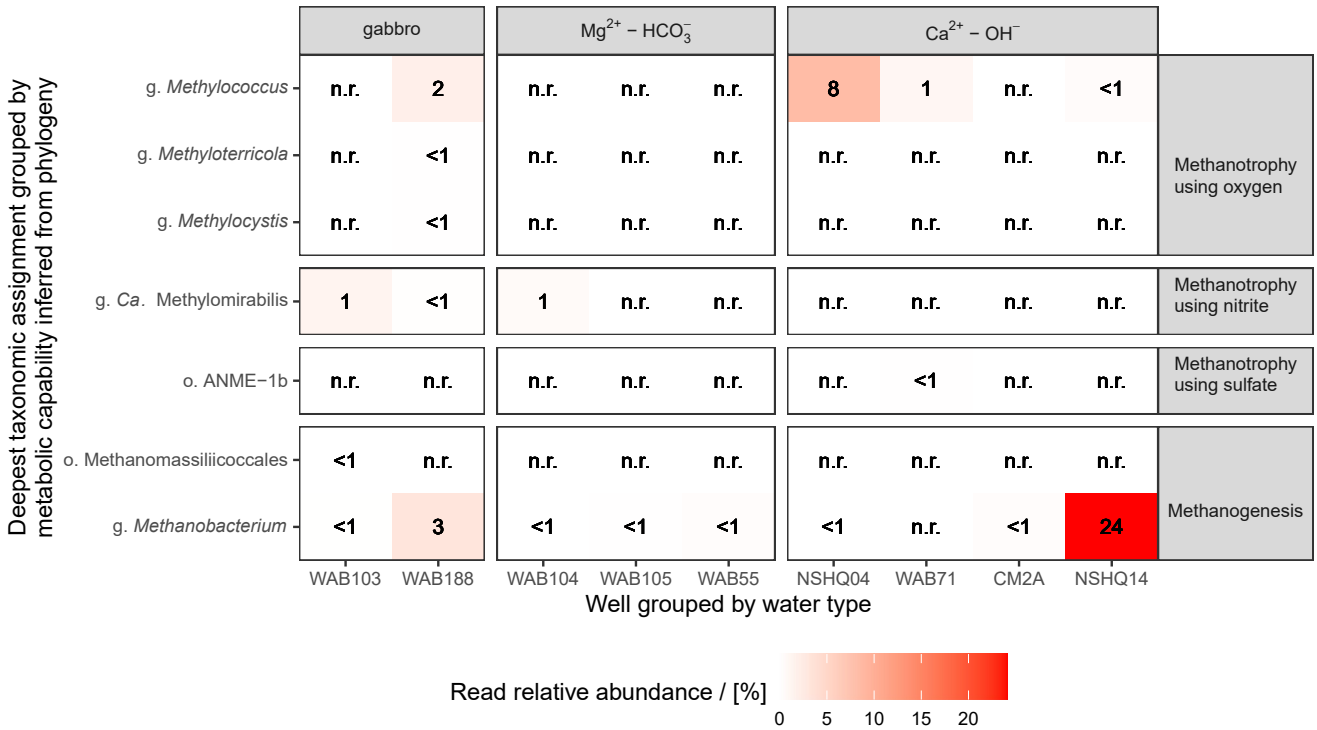

Figure 5. 16S rRNA gene read relative abundances of DNA extracted from Samail Ophiolite groundwaters sampled in 2018 affiliated with CH_4-cycling taxa. Read relative abundances are reported as percentages rounded to the ones place. Cases when a taxon was detected in a sample and was <1% read relative 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).

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 20m) prior to 2016 to larger, higher-flow pumps (maximum depth 90m). 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 O_2 than do shallower fluids.

