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5 **Distinct geochemistries of water-basalt-Fe⁰ reactions in the presence versus absence**
6 **of CO₂-driven microbial methanogenesis**
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26 **Abstract**

27 Microbial CO₂-driven methanogenesis, the biologically mediated conversion of
28 hydrogen and carbon dioxide to methane, is potentially one of the earliest metabolic
29 strategies to appear on Earth and likely originated in ecosystems sustained by water/rock
30 interaction. The potential for methanogens to influence secondary mineralization
31 pathways during water-rock reactions is not well known. Yet geochemical signatures of
32 the presence and activity of life in rock-hosted environments are of great interest in the

search for life and signs of life preserved on Earth and other planets. We designed a laboratory experiment to evaluate microbial growth and secondary mineralization pathways in the presence and absence of microbial methanogenesis. *Methanothermobacter thermoflexus*, a moderately thermophilic methanogen was supported by the continuous production of H₂ from water-basalt-Fe⁰ reactions at 55°C for over one year. As H₂ was converted to CH₄ in culture experiments, the H₂ concentration was maintained at 0.1 – 0.5 μM, orders of magnitude lower than the concentration in comparable abiotic experiments. The pH in active cultures was lower than in abiotic experiments. The concentration of CO_{2(g)}, and thus CO_{2(aq)}, was higher in the active cultures vs. abiotic and inactive culture experiments. These differences in chemistry between the two systems led to the prediction, from geochemical equilibrium models, of distinct Fe-bearing secondary phases stable in the active cultures (carbonate) vs. abiotic and inactive culture experiments (phyllosilicate). The application of synchrotron x-ray absorption spectroscopic techniques enabled the detection of rare and microscale Fe-bearing solid-phase reaction products. A phyllosilicate dominated the Fe-bearing secondary mineralogy of the abiotic and inactive culture experiments. In contrast, the phyllosilicate was not formed in the active cultures. However, a carbonate phase was not robustly detected. Surprisingly, an Fe-bearing pyroxene was detected in some cultures, and formed through an unknown pathway. Thus distinct secondary mineralization occurred in the presence vs. absence of microbial methanogenesis, whether it was directly or indirectly caused by *in-situ* biological activity.

Keywords: water-rock reactions; hydrogen; methanogenesis; mineralization pathway; synchrotron radiation

1. Introduction

The potential for subsurface chemolithoautotrophic life to survive and thrive solely on rock-derived H_2 has implications for our understanding of subsurface life on Earth today, the origin and evolution of life on early Earth, and the potential existence of life on other planets (e.g. Boston et al., 1992; Stevens and McKinley, 1995; Nealson et al., 2005; Hellevang, 2008; Russell et al., 2010; Edwards et al., 2012). In subsurface environments, dissolved or gaseous H_2 derived from high temperature ($>150^\circ\text{C}$) hydration of basalt or peridotite supports microbial ecosystems when transported to low(er) temperature sites colonized by H_2 -utilizing microorganisms such as at submarine hydrothermal vent chimneys (Kelley et al., 2002; Martin et al., 2008; Schrenk et al., 2010; Edwards et al., 2011) or terrestrial springs (e.g. Brazelton et al., 2013; Morrill et al., 2014; Suzuki et al., 2014). Alternatively, H_2 gas may be produced from low temperature ($\leq 100^\circ\text{C}$) water-rock reactions (Stevens and McKinley, 2000; Neubeck et al., 2011, 2014; Mayhew et al., 2013; Okland et al., 2014) and consumed *in situ* by H_2 -utilizing microorganisms (e.g. Stevens and McKinley, 1995). The existence of such *in situ* microbial ecosystems is dependent on the hydrogen generation potential of the host rock at temperatures $\leq 100^\circ\text{C}$, which is currently not well constrained.

In the absence of strong oxidants, the transformation of Fe(II) in primary minerals (e.g. silicates) to Fe(III) in secondary mineral phases (e.g. oxides and layer silicates such as magnetite and clays, respectively) hydrolyzes water and produces H_2 gas (e.g. Moody, 1976; Seyfried et al., 2007; McCollom and Bach, 2009). It has been proposed that H_2 generation and microbial H_2 consumption occurs within deep basaltic aquifers (Stevens and McKinley, 1995), although that proposal has been challenged on the basis of the

79 small and short-lived amounts of H₂ generated in experiments (e.g. Anderson et al.,
80 1998). Nevertheless, it has spurred a broad discussion of potential hydrogen-driven
81 subsurface lithoautotrophic microbial ecosystems (SLiMEs) that use geochemically
82 supplied electron donors (e.g. Chapelle et al., 2002; Takai et al., 2004; Nealson et al.,
83 2005).

