Molecular evidence for an active microbial methane cycle in subsurface serpentinite-hosted groundwaters in the Samail Ophiolite, Oman

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Abstract

Serpentinization can generate highly reduced fluids replete with hydrogen (H_2) and methane (CH_4), potent reductants capable of driving microbial methanogenesis and methanotrophy, respectively. However, CH_4 in serpentinized waters is thought to be primarily abiogenic, raising key questions about the relative importance of methanogens and methanotrophs in the production and consumption of CH_4 in these systems. Herein, we apply molecular approaches to examine the functional capability and activity of microbial CH_4 cycling in serpentinization-impacted subsurface waters intersecting multiple rock and water types within the Samail Ophiolite of Oman. Abundant 16S rRNA genes and transcripts affiliated with the methanogenic genus, *Methanobacterium*, were recovered from the most alkaline (pH > 10), H_2- and CH_4-rich subsurface waters. Additionally, 16S rRNA genes and transcripts associated with the aerobic methanotrophic genus, *Methylococcus*, were detected in wells that spanned varied fluid geochemistry. Metagenomic sequencing yielded genes encoding homologs of proteins involved in the hydrogenotrophic pathway of microbial CH_4 production and in microbial CH_4 oxidation. Transcripts of several key genes encoding methanogenesis/methanotrophy enzymes were identified, predominantly in communities from the most hyperalkaline waters. These results indicate active methanogenic and methanotrophic populations in waters with hyperalkaline pH in the Samail Ophiolite thereby supporting a role for biological CH_4 cycling in aquifers that undergo low temperature serpentinization.

Importance

Serpentinization of ultramafic rock can generate conditions favorable for microbial methane (CH_4) cycling, including the abiotic production of H_2 and possibly CH_4. Systems of low-temperature serpentinization are geobiological targets due to their potential to harbor microbial life and ubiquity throughout Earth's history. Biomass in fracture waters collected from the Samail Ophiolite of Oman, a system undergoing modern serpentinization, yielded DNA and RNA signatures indicative of active microbial methanogenesis and methanotrophy. Intriguingly, transcripts for proteins involved in methanogenesis were most abundant in the most highly-reacted waters that have hyperalkaline pH and elevated concentrations of H_2 and CH_4. These findings suggest active biological methane cycling in serpentinite-hosted aquifers, even under extreme conditions of high pH and carbon limitation. These observations underscore the potential for microbial activity to influence the isotopic composition of CH_4 in these systems, information that could help in identifying biosignatures of microbial activity on other planets.

Introduction

Life in deep subsurface environments is dependent on lithosphere-derived nutrients to drive metabolism and biosynthesis (i.e., chemosynthesis). Water-rock interactions are one potential source of nutrients that can be used by biological systems to generate chemical energy. During the hydration of olivine and/or pyroxene in ultramafic rocks, the oxidation of ferrous iron coupled to the reduction of water can generate molecular hydrogen (H_2) through the geological process of serpentinization (1, 2). Elevated dissolved H_2 concentrations can drive the reduction of inorganic carbon (CO_2) to generate formate ($HCOO^-$) and carbon monoxide (CO) (3), as well as methane (CH_4) and additional light hydrocarbons through abiotic reactions at low

temperature (<100 ºC) (5, 6). Serpentinization-impacted waters often have very low oxidation-reduction potentials, have pH values of 8 to greater than 12, and can have nM to mM concentrations of H_2 and CH_4 that can serve as electron donors to fuel microbial metabolism (7–13). Zones of active, low-temperature serpentinization exist beneath the water table within ophiolites, portions of oceanic crust and upper mantle that have been tectonically emplaced onto a continent. Ophiolites, such as the Samail Ophiolite in the Sultanate of Oman, provide an accessible venue to study the subsurface biosphere in bedrock environments undergoing serpentinization (8, 14).

Current data suggests CH_4 in ophiolites is generated abiotically at low temperatures or is primarily relict from early high temperature water/rock reactions that trapped fluids and gases in fluid inclusions, which are later released during weathering (6, 15). The abiotic sources of CH_4 in these systems are inferred by studies of stable isotope compositions showing CH_4 enriched in ^{13}C (6). Alternatively, several types of microorganisms can produce CH_4, including methanogenic Archaea that can generate CH_4 from a variety of substrates, including H_2/CO_2, CO, formate, a variety of methylated substrates, and acetate (16), many of which have been detected in waters that have been subjected to serpentinization (8, 10, 17–19). Although methanogens differ in their substrate use, all require the methyl-coenzyme M reductase (MCR) enzyme complex (encoded by *mcrABG*) for the terminal step of methanogenesis. Some H_2-dependent methanogens can also use formate as a source of electrons to reduce CO_2 instead of H_2 via the activity of formate dehydrogenase (encoded by *fdhAB*) (20). Conversely, anaerobic methanotrophs oxidize CH_4 with a variety of terminal electron acceptors including sulfate (SO_4^{2-}), nitrate (NO_3^-), nitrite (NO_2^-), and metals, likely via the reverse methanogenesis pathway (21). In addition to anaerobes, aerobic

93 methanotrophs catalyze the oxidation of CH_4 (to methanol) using the particulate or soluble
94 methane monooxygenase enzymes, encoded by the *pmoABC* and *mmoXYZ* genes, respectively
95 (22, 23). In the second step of CH_4 oxidation, methanol dehydrogenases (MDH) oxidize
96 methanol to formaldehyde. The *mxaF* gene encodes for the large subunit of the NAD-
97 independent MDH known to proteobacterial methanotrophs (24). These genes, therefore, can
98 serve as key putative markers of methanogenesis and methanotrophy in natural systems. The
99 presence of genes that encode for [NiFe]-hydrogenases and carbon cycling processes can
100 provide further insight into the specific electron donors capable of fueling these cells in an
101 environment.
102
103 Several environments impacted by the process of serpentinization show evidence of microbial
104 methanogenesis and methanotrophy. For example, the detection of key genes required for
105 methanogenesis and/or methanotrophy from the Voltri Massif (Italy), the Samail Ophiolite
106 (Oman), and the Santa Elena Ophiolite (Costa Rica) suggests that these processes are active in
107 these system (10, 19, 25) . The case for the presence of these organisms in ophiolites is bolstered
108 by detection of 16S ribosomal RNA genes affiliated with known CH_4 cycling organisms (7, 8,
109 10, 26). Methanotrophic ANME-1 archaea have been detected via high-throughput sequencing
110 methods in the Voltri Massif and Cabeço de Vide aquifers of Italy and Portugal (18, 19). Further,
111 the composition and ^{13}C enrichment of archaeal lipids from the Chimaera ophiolite of Turkey
112 provides evidence of archaeal methanogenesis under inorganic carbon limitation at that site (27).
113 Organisms collected from the Samail Ophiolite and the Cedars (California) show CH_4
114 production when amended with ^{14}C- or ^{13}C-labeled substrates in activity assays, respectively (12,
115 25), and the incubations conducted with organisms from the Samail Ophiolite also show labeled

substrate assimilation into biomass (25). Additionally, an enrichment culture of a methanogen of the genus *Methanobacterium* grown from alkaline waters of the Samail ophiolite was active over a pH range of 6.9 – 10.1 and showed an ability to use HCO_3^- and $CaCO_3$ as a C source (28). Yet, other sites, including the Coast Range Ophiolite Microbial Observatory (CROMO) (California) and the Tablelands ophiolite (Newfoundland, Canada), show no evidence of microbial methanogenesis, suggesting the presence of unknown factors that limit the distribution of CH_4 metabolisms at these sites (29, 30). Therefore, while incubation and cultivation studies show microbial activity when amended with substrate, they may not be representative of activity in the modern subsurface of ophiolites. Consequently, the environmental conditions conducive to methanogenic activity requires further investigation.