Consortia capable of anaerobic oxidation of CH_4 coupled to SO_4^{2-} reduction, including ANME, were not detected by 16S rRNA gene sequencing of samples obtained from NSHQ14 in 2018 (Figure 5), 2016, or 2014 (Miller et al., 2016; Rempfert et al., 2017), although sequences affiliated with order ANME-1b were detected in low abundance (<1% of reads) in samples obtained from NSHQ14 in 2017 and 2015 (Kraus et al., 2021; Rempfert et al., 2017). This scarcity of ANME may result from metabolic inhibition by high c_{H_2} in groundwaters at NSHQ14 and elsewhere in the Samail Ophiolite. It has been proposed that the thermodynamics of "reverse methanogenesis" require low c_{H_2} (e.g., ≤1nM in a marine cold seep environment; Boetius et al., 2000). Indeed, the bioenergetics of SO_4^{2-}-driven oxidation of CH_4 are less favorable than SO_4^{2-}-driven oxidation of H_2 or non-CH_4 organics, or other metabolisms such as methanogenesis or acetogenesis in the Samail Ophiolite (Canovas et al., 2017) and in deep continental settings where radiolytic H_2 accumulates (Kieft, 2016; Kieft et al., 2005; Moser et al., 2005).

While 16S rRNA gene sequences affiliated with anaerobic CH_4 oxidizing microbes have only occasionally been detected at NSHQ14, 16S rRNA gene sequences affiliated with the genus *Methylococcus*, which contains aerobic methanotrophs (Hanson & Hanson, 1996), have been detected in all samples from NSHQ14, ranging from 1% to <1% of reads in samples obtained from 2014 to 2018 (Figure 5; Kraus et al., 2021; Miller et al., 2016; Rempfert et al., 2017). Since the aerobic lifestyle of *Methylococcus* is at odds with that of the obligate anaerobe, *Methanobacterium*, it seems most likely that these 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 CH_4 cycle at this well is dominated by CH_4 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 ^{14}C-labeled HCO_3^- and ^{14}C-labeled formate to CH_4 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 $1.15 \cdot 10^5$ cells $\cdot$ 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 CH_4 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 1μmol $\cdot$ L^{-1} to 2μmol $\cdot$ 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 (Lang et al., 2018; McDermott et al., 2015). These relatively low formate concentrations in the ophiolite suggest that formate might be the primary limiting substrates for methanogenesis in $Ca^{2+} - OH^-$ waters, such as at NSHQ14. Coexisting hydrogenotrophic methanogens may produce CH_4 through direct uptake of $\sum CO_2$ in H_2-rich $Ca^{2+} - OH^-$ water, where kinetic inhibitions to abiotic $\sum CO_2$ reduction to CH_4 allow for a modest energy yield for hydrogenotrophic methanogens (Section 5; Leong & Shock, 2020). Methanogens using $\sum CO_2$ could benefit from greater chemical disequilibrium if they inhabit zones where deeply-sourced, H_2-rich $Ca^{2+} - OH^-$ water mixes with shallow, $Mg^{2+} - HCO_3^-$ water (Leong & Shock, 2020; Zwicker et al., 2018). In addition to direct uptake of $\sum CO_2$, 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 (<132nmol $\cdot$ 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_{CH_4}, $\Delta^{13}CH_3D$, and $\Delta^{12}CH_2D_2$ values that collectively indicate a substantial addition of microbial CH_4 to an otherwise abiotic pool of CH_4. 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 CH_4 at NSHQ14, the δD_{CH_4} data alone suggest that perhaps the majority of CH_4 at NSHQ14 formed through non-equilibrium processes, which include microbial methanogenesis. Thus, the high $\delta^{13}C$ of CH_4 at NSHQ14 suggests that the microbial component is more ^{13}C-enriched than microbial CH_4 formed in sedimentary environments, which typically ranges from –90‰VPDB to –50‰VPDB (Figure 3a; Milkov & Etiope, 2018). In cultures of a hydrogenotorophic strain of *Methanobacterium* provided $CaCO_3$(s) as a C source at pH ~9, Miller et al. (2018) observed suppressed apparent isotope effects during methanogenesis ($\alpha_{CO_2/CH_4} = 1.028$). The authors attributed this to the slow kinetics of carbonate dissolution at high pH and the near-total conversion of the resultant $CO_2(aq)$ to CH_4 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 $\sum CO_2$ is converted to CH_4, 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 CH_4 in hyperalkaline, serpentinizing settings. In such settings, the suppression of C isotope fractionation during methanogenesis is supported by observations of high $\delta^{13}C$ values (up to +14‰VPDB) of lipid biomarkers thought to be produced by methanogens at Chimaera, Turkey (Zwicker et al., 2018) and at Lost City (Bradley et al., 2009). Evaluation of these hypotheses will require further physiological studies of methanogens aimed at understanding substrate selection and limitation systematics in hyperalkaline, low-C conditions and the isotopic implications of these factors.