84 Methanogenesis, the conversion of hydrogen and carbon dioxide to methane, is
85 considered likely to be one of the earliest metabolisms on Earth (e.g. Woese et al., 1990;
86 Pace, 1997; Reysenbach and Shock, 2002; Martin and Russell, 2007). It is also plausible
87 that the earliest methanogens inhabited, and perhaps originated in, rock-hosted
88 environments such as hydrothermal vent systems (e.g. Pace, 1991; Martin et al., 2008;
89 Russell et al., 2010). Furthermore, it has been postulated that geochemical H₂ production
90 may have occurred on other planets and moons, including Mars, Europa, and Enceladus,
91 and as such may have supported extraterrestrial microbial methanogenesis (e.g. Boston et
92 al., 1992; McCollom, 1999; Ehlmann et al., 2010; Glein et al., 2015). Yet, very few
93 laboratory studies have focused on the affect of methanogenesis on microbial silicate
94 alteration in anoxic environments similar to subsurface systems (e.g. Hausrath et al.,
95 2007). However, studies with aerobic microbes indicate that microorganisms are an
96 important driver of the dissolution of Fe-silicate minerals and the precipitation of
97 secondary phases in water-rock systems (e.g. Kalinowski et al., 2000; Liermann et al.,
98 2000; Santelli et al., 2001; Benzerara et al., 2004; Daughney et al., 2004; Wu et al.,
99 2007). Thus, it is likely that microbial activity may have important effects in anoxic
100 water-rock systems.

Microbial methanogenesis is a sink for H₂ and CO₂ that may commonly influence the secondary mineralogy during water-rock reactions due to metabolic effects on the aqueous geochemistry. Thus, the objectives of this study were twofold, 1) to determine if microbial methanogenesis by a model methanogen (*Methanothermobacter thermoflexus*) could be sustained by the continual evolution of H₂ from water-basalt-Fe⁰ reactions over experimentally long time periods (over one year) via Reactions 1 and 2:

and 2) to determine if *in situ* microbial methanogenesis would measurably affect the chemistry of low temperature, anoxic, water-rock reaction systems and secondary mineralization pathways. We conducted a comprehensive characterization of the evolving aqueous, gaseous, and solid phase chemistry in culture and abiotic experiments. These proof-of-concept experiments utilized Fe⁰, which occurs in serpentinite systems, such as in the Samail Ophiolite, Oman, in the form of native iron alloys (e.g. awaruite (FeNi₃); Frost 1984) where it is highly unstable during late stage water-rock reactions. In our experiments, use of Fe⁰ gave rise to fluids poised at very low oxygen fugacities and produced significant H₂ to enable long term (1+ year) exploration of the effects of microbial methanogenesis on *in situ* H₂ production and secondary mineralogy in a water-rock system.

2. Materials and methods

2.1 Water-rock reaction environment

The constituents of each experiment are given in Table 1. Volcanic glass with a basaltic composition was chosen to represent ocean crust environments and provided all of the elemental components of basalt without the variety of mineral phases thus simplifying and facilitating the detection of any secondary mineral phases. The glassy Hawaiian basalt is composed of (in wt% oxide as determined by electron microprobe analyses) 51.87% SiO₂, 13.57% Al₂O₃, 11.43% CaO, 10.90% FeO, 6.65% MgO, 2.59% TiO₂, 2.37% Na₂O, 0.37% K₂O, and 0.06% Cr₂O₃. Electron microprobe, SEM, and optical microscopy was used to determine that in a subsample of the unreacted basalt (373 grains), 91% of the particles were basalt, approximately 8% were olivine phenocrysts (Fo₈₁), plagioclase and spinel were minor ($\leq 1\%$) phases and pyroxene was not detected. Basalt was ground and dry sieved to 53-425 μm . Fe⁰ (Acros Organics) was dry sieved and only the 1-2mm size fraction was used to maintain highly reducing conditions and produce measurable quantities of H₂ gas over long (1+ year) time periods. Fe(III)-oxides (ferrihydrite; synthesized by base titration of ferric chloride (Ryden et al., 1977)), sulfate, and CO₂ were present to provide several common electron acceptors found in anoxic subsurface environments. All experimental constituents were combined in 70 ml glass serum vials with 35ml of anaerobic, artificial sulfate-free seawater media (Templeton et al., 2005) purged with 80%/20% N₂/CO₂ gas. The vials were capped with an airtight butyl rubber stopper and incubated at 55°C. All components were sterilized

either by heating at 100°C in an oven for >24 hour or autoclaving and were kept sterile prior to assembly.

Control experiments were prepared with a known concentration of H₂ gas and incubated in the same way; these vials were used to test for diffusion of gas across the butyl rubber stopper over similar time scales and sampling frequency.