The Samail Ophiolite is the largest (approximately 15,000 km^3) and best exposed on Earth, with zones in the mantle peridotite section currently undergoing serpentinization largely below 60 ºC (8, 31–35). In 1983 – 1985 and 2004 – 2005, the Sultanate of Oman drilled several wells into the ophiolite, making it an accessible location to sample subsurface fracture waters and investigate the microbial contribution to CH_4 cycling in a low-temperature continental serpentinizing environment. Previous work has detected µM to mM concentrations of dissolved H_2 and CH_4 in aquifer waters, with the CH_4 in hyperalkaline waters displaying unusually high $\delta^{13}C$ values (up to +3‰ VPDB) that do not fall within typical ranges of microbial CH_4 (8, 28, 33, 36). This suggests either an abiotic origin for CH_4 or extensive biological production and/or consumption of CH_4 that is already enriched in $\delta^{13}C$. Here, we apply genomic and transcriptomic sequencing approaches to biomass collected from these same sites to better define the distribution and putative activity of microbial methanogens and methanotrophs within the Samail Ophiolite.

Results

Geochemical Characterization of Subsurface Fracture Waters. Fracture waters from five pre-existing wells in the Samail Ophiolite, Oman (Fig. 1), were sampled in February of 2017 for planktonic biomass for use in geochemical (Table 1) and DNA- and RNA-based-analyses. Well NSHQ14 was sampled at a depth of 50m (NSHQ14B) and 85m (NSHQ14C). Waters recovered from wells drilled in peridotite bedrock (NSHQ14 and WAB71) had hyperalkaline pH (pH > 10), with waters from NSHQ14C at 85 meters depth exhibiting the highest measured pH (11.3) and H_2 concentration (253 µM) of any of the sampled wells. The waters recovered from wells drilled near the "contact" or subsurface faulted boundary between gabbro and peridotite bedrock (NSHQ04, WAB55) had alkaline pH, with values of 10 for NSHQ04 and 9.2 for WAB55. The pH of gabbro-hosted well WAB188 was not measured in 2017 but previous observations recorded values of 8.7 and 7.6 in 2015 and 2016, respectively (7).

CH_4 was detected in every well, with the highest concentration (483 µM) measured in contact well NSHQ04. Dissolved inorganic carbon (DIC) was detected in low (<0.2 mM) concentrations in the hyperalkaline wells and in greater concentrations (up to 3 mM) in WAB188 and WAB55. Peridotite wells had lower concentrations of potential electron acceptors (e.g., SO_4^{2-}, NO_3^-, NO_2^-) relative to the contact wells (Table 1). Trace metal and non-metal elemental concentrations for all wells are in Table S1.

161 Previous studies grouped the waters in each of the wells sampled herein as either Type I, Type
162 II, or crust/mantle "contact" waters based on water geochemistry, specifically water pH and
163 concentrations of Ca and Mg (32, 34, 37–40). In agreement with prior classifications, NSHQ14,
164 WAB71, and NSHQ04 were dominated by Ca/OH^- waters typical of closed-system
165 serpentinization (termed Type II waters), WAB55 intersects Mg/HCO_3^-waters more typical of
166 open-system serpentinization (termed Type I waters), and gabbro-hosted WAB188 contains
167 waters typical of a contact well near the crust/mantle boundary.
168
169 ***Diversity of 16S rRNA Genes and Transcripts in Subsurface Fracture Waters.*** Biomass was
170 concentrated from subsurface Type I and II waters by filtration (0.22 µm) and processed for
171 DNA and RNA; the latter of which was converted to cDNA. The V4/5 hypervariable region of
172 both 16S rRNA genes and their transcripts (cDNA) were amplified, sequenced and clustered into
173 amplicon sequence variants (ASVs). An overview of the most abundant ASVs from each well
174 (Fig. S1), sequencing metrics (Table S2), and rarefaction curves of observed species richness
175 (Fig. S2) are in the supplemental information. DNA/RNA extraction, PCR, and the RT-negative
176 controls produced low numbers of sequence reads and did not resemble sampled well community
177 16S rRNA gene compositions (Fig. S3). Eukaryotic 18S rRNA sequence counts were low
178 (0.11% of all sequences from all wells) and these sequences were deemed contaminants in this
179 investigation due to the low read counts and compositional similarity to laboratory controls.
180
181 Numerous sequences were detected with close affiliation to known CH_4-producing and
182 consuming organisms. Based on homology to cultivars, the most abundant sequences
183 corresponding to methanogenic taxa were those affiliated with the genus *Methanobacterium*, a

methanogen within the Euryarchaeota phylum (41). This *Methanobacterium* 16S rRNA gene ASV was one of the most abundant sequences detected in both Type I and Type II well waters (Fig. S1). Other lesser abundant ASVs showed homology to other characterized methanogenic archaea. The most abundant sequences related to methanotrophs were those showing homology to *Methylococcus*, an aerobic methanotroph within the Gammaproteobacteria (42). Less abundant ASVs affiliated with putative anaerobic CH_4 oxidizing taxa were also detected in well water communities including ANME-1 (21) and *Candidatus Methylomirabilis* of the NC10 group (43).

Stark differences in ASV abundance were apparent between the DNA and cDNA fractions within samples. The mean abundance of 16S rRNA genes (DNA) and transcripts (cDNA) of two taxa putatively involved in CH_4 cycling is shown in Fig. 2. The mean percentage of sequences of ASVs affiliated with the most abundant methanogen, *Methanobacterium*, was greater in the cDNA fraction for NSHQ14 (Fig. 2a). At the 85m depth for NSHQ14, 47.1% of the cDNA reads were attributed to *Methanobacterium* compared to 13.3% of the DNA reads. Similarly, *Methanobacterium* constituted 34.5% of the cDNA and 10.7% of the DNA reads at the 50m depth. The proportion of *Methanobacterium* reads in NSHQ14 varied more widely in the cDNA (19.9 – 62.9%) than in the DNA (6.9 – 18.6 %) (Fig. 2b). In contrast to NSHQ14, the *Methanobacterium* ASVs represented less than 5% of the reads in any sample from other wells. Similarly, the *Methylococcus* affiliated ASV comprised a greater mean percentage of the cDNA (6.6 %, 2.8 %) when compared to the DNA (1.0 %, 0.4 %) in NSHQ14 at 50m and 85m. In hyperalkaline contact well NSHQ04, *Methylococcus* was 41.4% of the cDNA and 17.4% of the

206 DNA. The anaerobic methanotroph affiliated ASVs (ANME-1, *C. Methylomirabilis*) had low
207 read abundances (< 0.5%) in both the DNA fractions in hyperalkaline peridotite water samples.
208
209 ***Metagenomic Characterization of Subsurface Fracture Water Communities.*** Key genes
210 encoding proteins involved in methanogenesis were detected in assembled metagenomic
211 sequences from the Type II hyperalkaline waters of the peridotite-hosted wells NSHQ14 and
212 WAB71 (Fig. 3A). Metagenomic assemblies from both depth intervals at NSHQ14 harbored
213 genes coding for MCR (*mcrABCDG*) and tetrahydromethanopterin S-methyltransferase (MTR;
214 *mtrABCDEFGH*) operons. The MCR and MTR homologs were co-localized on the same contigs
215 recovered from both 50m and 85m depth intervals in NSHQ14 and the McrA sequences were
216 most closely affiliated with McrA from a cultivated *Methanobacterium* sp. (GenBank:
217 TMS43336.1). Genes for various [NiFe]-hydrogenases involved in supplying reductant or
218 balancing osmotic potential in methanogens (16) were also identified. These include active site
219 subunits of membrane-bound, ion translocating [NiFe]-hydrogenases (*mbhJL*), putative
220 bifurcating hydrogenases (*mvhADG*), and F_{420} or cytochrome reducing hydrogenases (*frhABG*,
221 *vhcD*, respectively) (44). Like MCR and MTR, these homologs were detected in assemblies from
222 both depths of NSHQ14 and showed close homology to various cultivated *Methanobacterium* sp.
223 CH_4 cycling genes identified in metagenomes with homology to proteins from
224 *Methanobacterium* sp. (with an e-value of $< 1 \times 10^{-6}$, > 30% amino acid identity over > 50% of
225 the length) are shown in Fig. 3B.
226
227 In metagenomic assemblies from the peridotite-hosted well WAB71, only an amino acid
228 sequence for *mcrG* was detected and it exhibited homology to *mcrG* of a *Methanophagales* sp. of