While the data support substantial microbial CH_4 and abiotic, fluid inclusion-derived CH_4 in NSHQ14 fluids, we find less evidence for abiotic CH_4 production at the low temperatures that pervade the modern weathering horizon in the ophiolite. Below 100°C, access of gas-phase H_2 and CO_2 or CO to the catalytic metals

Ru or Rh is required for CH_4 to form at appreciable rates (Etiope & Ionescu, 2015; Jacquemin et al., 2010; McCollom, 2016; Thampi et al., 1987). It has been proposed that the spatial concentration of potentially catalytic Ru-rich chromites in chromitites is important for catalysis of low-temperature CO_2 reduction to CH_4 in ophiolites (Etiope et al., 2018; Etiope & Ionescu, 2015). While peridotites in Oman ubiquitously contain a few percent distributed chromite (Hanghøj et al., 2010), massive chromitites were not reported in lithologic descriptions of cores or drill cuttings from NSHQ14 or any of the six additional wells ranging from 300m to 400m depth that have been drilled in the same catchment by the Oman Drilling Project (Kelemen et al., 2020). Nor are chromitites notably abundant in outcrop within this catchment. Further, although some flow paths of meteoric water through the ophiolite may result in saturation in H_2 and separation of a free gas phase (Canovas et al., 2017), the depth to water is <20m in all wells in the catchment of NSHQ14, suggesting water-saturated conditions in the subsurface. Moreover, if free $H_2(g)$ were generated at high extents of reaction progress, co-existing $CO_2(g)$ would be extremely scarce due to precipitation of carbonate minerals and high pH (Etiope & Ionescu, 2015; Leong & Shock, 2020). It has been proposed that CH_4 in ophiolites can form through reduction of $CO_2(g)$ from non-atmospheric sources such as magma, the mantle, or sedimentary carbonate formations (Etiope & Ionescu, 2015). A magmatic/mantle CO_2 source is not supported at NSHQ14 because excess He above air saturation in groundwaters from this well has a dominantly radiogenic isotopic composition that is distinct from mantle-derived He (Vankeuren et al., 2019). Further, although sedimentary carbonates are present in the vicinity of NSHQ14 and elsewhere in the ophiolite (Boudier & Coleman, 1981; de Obeso & Kelemen, 2018), there is no clear mechanism to liberate $CO_2(g)$ from mineral carbonates and transfer that $CO_2(g)$ to catalytic sites of reaction on chromites where $H_2(g)$ is also present. Thus, the apparent lack of massive chromites and free gaseous potential reactants suggest that the subsurface surrounding NSHQ14 is not conducive to low-temperature abiotic CH_4 production. While substantial abiotic, low-temperature CH_4 production in the catchment of NSHQ14 seems unlikely, NSHQ14 groundwaters could be mere carriers of CH_4 that was produced elsewhere in the ophiolite under gaseous conditions and that has subsequently migrated into the aquifer. Some studies of CH_4 origin in other peridotite bodies have favored such a hypothesis (Etiope et al., 2016; Marques et al., 2018). However, it is not clear how this hypothesis could be tested in the case of the NSHQ14, nor how it addresses the issue of CO_2 source.

In summary, isotopic and microbiological data lead us to conclude that the high concentrations of CH_4 ($10^2\ \mu mol \cdot L^{-1}$) in groundwaters accessed by NSHQ14 primarily result from microbial methanogenesis and the release of abiotic CH_4 from fluid inclusions. The known presence of CH_4-bearing fluid inclusions in the Samail Ophiolite and our finding of high $\delta^{13}C$ values of CH_4, C_2H_6, and C_3H_8 that overlap with values reported from seafloor hydrothermal vents where CH_4 formed at >270°C in fluid inclusions predominates suggest a similar source in the ophiolite. However, deficits in $^{12}CH_3D$, $^{13}CH_3D$, and $^{12}CH_2D_2$ relative to equilibrium indicate the production of additional CH_4 at low temperatures. The $^{13}CH_3D$ deficit in particular is more compatible with a microbial origin than a low-temperature abiotic origin. Moreover, genomic, transcriptomic, and physiological data show that methanogens are abundant and active in aquifers accessed via NSHQ14. Organic geochemical and cultivation data from the literature suggest that C isotope effects of methanogenesis may be suppressed under C-limited conditions in serpentinizing settings. That genes associated with methanogens coexist with a smaller abundance of genes associated with methanotrophs (particularly aerobes) in NSHQ14 groundwaters suggests that some of the CH_4 has undergone microbial oxidation, which would further help explain the high $\delta^{13}C$ values of CH_4 at this well.

4.2.3. Abundant Microbial CH_4 Produced under C-Limited Conditions and Substantial Microbial CH_4 Oxidation in the Ca^{2+} − OH^- Waters of Well NSHQ04

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

Primary differences in fluid composition between NSHQ04 and NSHQ14 include lower pH by ~1 and higher $c_{\sum Ca}$ and $c_{\sum Si}$ at NSHQ04 (Tables 1 and 3; Fones et al., 2019; Miller et al., 2016; Rempfert et al., 2017; Vankeuren et al., 2019). These differences could be related to the scarcity of fresh, near-surface olivine at NSHQ04, which may result in a greater influence of pyroxene serpentinization at NSHQ04 (Miller et al., 2016).

Low-temperature pyroxene serpentinization generally continues after olivine is exhausted, and leads to higher $c_{\sum Si}$ and, depending on pyroxene chemical composition, can also lead to higher $c_{\sum Ca}$ and lower pH (Bach et al., 2006; Leong & Shock, 2020). The relatively low pH and high $c_{\sum Si}$ could also stem from mixing of $Ca^{2+} - OH^-$ waters with gabbro- or atmosphere-influenced fluids.

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

While H_2 has only been transiently detected at NSHQ04, c_{CH_4} at this well has consistently been the highest among our sample sites ($144\,\mu mol \cdot L^{-1}$ in 2018 and $483\,\mu mol \cdot L^{-1}$ in 2017. In comparison to NSHQ14, CH_4 at NSHQ04 is more ^{13}C- and D-enriched (mean weighted by sample year $\delta^{13}C = +3.3‰$VPDB, $s = 1.8‰$; $\delta D = -220‰$VSMOW, $s = 11‰$; $n = 4$; Table 2 and Figure 3a). Fluids sampled from NSHQ04 are in $CH_4 - H_2O$ H isotopic disequilibrium and intra-CH_4 disequilibrium (Figures 3b and 3d), which is also true of fluids from NSHQ14. However, CH_4 sampled from NSHQ04 has distinctly negative $\Delta^{12}CH_2D_2$ (−24.502‰) and low $\Delta^{13}CH_3D$ (mean weighted by sample year of 0.36‰, $s = 0.32‰$, $n = 3$; Table 2). As such, CH_4 from NSHQ04 plots squarely among methanogen culture samples in $\Delta^{13}CH_3D/\Delta^{12}CH_2D_2$ space (Figure 3d), suggesting that CH_4 is dominantly microbial at NSHQ04. Moreover, alkane gases dissolved in waters pumped from NSHQ04 exhibited a $C_1/(C_2 + C_3)$ ratio of $5.4 \cdot 10^3$ in 2018, which is higher than other wells in this study (Table 4 and Figure 3c), further supporting a major component of microbial CH_4 at NSHQ04.