229 the ANME-1 group (Genbank: RZN33282.1). Among the communities in Type I well waters,
230 those from WAB188 showed the greatest capability for hydrogenotrophic methanogenesis (Fig.
231 3A). The operons encoding MCR (*mcrABCDG*) and MTR (*mtrABCDEFGH*) were co-localized
232 on a single contig, while the MCR II (*mrtBDGA*) operon was found on a separate contig, and
233 homologs of [NiFe]-hydrogenases (*mvhDG*, *frhABG*, *vhcG*, and *mbhJL*) were detected.
234 Homologs of each protein were closely related to cultivated *Methanobacterium* sp. (mcrA:
235 Genbank WP_048081846.1, mrtA: Genbank AXV36901.1). In comparison, metagenomic
236 assemblies from WAB55 (Type I) were found to only contain homologs of *mcrCG* (related to
237 *Methanobacterium*, Genbank WP_048081846.1) and assemblies from NSHQ04 only encoded
238 *mtbB* homologs most closely related to halophilic methanogenic taxa (*Methanonatronarchaeum*
239 *thermophilium*, Genbank OUJ19070; and *Methanohalophilus* sp., Genbank OBZ35607.1). These
240 findings point to methanogens being less abundant in WAB55 and NSHQ04, as compared to
241 WAB188 and NSHQ14, thereby leading to incomplete representation of pathways involved in
242 their energy metabolism in metagenomic assemblies.
243
244 Homologs of *fdhAB* encoding the subunits of the formate dehydrogenase enzyme were detected
245 in all well metagenomes, with some sequences homologous to *Methanobacterium* strains (Fig.
246 3). Homologs of carbonic anhydrase (CA) genes (*can*, *cynT*, *cah*) were detected in all
247 metagenomes. Most CA sequences were identified most closely to non-
248 methanogenic/methanotrophic organisms but CA sequences from NSHQ14 and WAB188 were
249 found to be most closely related to methanogens and methanotrophs, including
250 *Methanobacterium* and *Methylococcus* strains.
251

252 Homologs of protein coding genes associated with methanotrophy were detected in metagenomic
253 sequences, consistent with 16S rRNA gene and transcript data suggesting the potential
254 importance of methanotrophy in the Samail Ophiolite. Homologs of *pmoABC* were detected in
255 every well (Fig. 3A), often with one or more slightly divergent copies. These homologs were
256 most closely related to those identified in previously characterized aerobic *Methylococcus* sp.
257 Homologs of protein coding genes affiliated with soluble methane monooxygenases (*mmoXYZ*)
258 or of the large subunit of methanol dehydrogenase (*mxaF*) were not detected in any metagenome
259 from the subsurface water communities.
260
261 ***Metatranscriptomic Characterization of Subsurface Fracture Water Communities.*** Transcripts
262 of genes involved in various CH_4-cycling processes were detected in subsurface waters from the
263 Samail ophiolite. Transcript abundances were normalized to counts per million reads (CPM) and
264 lowly expressed transcripts (<1 CPM in all samples) were removed as possible contaminants.
265 The results for all transcripts investigated are in Table 2.
266
267 The abundance of transcripts in CPM for MCR (*mcrABG*), [NiFe]-hydrogenases (*mvhADG*,
268 *frhABG*, *vhcADG*, *vhuADGU*, *mbhJL*), *pmoABC*, CA, and *fdhAB* are shown in Fig.4. MCR was
269 expressed in NSHQ14, WAB188, and WAB71. NSHQ14 extracts contained the greatest
270 expression of MCR transcripts, with 510.6 CPM at the 50m depth interval. To further evaluate
271 the energy metabolism of putative methanogens in the Samail Ophiolite, the abundance of
272 transcripts affiliated with [NiFe]-hydrogenases was examined. After filtering out transcripts with
273 low normalized expression (<1 CPM), homologs of [NiFe]-hydrogenases common to
274 characterized methanogens were detected primarily in NSHQ14C and NSHQ14B with CPM of

21.7 and 16.1, respectively (Table 2, Fig. 4). No transcription of energy-converting [NiFe]-hydrogenases was detected. Transcripts for formate dehydrogenases (*fdhAB*) were most abundant in NSHQ04 (103.7 CPM) and NSHQ14C (55 CPM). Transcripts for *fdhAB* and for [NiFe]-hydrogenases were not highly expressed relative to MCR in NSHQ14.

The carbon monoxide dehydrogenase (CODH) and carbonic anhydrase (CA) enzymes could generate inorganic carbon from CO or carbonates as another source of CO_2 for H_2-dependent methanogens and autotrophs under carbon limitation in serpentinizing environments. Transcripts affiliated with Ni-containing CODH and Mo-containing CODH homologs were detected throughout the ophiolite waters. NSHQ14C contained the largest CPM of both Ni-CODH and Mo-CODH with 32.8 and 29.7 CPM, respectively. CA transcripts were similarly observed across all well extracts, with the greatest expression at the 85m depth interval (757 CPM) and 50m depth interval (363 CPM) of NSHQ14.

Aligned with the presence of genes encoding the three subunits of particulate methane monooxygenases (*pmoABC*) in metagenomic assemblies from all wells examined, transcripts for *pmoABC* were detected in extracts from all wells with the greatest expression in NSHQ04 (1,650.9 CPM) and NSHQ14C (72.2 CPM). Transcripts for *mxaF* were similarly expressed with 513.1 CPM in NSHQ04 and 18.3 CPM in NSHQ14C. No transcripts of soluble CH_4 monooxygenase subunit genes (*mmoXYZ*) were detected in transcriptomes from any well.

Discussion

298 ***Active Microbial Methanogenesis in Subsurface Waters of the Samail Ophiolite***. The
299 enrichment of *Methanobacterium* in the cDNA fraction relative to the DNA fraction of 16S
300 rRNA in extracts from NSHQ14 suggests this organism is active. This finding agrees with
301 previous studies of planktonic communities from this well showing $^{14}CH_4$ production from ^{14}C-
302 labeled bicarbonate (25) and the detection of *Methanobacterium* affiliated 16S rRNA genes. (7,
303 8). However, cDNA and DNA comparisons can be impacted by differences in sequencing library
304 sizes, affecting the ratio of *Methanobacterium*-affiliated SSU rRNA genes. In addition to SSU
305 rRNA, genes for the biosynthesis of MCR were detected on single contigs assembled from
306 NSHQ14 and WAB188 metagenomes that share close homology to *Methanobacterium* sp. The
307 homology of this MCR to *Methanobacterium* sp. was previously reported by Fones et al. 2019,
308 where the authors found low homolog counts per Mbp assembled for MCR. In this work, these
309 single contigs of *Methanobacterium* MCR have > 2,000 fragments per kilobase of exon per
310 million reads (FPKM) indicating adequate sequencing coverage and *Methanobacterium* as the
311 primary methanogenic strain. Metagenomic sequences can offer evidence of functional
312 capability but not cellular activity as these genes may be from dormant or dead organisms.
313 Microbial methanogenic activity was evidenced by detection of MCR transcripts in NSHQ14.
314 Transcription of *mcr* genes has been correlated with methanogenic activity in other environments
315 and incubation experiments containing *Methanobacterium* (45–47). Collectively, these molecular
316 observations suggest *Methanobacterium* to be an active organism in the hyperalkaline (pH 11.3)
317 waters of NSHQ14 and thus represents an important extension of the pH spectrum (4.5 – 10.2)
318 (48) where methanogenesis is commonly observed.
319