Microbial CH_4 production at NSHQ04 is also indicated by microbiological data.16S rRNA gene sequences affiliated with *Methanobacterium* have been detected in DNA extracted from biomass filtered from waters pumped from NSHQ04, albeit in low relative abundance (<1% of reads in 2018; Figure 5; also detected in <1% of reads in 2014, but not detected in 2015 and 2017 (Kraus et al., 2021; Miller et al., 2016; Rempfert et al., 2017). The apparent low relative abundance of *Methanobacterium* at NSHQ04 could have resulted from the relatively shallow depth from which samples were collected at NSHQ04 due to well obstruction and the consequential sampling of groundwaters that may have experienced atmospheric O_2 infiltration. High relative read abundances of sequences affiliated with aerobes and transient H_2 across years of sampling NSHQ04 suggest that zones of the aquifer that are not always anoxic were accessed. These conditions may restrict methanogen abundance to greater depths than were sampled, but not constrain the upward diffusion of the product of their metabolism, CH_4. Nevertheless, fluids obtained from NSHQ04 have yielded robust cultures of *Methanobacterium* (Miller et al., 2018). In addition, high relative abundances of 16S rRNA gene reads of DNA extracted from biomass in waters sampled from NSHQ04 were related to an aerobic methanotroph of the genus *Methylococcus* (8% of reads in 2018; inter-annual mean of 11%; Figure 5; Kraus et al., 2021; Miller et al., 2016; Rempfert et al., 2017). Greater aerobic methanotrophy at NSHQ04 relative to NSHQ14 may have contributed in part to the lower $\Delta^{13}CH_3D$ and $\Delta^{12}CH_2D_2$ and higher $\delta^{13}C$ and δD of CH_4 sampled from NSHQ04.

Methanotrophic activity at NSHQ04 is consistent with the observed ^{13}C-depletion in $\sum CO_2$ at NSHQ04 (−29.7‰VPDB $\delta^{13}C$; Table 2) relative to the other studied wells because environments of active methanotrophy often have ^{13}C-depleted $\sum CO_2$ (Barker & Fritz, 1981; Michaelis et al., 2002). Indeed, $\delta^{13}C_{\sum CO_2}$ at NSHQ04 is compatible with aerobic oxidation of CH_4 of ~0‰VPDB $\delta^{13}C$ (Barker & Fritz, 1981; Feisthauer et al., 2011). Alternatively, ^{13}C-depletion in $\sum CO_2$ could be explained by kinetic isotope fractionation during hydroxylation of atmospheric CO_2 upon contact with $Ca^{2+} - OH^-$ water, which has been interpreted as the cause of $\delta^{13}C$ as low as −27.21‰VPDB in Ca-rich carbonates from hyperalkaline seeps in the Samail Ophiolite (Clark et al., 1992; Falk et al., 2016; Kelemen et al., 2011). Considering the relatively shallow sampling depth at NSHQ04 in 2018 (Table 1), it is plausible that the sampled groundwaters continuously interact with

atmospheric CO_2. Although the relative influences of methanotrophy and atmospheric CO_2 hydroxylation cannot be determined based on the available data, both processes could affect $\delta^{13}C_{\sum CO_2}$ at NSHQ04.

In summary, low $\Delta^{13}CH_3D$ and $\Delta^{12}CH_2D_2$, high $C_1/(C_2 + C_3)$, the presence of *Methanobacterium* that were readily cultured, and high 16S rRNA gene relative abundances of *Methylococcus* lead us to conclude that microbial production and consumption of CH_4 are the dominant factors controlling CH_4 concentration and isotopic composition at NSHQ04.

4.2.4. H_2-Limited Microbial Methanogenesis with Classic C Isotope Effect Expressed at Well WAB188

WAB188 is situated 2km down-gradient from NSHQ04 and is set in gabbro on the opposite side of a fault from NSHQ04 (Figure 1 and Table 1; Figure S1). Fluids pumped from WAB188 have had variable pH (8.72 to 5.75) and oxidation-reduction potential (f_{O_2} of 10^{-61} bar to 10^{-34} bar and *Eh* of −220mV to +214mV) across four years of sampling (Table 1; Fones et al., 2019; Rempfert et al., 2017). WAB188 has consistently had major ion compositions similar to the gabbro-hosted well WAB103, except that WAB188 has had higher $c_{\sum Ca}$ (Table 3; Fones et al., 2019; Rempfert et al., 2017). H_2 has occasionally been detected in fluids pumped from WAB188 (c_{H_2} = 0.992 μmol · L^{-1} in 2017), and CH_4 has consistently been detected at moderate concentrations (c_{CH_4} = 1.83 μmol · L^{-1} in 2017 and 0.917 μmol · L^{-1} in 2018; Table 4; Fones et al., 2019; Rempfert et al., 2017). The high $c_{\sum Ca}$ 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 (Hoehler, 2004; McCollom, 1999), but may also be affected by inputs of fresh rainwater and/or H_2-bearing $Ca^{2+} - OH^-$ 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; Kraus et al., 2021; Rempfert et al., 2017). 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; Kraus et al., 2021; Rempfert et al., 2017). Furthermore, 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 $Ca^{2+} - OH^-$ fluids from NSHQ14 and NSHQ04, the circumneutral fluids from WAB188 have ~10^2 to ~10^3 times higher $c_{\sum CO_2}$ (inter-annual mean of 2910 μmol · L^{-1}, s = 620 μmol · L^{-1}, n = 3; Table 3) and ~75‰ lower $\delta^{13}C_{CH_4}$ (inter-annual mean $\delta^{13}C$ = −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 $C_2 - C_6$ 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