320 SSU rRNA gene sequencing from other deep subsurface ecosystems (e.g., (7, 19, 49–51)) that
321 have geochemical similarity to NSHQ14 and WAB188 suggests *Methanobacterium* is a
322 cosmopolitan organism in other highly reduced, high pH environments. For example, in the pH
323 9.1, H_2- and CH_4-containing waters of a deep fault within the Driefontein Mine of South Africa,
324 *Methanobacterium* co-dominate the microbial community with a sulfate-reducing bacterium
325 (51). SSU rRNA sequences affiliated with members of the family Methanobacteriaceae (of
326 which *Methanobacterium* belongs to) have also been identified in pH 11.5 springs of the Santa
327 Elena ophiolite, yet ANME–1 organisms dominate the archaeal communities in these waters
328 (10). Likewise, 16S rRNA gene and metagenomic data suggest the presence of
329 *Methanobacterium* in hyperalkaline waters (pH 11.8 – 12.3) in the Voltri Massif of Italy;
330 however, subsequent incubation experiments did not confirm methanogenic activity (19). The
331 transcriptional evidence presented herein confirms methanogenic metabolic activity at pH 11.3, a
332 unit above the pH of 10.2 at which methanogenesis is typically observed (48). In the context of
333 previous observations of biological reduction of HCO_3^- to CH_4 in incubations from NSHQ14C
334 (25), these data strongly point to a microbial contribution to CH_4 in waters impacted by
335 serpentinization in the Samail Ophiolite, in particular in hyperalkaline, highly reacted waters
336 with dissolved inorganic carbon levels below detection.
337
338 Genes and transcripts of the energy-converting [NiFe]-hydrogenases Eha/Ehb typical of
339 *Methnobacterium* sp. were not detected. However, genes for another energy-converting [NiFe]-
340 hydrogenase (*mbhJL*) and a subtype of the hydrogenase Mvh (*vhcD)* displayed homology to
341 *Methanobacterium* sp. though these genes are not found in this cytochrome-lacking methanogen
342 (16). This may indicate an annotation mischaracterization for Mbh and Vhc in our study.

343 Interestingly, while [NiFe]-hydrogenase genes were identified in metagenomic assemblies in
344 Samail Ophiolite waters, transcripts corresponding to these genes were not in high abundance.
345 [NiFe]-hydrogenases are requisite for hydrogenotrophic methanogenesis (16) and thus the
346 evidence for their low transcription CPM is surprising. It is possible that this observation is
347 attributable to limitations and/or sequencing biases imposed by the low biomass associated with
348 these samples or that [NiFe]-hydrogenase transcripts are inherently less stable and thus degrade
349 rapidly.
350
351 A subset of methanogens can use the formate dehydrogenase enzyme (encoded by *fdhAB*) to use
352 formate as a methanogenic substrate as an alternative to using hydrogenases to activate H_2 as a
353 source of electrons for methanogenesis (20). Furthermore, Fones et al. (25) found that life in
354 alkaline Samail ophiolite waters is likely carbon limited rather than energy limited, and formate
355 may be a favored carbon source in alkaline waters. The hydrogenotrophic *Methanobacterium* sp.
356 may therefore use formate instead of H_2/CO_2 as a methanogenic substrate under carbon
357 limitation and abundant H_2 in this environment. Transcripts of *fdhAB* had higher CPM than
358 [NiFe]-hydrogenases in hyperalkaline NSHQ14 but were not highly expressed relative to MCR
359 transcripts in this well. This finding suggests formate marginally augments the energy
360 metabolism of methanogens in NSHQ14 and may point to a different and yet to be defined
361 mechanism of generating reductant in methanogens (52).
362
363 Other compounds such as CO or mineral carbonates might be a source of inorganic carbon for
364 autotrophs in serpentinizing environments. Homologs of CODH were detected in NSHQ14
365 metagenomes previously (25), but low abundances of transcripts for these genes in the

366 communities examined indicate that CO is not a predominant source of carbon or energy for the
367 microbial populations. Alternatively, mineral carbonates may provide a source of carbon for
368 autotrophs. Mineral carbonate dissolution in seawater has been correlated to activity of
369 microbial carbonic anhydrase (CA) (53), an enzyme that catalyzes the interconversion of
370 carbonic acid with HCO_3^- and H^+ (54). CA may therefore play a role in liberating inorganic
371 carbon from mineral carbonates to make it bioavailable by shifting the equilibrium dissolution of
372 carbonate toward HCO_3^-. Of the three (α, β, γ) classes of CA, genes for the β and γ types have
373 been identified in strains of the hydrogenotrophic methanogen *Methanobacterium*
374 *thermoautotrophicum* and acetoclastic methanogen *Methanosarcina thermophilia*, respectively
375 (55). In *M. thermoautotrophicum*, where CO_2 is in demand for hydrogenotrophic
376 methanogenesis, the β CA could be used to interconvert HCO_3^- and CO_2 and/or concentrate CO_2
377 near the formylmethanofuran dehydrogenase for the first step of methanogenesis, analogous to β
378 CA and the CO_2-fixing enzyme in photosynthetic organism (56).
379
380 In NSHQ14, the CPM of CA transcripts increased with increasing depth and pH, indicating CA
381 may be generating the carbon needed by autotrophic methanogens as the water becomes more
382 alkaline. Consistent with a potential for CA in mitigating inorganic carbon limitation for
383 autotrophs in serpentinizing systems, homologs of this gene were detected in genomes of
384 *Serpentinomonas* strains isolated from a hyperalkaline serpentinization site in The Cedars,
385 California (57). Indeed, this strain was shown to be capable of autotrophic growth on solid
386 calcium carbonate ($CaCO_3$), H_2, and O_2 (57). Furthermore, Miller et al. (28) observed a
387 *Methanobacterium* strain cultured from the Samail ophiolite to be capable of growth on H_2 and
388 solid $CaCO_3$ as the sole carbon source. Collectively, these observations and CA transcription

389 suggest that dissolution of carbonates may be a source of inorganic carbon for methanogens in
390 high pH, carbon-limited environments such as NSHQ14.
391
392 Alternatively, carbonic anhydrase activity may be driven by microorganisms scavenging
393 bicarbonate introduced into NSHQ14 via limited mixing of Type I and II waters within the
394 aquifer or small amounts of atmospheric CO_2 introduced during sample collection. Zwicker et al.
395 (27) describes this scenario where a mixture of Type I and II fluids with minimal CO_2 is the
396 carbon source for methanogens in the Chimaera ophiolite of Turkey. Furthermore, the isotopic
397 composition of archaeal lipid biomarkers from the C-limited conditions of the Chimaera
398 ophiolite show enrichment in ^{13}C, and the authors conclude that microbially-produced CH_4 may
399 contribute to the abiotic CH_4 pool in that system (27). Similarly, the high H_2 concentrations in
400 NSHQ14 likely make scavenging any available bicarbonate via CA favorable for autotrophs and
401 methanogens.
402
403 ***Methanotrophy in Subsurface Waters of the Samail Ophiolite.*** All well waters contained SSU
404 rRNA gene sequences affiliated with the aerobic bacterial methanotroph *Methylococcus*, with the
405 greatest relative abundance observed in the CH_4-rich groundwaters of NSHQ04. This is
406 consistent with detection of *pmo* genes and their transcripts in NSHQ04 and, to a lesser extent,
407 NSHQ14. Notably, NSHQ04 was sampled shallowly at 6m due to blockage within the well.
408 Waters sampled from this depth might have been infused with atmospheric oxygen to a greater
409 extent than waters from other wells that were sampled much deeper, and this may have led to the
410 increased relative proportions of aerobic methanotrophs relative to methanogens in samples
411 collected in NSHQ04. Consistent with this hypothesis, previous sequencing work on 16S rRNA