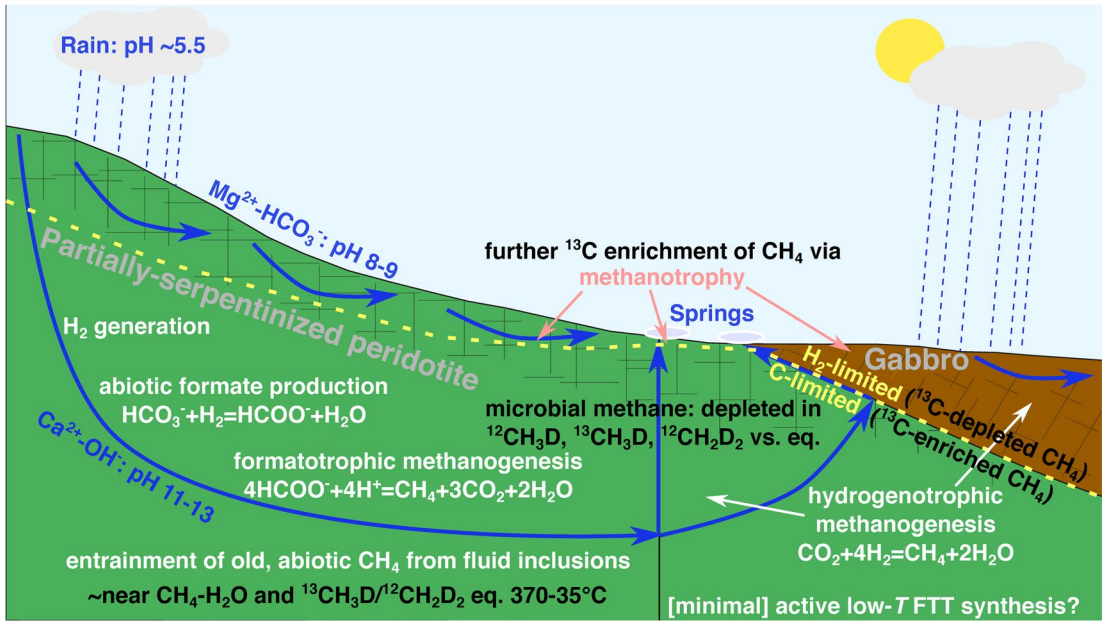

Figure 6. Conceptual model of CH_4 dynamics in Samail Ophiolite. Cross section after Neal and Stanger (1985), Dewandel et al. (2005), and Rempfert et al. (2017). Groundwater flow is depicted with blue arrows. Cross-hatching illustrates fissured zone of aquifer, extending to ~50m depth. A deep tectonic 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.

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 $c_{\sum CO_2}$ and c_{H_2} similar to WAB188 in 2017 (Section 4.2.1; Table 5). Further, the apparent α_{CO_2/CH_4} at WAB188 (based on measured $\delta^{13}C_{\sum CO_2}$ 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 α_{CO_2/CH_4} observed for parallel cultures under CO_2-poor conditions (Miller et al., 2018). In sum, the conditions at WAB188 contrast starkly with those that prevail in Ca^{2+} – OH^- fluids, where C substrates for methanogenesis are often more scarce than H_2. These differences may be reflected in the inverse relationship between $c_{\sum CO_2}$ and $\delta^{13}C_{CH_4}$ across fluids from wells WAB188, NSHQ14, and NSHQ04 (Figure S5), which is consistent with an effect of C availability on the apparent C isotope effect of microbial methanogenesis.

5. Conclusions

Through integration of isotopic, microbiological, and hydrogeochemical data, we conclude that substantial microbial CH_4 is produced under varying degrees of C or H_2 limitation in subsurface waters of the Samail Ophiolite and mixes with abiotic CH_4 released from fluid inclusions (Figure 6). Across subsurface fluids ranging in pH from circumneutral to 11.39, microbial CH_4 production is evidenced by 16S rRNA gene sequencing and other microbiological data indicating that methanogens are widespread and active in groundwaters in the ophiolite. We propose that CH_4 produced by these microbes constitutes a substantial portion of the total CH_4 pool, which is consistent with our finding of $^{13}CH_3D$ and $^{12}CH_2D_2$ relative abundances significantly less than equilibrium. Using a simple thermodynamic model, we find that formatotrophic methanogenesis may become more energetically favorable than hydrogenotrophic methanogenesis as Mg^{2+} – HCO_3^- waters transition to $Ca^{2+}OH^-$ waters where $CO_2(aq)$ is extremely scarce, despite relatively low formate concentrations of ~ $1\mu mol \cdot L^{-1}$ across fluid types (Rempfert et al., 2017). This lends geochemical support to recent microbiological findings that independently indicate that the activity of formatotrophic methanogens increases relative to hydrogenotrophic methanogens as groundwater pH increases in the ophiolite (Fones et al., 2020).

In addition, an abiotic, fluid inclusion-derived source of CH_4, C_2H_6, and C_3H_8 is inferred from the widespread occurrence of CH_4 in fluid inclusions in peridotites, including those in Oman, and is supported by the relatively ^{13}C-enriched compositions of CH_4, C_2H_6, and C_3H_8 measured in gases exsolved from peridotite-hosted groundwaters in this study. The measured $\delta^{13}C$ values overlap with those of CH_4, C_2H_6 and C_3H_8 from seafloor hydrothermal vents where fluid inclusions are the dominant source of these alkanes, suggesting similar CH_4 sources across these environments. In contrast, abiotic, low-temperature reduction of CO_2 to CH_4 appears less likely to contribute substantially to the CH_4 pool in the study area due to a scarcity of conditions favorable to catalysis, namely, access of gas-phase H_2 and CO_2/CO to Ru-bearing chromites.

Further, we note an inverse relationship between $c_{\sum CO_2}$ and $\delta^{13}C_{CH_4}$ across groundwaters bearing microbiological evidence of methanogenic activity. This finding supports the hypothesis that the apparent C isotope fractionation between the C substrate used by methanogens and the CH_4 they produce is suppressed when the C substrate is limiting. Thus, our finding that $\delta^{13}C_{CH_4}$ varies by 90‰ in the Samail Ophiolite suggests that, in some settings, $\delta^{13}C_{CH_4}$ may be a powerful indicator of transitions from H_2-limited to C-limited conditions for microbial methanogenesis, rather than a discriminant between microbial versus abiotic CH_4. The 16S rRNA gene sequencing data also indicate the presence of microbes capable of CH_4 oxidation, particularly those that can use O_2 as an oxidant. This oxidation may also contribute in part to the ^{13}C-enriched composition of CH_4 in the ophiolite, which is considered unusual for CH_4 with a substantial microbial component.

This study supports the premise that H_2 produced from water/rock reaction can fuel microbial life, even under challenging conditions of high pH and low oxidant availability. By identifying where and how microbial methanogenesis can reasonably be expected to occur in H_2-rich, subsurface environments, this work complements theoretical models in guiding the search for rock-hosted life, including extraterrestrial life. For example, our findings substantiate predictions that microbial methanogenesis could occur in the reduced, alkaline ocean of Saturn's moon, Enceladus (Glein et al., 2015; McKay et al., 2008; Waite et al., 2017) and in the Martian subsurface (Kral et al., 2014).

Data Availability Statement

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

Acknowledgments
This research was directly supported by the Rock-Powered Life NASA Astrobiology Institute (NNA15BB02A). This research also used samples and/or data provided by the Oman Drilling Project. The Oman Drilling Project (OmanDP) has been possible through co-mingled funds from the International Continental Scientific Drilling Project (ICDP), the Sloan Foundation–Deep Carbon Observatory (Grant 2014-3-01, Kelemen PI), the National Science Foundation (NSF-EAR-1516300, Kelemen PI), the NASA Astrobiology Institute (NNA15BB02A), the German Research Foundation (DFG), the Japanese Society for the Promotion of Science (JSPS), the European Research Council, the Swiss National Science Foundation, JAMSTEC, the TAMU-JR Science operator, and contributions from the Sultanate of Oman Ministry of Regional Municipalities and Water Resources, the Oman Public Authority of Mining, Sultan Qaboos University, CRNS-Univ. Montpellier II, Columbia University, and the University of Southampton. Work at LBNL was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, Chemical Sciences, Geosciences, and Biosciences Division, under Award Number DE-AC02-05CH11231. We thank the Ministry of Regional Municipalities and Water Resources in the Sultanate of Oman (particularly Said Al Habsi, Rashid Al Abri, Salim Al Khanbashi, and Haider Ahmed Mohammed Alajmi) for allowing access to wells and logistical support, Zaher Al Sulaimani and Mazin Al Sulaimani from the Oman Water Centre and AZD Engineering for their technical and logistical support, Jude Coggon for coordinating Oman Drilling Project activities, Benoît Ildefonse for sharing geologic map data, Eric Ellison and Kaitlin Rempfert for their assistance in the field and laboratory, Elizabeth Fones for sharing biomass samples, Emily Kraus for critical discussion of Oman CH_4 cycle processes, and Noah Fierer, Jen Reeves, Corinne Walsh, Matthew Gebert, and Angela Oliverio for assisting with DNA sequencing and interpretation.

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