412 genes recovered from deeper within this well, prior to the blockage, detected sequences affiliated
413 with *Methanobacterium* (8, 28). Thus, aerobic methanotrophs are components of communities in
414 subsurface waters of Oman; most notably in more oxidizing waters near zones of mixing
415 between aquifer waters and/or the atmosphere.
416
417 In addition to aerobic methanotrophy, DNA sequencing suggests the possibility of anaerobic
418 CH_4 oxidizing organisms in several of the communities sampled from the Samail Ophiolite.
419 Several sequences with homology to the ANME–1 group of Archaea, organisms putatively
420 involved in anaerobic oxidation of CH_4 (AOM) (21), were detected in the hyperalkaline wells
421 WAB71 and NSHQ14. Likewise, *mcrG* sequences with homology to ANME–1 were also
422 detected in WAB71. The lack of a full complement of MCR homologs for putative ANME–1
423 organisms in these wells is likely attributable to their low abundance, as gauged by 16S rRNA
424 gene and transcript sequencing, which likely limited their representation in our metagenomic
425 sequences. Nonetheless, the presence of ANME in serpentinization-impacted waters is consistent
426 with previous reports of the evidence of these guilds in Chimaera, Santa Elena, and Cabeço de
427 Vide serpentinizing environments (10, 18, 27). However, the lack of detected transcripts
428 affiliated with these organisms and their genes and their limited representation in metagenomic
429 sequences, together, suggest that AOM is of minimal importance in the waters in the Samail
430 ophiolite.
431
432 ***A Subsurface CH_4 Cycle Impacted by Microbial Activity.*** Dissolved CH_4 in ophiolite waters is
433 often enriched in ^{13}C and this has been used to suggest that this CH_4 is primarily abiogenic (58,
434 59). However, methanogens can produce CH_4 enriched in ^{13}C under inorganic C limited

435 conditions. For example, a *Methanobacterium* strain isolated from NSHQ04 (pH 10) was shown
436 to generate CH_4 that was markedly enriched in ^{13}C (−28‰ VPDB) when using calcium
437 carbonate mineral (−0.1‰ VPDB) as the sole C source, in particular when cultivated in medium
438 with pH values greater than 9 (28). Previous CH_4 isotopic measurements of NSHQ14 waters
439 reported a $\delta^{13}C$of up to +3‰ VPDB (8) and our study found transcription of carbonic anhydrase
440 specific to this well. The dominance of *Methanobacterium* in our analysis of SSU rRNA genes
441 and MCR transcripts in NSHQ14 (pH 11.3) indicates that methanogenesis is occurring at high
442 environmental pH values in this system and it is possible that these cells are using C liberated
443 from carbonate minerals as a carbon source. The use of carbon liberated from carbonate minerals
444 by methanogens under hyperalkaline, DIC-limited conditions may thus be contributing to the
445 environmental CH_4 pool, yet their contribution may be obscured by the unusual isotopic
446 signatures associated with this environment.
447
448 The opposing process of microbial methanotrophy can impact the C isotopic composition of
449 CH_4 by preferentially using $^{12}CH_4$ leading to ^{13}C enrichment (60). Like the Samail Ophiolite
450 waters, those of the Santa Elena ophiolite host CH_4 that is unusually enriched in ^{13}C and contain
451 methanogenic and methanotrophic microorganisms, including ANME–1 and *Methanobacterium*
452 sp. (10). The detection of CH_4 that is enriched in ^{13}C combined with evidence for potential
453 methanogens/methanotrophs in geographically distinct ophiolites warrants further studies
454 focused on the interplay between organisms involved in CH_4 cycling and their effect on the $\delta^{13}C$
455 of CH_4 in environments influenced by serpentinization.
456

457 Additional work is also needed to better understand potential electron donors that fuel
458 methanogenesis in environments that are impacted by the process of serpentinization. The high
459 concentration of H_2 in some serpentinizing environments has been used to suggest these systems
460 are not only conducive to hosting robust communities of hydrogenotrophic methanogens but may
461 have also been prime environments for the origin of this process (61). This argument was based
462 on the extremely low reduction potentials associated with waters in active serpentinizing
463 systems, a feature that should allow for the facile reduction of low potential ferredoxin (Fd) with
464 H_2. Reduced, low potential Fd is required during the reduction of CO_2 to formylmethanofuran
465 during the first step of autotrophic methanogenesis (62). However, transcripts for [NiFe]-
466 hydrogenases that can catalyze reduction of Fd with H_2 in *Methanobacterium* (group 4 Eha/Ehb
467 or group 3c Mvh) (44)) were detected in low abundance in our analysis of the NSHQ14 RNA
468 pool, pointing toward the potential importance of other electron donors (e.g., formate, CO)
469 capable of fueling methanogenesis in environments impacted by serpentinization.
470
471 The process of serpentinization creates additional challenges for autotrophs, including
472 methanogens. Serpentinization generates waters with high pH and Ca, which leads to low
473 aqueous DIC in systems closed to atmospheric CO_2. Nonetheless, data presented here indicate
474 that autotrophic *Methanobacterium* are active in such conditions, which indirectly shows that
475 they are meeting demands for cytoplasmic CO_2 in a yet to be defined mechanism. Carbonic
476 anhydrase transcripts within NSHQ14 indicate that cells may be capable of interconverting
477 bicarbonate introduced from fluid mixing or liberated from dissolution of carbonate minerals to
478 meet CO_2 demands. However, the source of bicarbonate remains unknown and it is unclear
479 whether carbonate dissolution rates are sufficient to meet this CO_2 demand through equilibration

480 or if cells actively promote dissolution. These possibilities are likely to have an influence on the
481 isotopic composition of CH_4 produced during methanogenesis. Additional physiological studies
482 of these organisms and their mechanisms of acquiring cytoplasmic CO_2 need to be conducted.
483
484 **Materials and Methods:**
485
486 ***Site Description and Geochemical Characterization of Subsurface Waters.*** Five pre-existing
487 water wells drilled in the mantle section of the Samail ophiolite by the Oman Ministry of
488 Regional Municipalities and Water Resources were sampled in February of 2017. Well waters
489 were collected for geochemistry and cellular biomass from borehole NSHQ14 at 50m
490 (NSHQ14B) and 85m (NSHQ14C), and boreholes WAB188, WAB71, and WAB55 using a
491 Grundfos SQ2-85 submersible pump (Grundfos Pumps Corp., Denmark, Netherlands) and a
492 splitting manifold with field-washed Tygon tubing. Borehole NSHQ04 was sampled with a small
493 Typhoon® pump (Proactive Env. Products, Bradenton, FL).
494
495 At each well, the pump, manifold, tubing, and filter housing were field washed by running the
496 pump for 20-30 minutes (approximately ≥ 100L throughput). Well waters were then passed
497 through a 0.22µm polycarbonate filter (MilliporeSigma, Burlington, MA) and collected in 15 ml
498 Falcon™ tubes (Corning Inc., Corning, NY) for analyses of anion and cation concentrations,
499 with the latter acidified with nitric acid (for a solution pH < 2) in the field at the time of
500 collection. Cations and anions were quantified using inductively coupled plasma atomic emission
501 spectroscopy (ICP-AES; Optima 5300, Perkin-Elmer, Fremont, CA) and ion chromatography
502 (IC; ICS-90, Dionex, Sunnyvale, CA), respectively, at the Colorado School of Mines. For DIC

503 analyses, 6 ml aliquots of water were transferred from the sample collection vials (blue butyl-
504 stoppered borosilicate glass) to 12.0 ml helium purged Labco Exetainer® tubes. To convert DIC
505 species to CO_2 for analysis, 0.5 ml of boiled 85% H_3PO_4 were added to the samples while still
506 hot. Standards were made by weighing out $CaCO_3$ in varying amounts to Exetainer® tubes,
507 which were subsequently flushed with He and injected with 6 ml of boiled MilliQ water while
508 still hot. Acidification was performed at the same time and using the same methods for standards
509 and samples. Standards and samples were centrifuged and then mixed on a shaker table for 12-18
510 hours to homogenize and equilibrate CO_2. Headspace CO_2 was then introduced via a Thermo
511 Fisher GasBench II to a Thermo Delta V Plus isotope ratio mass spectrometer for analysis.
512
513 Gas sampling was conducted using the bubble strip method (modified from (63)). Details on
514 bubble strip gas sampling are available at http://dx.doi.org/10.17504/protocols.io.2x5gfq6. Gas
515 concentrations were measured using an SRI 8610C gas chromatograph (GC) with N_2 as the
516 carrier gas. H_2, CO, CH_4, and CO_2 were separated with a 2 m by 1 mm ID micropacked
517 ShinCarbon ST column. Peak intensities were measured concurrently using a thermal
518 conductivity detector (TCD) and a flame ionization detector (FID) and calibrated with standard
519 gas mixes (Supelco Analytical, Bellefonte, PA, USA, accuracy ±2 %). Measurement
520 repeatability expressed as relative standard deviation is 5 % over most of the calibrated range.
521 We define the limit of quantitation as the signal at which the relative standard deviation increases
522 to 20 %.
523
524 ***Biomass Collection, DNA/RNA Extraction, Quantification, and SSU rRNA Gene and***
525 ***Transcript Sequencing***. Biomass was collected onto 0.22µm polycarbonate filters. Once filters

began to clog or appeared to hold particulates, they were removed from the housing and suspended in bead tubes with DNA/RNA Shield Lysis/Stabilization Solution (Zymo Research, Inc., Irvine, CA), which stabilizes nucleic acids at room temperature over several weeks. Samples were shipped to the Colorado School of Mines where cells were lysed by bead beating for a total of 5 minutes with rests (in intervals of 1 min. lysis, 1 min. rest) to cool the sample tubes to prevent RNA degradation. DNA and RNA were extracted in parallel using the Zymo Research Microbiomics Soil/Fecal DNA MiniPrep Extraction kit (Zymo Research, Inc.) following the manufacturer's protocol. DNA was quantified post-extraction by Qubit™ dsDNA HS assay (ThermoFisher Scientific, Waltham, MA). Recovered DNA and RNA were stored at -80ºC.

A portion of the extracted RNA from each sample was converted to complementary DNA (cDNA) via reverse-transcription polymerase chain reaction (RT-PCR) with the qScript™ XLT One-Step RT-PCR Kit (Quanta Biosciences, Beverly, MA). Each 25µl PCR reaction contained One-Step HiFi PCR ToughMix (1x concentration), One-Step RT master mix (1x), 200nM of the forward primer and 200nM of the reverse primer (described below), nuclease-free water, and 10µl of RNA sample template. A reaction in which no One-Step RT master mix was added served as a negative control for the activity of the reverse transcriptase to ensure no extraneous DNA was being amplified. Reactions were run in a thermocycler with the lid pre-heated to 105 ºC, two initial steps of 48 ºC for 20 minutes and 94 ºC for 3 minutes, followed by 30 cycles of 94 ºC for 45 seconds, 50 ºC for 45 seconds, and 68 ºC for 90 seconds, ending with a final extension of 68 ºC for 5 minutes and a hold at 4 ºC until removal from the thermocycler.

549 SSU rRNA genes were amplified from each DNA and cDNA sample via PCR with primers that
550 span the V4 and V5 hypervariable regions of the 16S rRNA to produce gene fragments of ~400
551 bp and ~600 bp for Bacteria/Archaea and Eukarya, respectively. The 515-Y M13 and 926R
552 primer set (modified from (64)) most evenly amplifies this region of SSU rRNA from all three
553 domains of life. The primers and PCR conditions used in this study are described previously
554 (65). Technical replicate reactions for each sample, five extraction negative controls, and three
555 negative PCR controls (no sample added) were amplified as well. Technical replicates were
556 pooled and purified using Kapa Pure Beads (Kapa Biosystems, Wilmington, MA) at a 1.0x ratio
557 of beads to sample volume to retain any fragments ≥ 250bp in length. Barcoding of sequences
558 was carried out on the purified PCR products using a limited 6-cycle PCR (65). Replicate
559 barcode reactions were pooled and purified with Kapa Beads before quantification with the
560 Qubit™ dsDNA HS assay. Final products were pooled in equimolar amounts before being
561 concentrated to a final volume of 80µl on an Ultracel-30K membrane (Millipore Sigma,
562 Billerica, MA) within an Amicon® Ultra 0.5 ml centrifugal filter (Millipore Sigma). Extraction
563 blanks (no sample added) and negative PCR control reactions (no template added) were included
564 in this sequenced pool. The prepared DNA/cDNA library was sequenced on an Illumina MiSeq
565 (Illumina Inc., San Diego, CA) at the Duke Center for Genomic and Computational Biology
566 (https://www.genome.duke.edu) using V2 PE250 chemistry. Sequences produced from this effort
567 are available on the Short Read Archive (NCBI) database under accession PRJNA560313.
568
569 Resultant FASTQ sequence files were demultiplexed and trimmed with Cutadapt (66). Reads
570 were filtered by error rates, amplicon sequence variants (ASVs) were identified, and read pairs
571 merged to construct a sequence table with DADA2 in R (67, 68). Chimeric sequences were

572 removed before taxonomic assignment against the SILVA r138 database (69). The protocol and
573 resultant files for this effort are available at https://github.com/danote/Samail_16S_compilation
574 as "OM17".. The phyloseq and ggplot2 software packages were used in analysis and
575 visualization of the sequence table (70, 71).
576
577 ***Metagenomic and Metatranscriptomic Library Preparation and Sequencing***. Metagenomic and
578 metatranscriptomic libraries were prepared from six DNA samples from the five wells examined:
579 WAB55, WAB188, WAB71, NSHQ04, and NSHQ14, with separate libraries made for two
580 different depths (50 m and 85m) of NSHQ14. Metagenomic library preparation was conducted
581 using the NexteraXT library preparation kit (Illumina Inc.) according to manufacturer's
582 instructions with 1 ng template DNA as input. Metatranscriptomic libraries were generated by
583 first incubating 10µl of RNA templates in a reaction mix of 12.5µl qScript^{TM} XLT One-Step RT-
584 qPCR ToughMix (1x final concentration) (QIAGEN, Beverly MA), 200nM of random hexamer
585 primers, 1µl of 25x qScript XLT One-Step reverse transcriptase (RT), and 1.25µl of nuclease
586 free water. A RT-negative control was run with the same components but an additional 1µl of
587 nuclease-free water was added instead of the RT enzyme. Technical replicate reactions of 25ul
588 were produced for each sample and RT-negative control and pooled after reverse transcription
589 and amplification in separate thermocyclers with pre-heated bonnets. The reactions underwent 20
590 minutes at 48 ºC, 3 minutes at 94 ºC, followed by 30 cycles of 94 ºC for 45 seconds, 50 ºC for 45
591 seconds, and 68 ºC for 90 seconds, ending with a final extension of 5 minutes at 68 ºC and a
592 short 4 ºC hold. Library amplification and fragment size distribution were confirmed on an
593 Agilent 2100 Bioanalyzer with the Agilent DNA 7500 assay (Agilent Technologies, Santa Clara
594 CA) for all libraries. Libraries were then pooled at an equimolar ratio and sequenced on an

595 Illumina HiSeq 2500 using V2 PE250 Rapid Run chemistry at the Duke Center for Genomic and
596 Computational Biology. Metagenomic sequences are available in the MG-RAST database under
597 accession numbers mgm4795805.3 – mgm4795809.3 and mgm4795811.3.
598
599 Raw metagenomic sequence read adapters were removed using PEAT (72) and individual
600 samples were assembled using MEGAHIT (73) with a maximum kmer of 141. This work used
601 the Extreme Science and Engineering Discovery Environment (XSEDE) (74) resource Comet at
602 the San Diego Supercomputer Center through allocation TG-BIO180010. Protein coding regions
603 were identified and annotated with Prokka v1.12 and Prodigal v.2.6.3 with an e-value threshold
604 of 1×10^{-6} (75, 76) and the output translated protein files were also annotated with GHOSTX
605 (77) to search for homologs involved in various methanogenesis and methanotrophic pathways
606 (described in detail below). Protein sequences with homology to those involved in
607 methanogenesis and methanotrophic pathways were subjected to reciprocal BLASTp analysis
608 (78) to check annotation accuracy and homology to known methanogens/methanotrophs.
609 Homology was determined if the query amino acid sequence was >30% identical over >50% of
610 its length, with an e-value below 1×10^{-6}. Homolog counts were normalized by exon length and
611 sequencing depth to fragments per kilobase of exon per million reads (FPKM) to ensure adequate
612 coverage indicating a non-contaminant.
613
614 Metatranscriptomic sequences were trimmed and *de novo* co-assembled into transcripts with
615 Trinity v2.8.6 (79–81). Sequence reads from each sample were aligned to the assembled
616 transcripts with RSEM and counted to generate an expression matrix (82). Coding regions of
617 transcripts were identified with Transdecoder and annotated with Trinotate against the NCBI and

618 SwissProt databases and against the Pfam database using HMMER v3.3 (hmmer.org) using
619 default parameters and an e-value threshold of 1×10^{-6}. (81, 83–85). Top hits were used as
620 transcript identities. Exploratory analyses of transcript expression used R with the "edgeR" and
621 "limma" packages (67, 86, 87). Transcript counts were normalized by the trimmed-mean of M-
622 values method (TMM). Transcripts with less than or equal to 10 counts per million (CPM) were
623 removed and transcript counts were renormalized by the TMM method for comparison of counts
624 across samples (88). Metatranscriptomic sequences are available at the Short Read Archive
625 (NCBI) database under accession PRJNA560313 (SRR11431188 – SRR11431193).
626
627 Metagenome and metatranscriptomes were queried for key genes associated with
628 methanogenesis and methanotrophic pathways including genes for methyl coenzyme M
629 reductases (*mcrABGCD, mcrIIABGCD*) (89), the subunits of [NiFe]-hydrogenases (16), formate
630 dehydrogenase (*fdhAB*) (20), acetate kinase and phosphate acetyltransferase (*ackA, pta*) for
631 acetoclastic methanogenesis (90), methylotrophic methanogenesis genes (*mtaA, mtbB, mtmB,*
632 *mttB, mtsA*) (10, 91), particulate and soluble methane monooxygenases (*pmoABC, mmoXYZ,*
633 respectively) (22, 23), and the large subunit of methanol dehydrogenase (*mxaF*) (24). Transcripts
634 of genes potentially involved in producing alternative inorganic carbon sources for methanogens
635 including carbonic anhydrases (*can, cynT, cah*) (55) and carbon monoxide dehydrogenases
636 (*cooS, cdhAB, coxL, cutL*) (25) were also examined.
637

638 **Data Availability**

639 Unprocessed demultiplexed sequences produced for the SSU rRNA and metatranscriptomic
640 analyses are available at the Short Read Archive (NCBI) database under BioProject accession

641 PRJNA560313 (https://www.ncbi.nlm.nih.gov/sra/PRJNA560313). SSU rRNA sequences are
642 accessions SRR12495563 – SRR12495576 and metatranscriptomic sequences are accessions
643 SRR12816294 – SRR12816299 in the SRA. Metagenomic sequences are available in the MG-
644 RAST database under accession numbers mgm4795805.3 – mgm4795809.3 and mgm4795811.3
645 (https://www.mg-rast.org/linkin.cgi?project=mgp85625).

646

647 **Conflict of Interest:**

648 The authors declare that the research herein was conducted in the absence of any commercial or
649 financial relationships that could act as a potential conflict of interest.

650

651 **Acknowledgements:**

652 The authors thank the Ministry of Regional Municipalities and Water Resources in the Sultanate
653 of Oman (particularly Engineer Said Al-Habsi, Dr. Rashid Al-Abri, Engineer Salim Al-
654 Khanbashi, Abdullah Al-Kasbi and Said Al Mangji), and the Oman 2017 BIO Team for
655 insightful discussion, collaboration, and sample collection. This work was supported by the
656 NASA Astrobiology Institute "Rock-Powered Life" NAI (NNA15BB02A). The funding agency
657 had no role in study design, data collection and interpretation, or the decision to submit the work
658 for publication.

659

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940 Table 1. Geochemical composition of waters sampled from wells that intersect peridotites or that
941 lie at the boundary of peridotites and gabbros in the Samail Ophiolite in 2017. For comparison,
942 data is presented on the geochemical composition of a single sample of rainwater collected from
943 the Samail Ophiolite in 2017. ∑ indicates the value is the sum of all species of an element.
944 Values below the limit of quantification (BLOQ) are indicated and dashes (-) indicate that
945 measurements were not taken. Concentrations of PO_4^{2-} was below the limit of quantification in
946 all samples. The pH of NSHQ04 was taken with a colorimetric indicator strip. Measurement of
947 the pH of WAB188 was not possible in 2017 and previous measurements recorded in 2015 and
948 2016 were 8.7 and 7.6, respectively (7).

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	NSHQ14		WAB71	NSHQ04	WAB55	WAB188	Rain	LOQ
Well type	Peridotite		Peridotite	Contact	Contact	Contact		
Pump depth (m)	50	85	70	5.8	30	78		
pH	11.05	11.28	10.59	10	9.22	NA	-	
Temp. °C	34.4	36.3	-	-	-	-	-	
Eh (mV)	−415	−253	-	-	-	214	-	
H_2 (µM)	32.5	253	0.59	BLOQ	BLOQ	0.99	-	0.05
CH_4 (µM)	53.5	106	14.8	483	0.106	1.83	-	0.015
DIC (mM)	0.05	0.13	0.12	0.04	2.90	3.00	-	0.02
CO (µM)	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	-	0.28
∑ Na (mM)	8.35	10.21	4.95	10.40	4.12	3.49	0.25	$5.85x10^{-3}$
∑ Ca (mM)	3.66	4.34	4.07	7.79	0.05	1.33	0.52	$1.6x10^{-4}$
∑ Mg (mM)	0.01	0.02	BLOQ	0.02	2.75	1.44	0.15	$5.9x10^{-5}$
∑ Mn (µM)	$7.83x10^{-3}$	$3.3x10^{-2}$	BLOQ	0.03	0.02	0.14	$1.0x10^{-4}$	$7.38x10^{-4}$
∑ Al (mM)	BLOQ	$2.0x10^{-3}$	$1.8x10^{-3}$	$2.0x10^{-3}$	BLOQ	BLOQ	$1.0x10^{-3}$	$7.6x10^{-4}$
∑ Fe (mM)	$1.5x10^{-4}$	$1.8x10^{-3}$	$1.6x10^{-4}$	$8.2x10^{-4}$	$2.5x10^{-3}$	$3.8 x10^{-4}$	$3.6x10^{-4}$	$6x10^{-6}$
∑ Si (mM)	$8x10^{-3}$	$6x10^{-3}$	$2.1x10^{-2}$	$3.6x10^{-2}$	$3 x10^{-3}$	0.37	0.08	$4x10^{-4}$
∑ K (mM)	0.20	0.25	0.25	0.29	0.21	$3.9x10^{-2}$	$7.1x10^{-2}$	$8.3x10^{-4}$
NH_4^+ (µM)	14.2	13.0	100.0	55.5	BLOQ	BLOQ	-	1.0
SO_4^{2-} (mM)	0.13	$2x10^{-3}$	0.04	0.68	0.88	1.13	0.17	$1.04x10^{-3}$
NO_3^- (mM)	BLOQ	BLOQ	BLOQ	BLOQ	0.14	0.12	0.26	$1.61x10^{-3}$
Cl^- (mM)	14.28	16.20	11.59	BLOQ	7.24	5.04	0.40	$2.82x10^{-3}$
Br^- (mM)	0.02	$2.5x10^{-2}$	$1.2x10^{-2}$	$2.7 x10^{-2}$	$5 x10^{-3}$	$2 x10^{-3}$	BLOQ	$1.79x10^{-4}$

967 Table 2. Genes targeted to investigate CH_4-cycling metabolisms in the Samail ophiolite.

968 Transcript counts per million reads (CPM) from metatranscriptomes are given for each gene and

969 well.

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Gene Target	Name:	Enzymatic function / Pathway in CH_4 cycling	Reference	CPM NSHQ14C	NSHQ14B	WAB71	NSHQ04	WAB55	WAB188	NoRT
mcrABG, mrtABG	Methyl-coenzyme M reductase I and II subunits	Catalyzes the final step in methanogenesis	Reeve *et al.* 1997	236.3	510.6	0.2	0	0	9.4	0
mvhADG	F_{420}-non-reducing hydrogenase subunits	Cytoplasmic [NiFe]-hydrogenase	Thauer *et al.* 2010	0.5	0	0	4.1	0	0	0
frhABG	F_{420}-reducing hydrogenase subunits			10.1	6.3	0.1	0	0	0.3	0.2
vhcADG	F_{420}-non-reducing hydrogenase vhc subunits			5.2	1.5	0.8	4.4	0	3.4	0
vhuADGU	F_{420}-non-reducing hydrogenase vhu subunits			0	5.1	0	0	0	2.7	0
mbhJL	Energy-converting [NiFe]-hydrogenase mbh subunits	Membrane-associated energy-converting [NiFe]-hydrogenase		6.0	3.2	0	0	0	0	0
ehaNO	Energy-converting [NiFe]-hydrogenase eha subunits			NA	NA	NA	NA	NA	NA	NA
echCE	Energy-converting [NiFe]-hydrogenase ech subunits			NA	NA	NA	NA	NA	NA	NA
ebhMN	Energy-converting [NiFe]-hydrogenase ebh subunits			NA	NA	NA	NA	NA	NA	NA
fdhAB	Formate dehydrogenase subunits	Catalyzes the oxidation of formate to reduce CO_2 to CH_4	Shuber *et al.* 1986	55.0	41.7	6.9	103.7	3.3	24.7	4.5
ackA	Acetate kinase	Acetoclastic methanogenesis	Fournier *et al.* 2008	3.1	1.5	3.0	305.2	7.1	4.4	4.2
pta	Phosphate acetyltransferase			2.7	8.7	14.3	15.8	17.1	18.8	12.7
mtaA	Methylcobamide:CoM methyltransferase	Methylotrophic methanogenesis	Crespo-Medina *et al.* 2017	0	0	0	0	0	5.4	0
mtbB	Dimethylamine methyltransferase			0	0	0	0	0	0	0
mtmB	Monomethylamine methyltransferase			0	0	0	0	0	0	0
mttB	Trimethylamine methyltransferase		Paul *et al.* 2000	0	0	0	14.6	0	1.7	1.8
mtsA	Methylated-thiol-coenzyme M methyltransferase			NA	NA	NA	NA	NA	NA	NA
pmoABC	Particulate CH_4 monoxygenase subunits	Catalyzes the oxidation of CH_4 to methanol	Stainthorpe *et al.* 1990	72.2	37.2	1.8	1650.9	14.2	38.4	0
mmoXYZ	Soluble CH_4 monooxygenase subunits		Csaki *et al.* 2003	NA	NA	NA	NA	NA	NA	NA
mxaF	Methanol dehydrogenase alpha subunit	Catalyzes the oxidation of methanol to formaldehyde	Lau *et al.* 2013	18.3	5.3	5.0	513.1	8.4	6.6	3.3
cynT, cah, can	Carbonic anhydrase	Catalyzes the interconversion of HCO_3^- + H^+ to CO_2 + H_2O	Smith *et al.* 1999	757.3	363.1	16.0	8.1	0.8	12.5	10.6
cdhAB	Ni-containing CO dehydrogenase subunits	NiCODH, catalyzes the reversible oxidation of CO to CO_2	Fones *et al.* 2019	32.8	9.6	0.9	25.2	0	3.0	0
cooS	Ni-containing CO dehydrogenase									
cutSML	Mo-containing CO dehydrogenase subunits	MoCODH, catalyzes the reversible oxidation of CO to CO_2		29.7	16.7	13.1	16.6	7.6	26.8	4.5
coxSML	Mo-containing CO dehydrogenase subunits									

971 Figure 1. Geological map of a portion of the Samail Ophiolite (after Nicolas *et al*., 2000)
972 showing sampling locations in the Wadi Tayin massif (adapted from Nothaft *et al*., 2020) (92).
973
974 Figure 2. (A) Mean relative abundance of the most abundant taxa putatively involved in CH_4
975 production and consumption in the cDNA and DNA of biomass collected from well waters of the
976 Samail Ophiolite. (B) Percentage of the top putative methanogenic and methanotrophic
977 organisms inferred by SSU rRNA sequence abundance of ASVs with homology to
978 *Methanobacterium* (red) and *Methylococcus* (blue). Each point represents a biological replicate.
979
980 Figure 3. (A) : Fragments per kilobase of exon per million reads (FPKM) of key functional genes
981 of interest for CH_4 cycling metabolisms. The methyl-coenzyme M I *(mcrABGCD*), methyl-
982 coenzyme M II (*mrtABGD*), formate dehydrogenase(*fdhAB*), carbonic anhydrase (CA),
983 particulate methane monooxygenase (*pmoABC*), and methanogenic [NiFe]-hydrogenases (*frh,*
984 *mvh, mbh, vhc*) enzymes from assembled metagenomes are shown. Notably, FPKM values are
985 comparable within each sample (shown by color) but not across samples. Wells are ordered by
986 decreasing fluid pH. (B) FPKM of CH_4 cycling genes that are homologous to proteins from
987 *Methanobacterium* sp. (e-value of $< 1 \times 10^{-6}$, > 30% amino acid identity over > 50% of the
988 length).
989
990 Figure 4: Transcript counts per million reads (CPM) for genes of interest in CH_4-cycling
991 normalized by the TMM method. Reads with homology to transcripts for methyl coenzyme M
992 reductase (*mcrABGCD)* and the co-localized tetrahydromethanopterin S-methyltransferase
993 subunits (*mtrACDEH*), particulate CH_4 monooxygenase *(pmoABC)*, formate dehydrogenase

alpha subunit (*fdhA*), and carbonic anhydrase (CA) are shown. A negative control wherein no reverse transcriptase was added to the PCR reaction had no transcripts for any gene within this subset.