2.2 Inoculum

A pure culture of *Methanothermobacter thermoflexus* strain IDZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) 7268), a moderately thermophilic methanogenic Archaea with a pH optimum of 7.9-8.2 (Kotelnikova et al., 1993; Wasserfallen et al., 2000), was chosen as the inoculum because it: 1) utilizes H₂ for growth, producing CH_{4(g)} (the concentration of which can be tracked over time); 2) tolerates pH>8 and seawater salinity; and 3) exists as long rods and chains of rods that are visible by light and fluorescence microscopy even when embedded among mineral particles. The pure culture of *M. thermoflexus* was obtained from DSMZ and, in our laboratory, was transferred into fresh media (DSMZ media #141) with an 80%/20% CO₂/H₂ headspace overpressured to 2 atm. After a year of incubation, the 16S rDNA from this culture was extracted, sequenced using primers 21f and 948r, and cloned. The 690 base pairs that were successfully sequenced were compared to the BLAST library and found to be 98% similar to *M. thermoflexus* IDZ as purchased. The reaction vials (35 ml media + rock) were inoculated with an aliquot (0.5 ml) of the culture grown in DSMZ media in our lab.

2.3 Fluorescence Microscopy

Methanothermobacter thermoflexus cultures and abiotic experiments were frequently visualized using a Zeiss Axio Imager Z.1 fluorescence microscope to verify the continued existence of *M. thermoflexus* in the cultures (data not shown). Aliquots (~50 µl) of experiments were aseptically sampled using sterile needles (21 gauge) and syringes. Aliquots were deposited directly onto microscope slides and cells were detected using the autofluorescence of the methanogenic coenzyme F420 with excitation at 390-420 nm and emission at 450 nm.

2.4 Cell Enumeration

The cellular concentration of the pure culture grown in DSMZ media was $\sim 4.8 \times 10^6$ cells/ml. Transfer of 0.5 ml of this pure culture into the reaction vials (35 ml media) resulted in a starting cellular concentration in the culture reaction vials of $\sim 6.8 \times 10^4$ cells/ml. Fluorescence microscopy verified the persistence of living cells over time but accurate direct cell counts could not be conducted because of close cell-mineral associations. Removal of cells from mineral particles for direct cell counting was not attempted due to the common problems of incomplete separation and cell loss that result in inaccurate assessment of cell numbers (maximum yield $\sim 10\%$) (e.g. Riis et al., 1998; Gough and Stahl, 2003).

2.5 Concentrations of gases

Analysis of H₂, CH₄, and CO₂ concentrations in the headspace was performed by gas chromatography (GC). Headspace gases were aseptically extracted using sterile

needles and a gas-tight syringe and replaced with an equal volume of 0.2 μm filter sterilized O_2 -free N_2 gas. For detection of H_2 , the sample was injected into an SRI 310C Gas Chromatograph equipped with an Alltech Molecular Sieve (5A 80/100) 6' x 0.085" ID column and a thermal conductivity detector (TCD) with N_2 as the carrier gas. For detection of CO_2 and CH_4 , the sample was injected into an 8610C SRI Gas Chromatograph equipped with a PORAPAK Q 6' x 0.085" ID column with He as the carrier gas. A flame ionization detector (FID) was used for measurements of CH_4 and a TCD was used to detect CO_2 . Headspace concentrations are converted to aqueous concentrations using the Ideal Gas Law and Henry's Law using coefficients, adjusted for the experimental temperature, from the Sander Compilation (Sander, 1999). Replicate samples from a single experiment were not measured at each time point because of the limited available sample volume.

2.6 Concentrations of aqueous species

Dissolved concentrations of aqueous Fe, Si, Mn, Mg, and Ca were measured using an Inductively Coupled Optical Emission Spectrometer (ICP-OES). Media (1.1 ml) was aseptically removed from the experiments and filtered through a 0.2 μm sterile Millipore Millex filter, acidified with trace metal grade nitric acid (67-70% as HNO_3 , Fisher Scientific; final pH = 2), and diluted 10x to reduce interference in the measurements caused by the high concentration of total dissolved solids (TDS) in the seawater-based media. Spiked matrix blanks were measured to further diminish error associated with the TDS of the media. A minimal amount of media was removed in an effort to minimize the perturbation to the reaction system at each sampling time. Thus,

replicate measurements on a single experiment were not conducted. To further minimize sample loss, and because of the difficulty in measuring pH in-situ in anaerobically sealed vials, acidity was semi-quantitatively assessed using pH paper with a range of 5-10 pH units with a standard error of <0.5. To maintain a constant media volume and to avoid pulling a vacuum in the system, the media removed was replaced with an equal volume of sterile, anaerobic media.

2.7 Synchrotron microprobe μ XRF mapping and μ XANES

2.7.1 Sample preparation

Reacted mineral particles from culture and abiotic experiments were prepared for optical imaging and spatially resolved synchrotron radiation x-ray fluorescence mapping and spectroscopy. For each synchrotron analysis, in an anaerobic chamber, 1 ml of mineral-bearing media was removed aseptically using a 23 gauge needle (we observed a preferential selection of particles <100 μ m and due to size, the mm-sized Fe⁰ particles were not selected) diluted in anaerobic MilliQ water to remove salts and obtain a reasonable particle density, applied directly to a pure quartz microscope slide (ESI Co.) and dried at room temperature overnight. Epoxy (Buehler) was applied to the dried sample and it was removed from the anaerobic chamber and allowed to harden at 55°C. Epoxy above the grain surfaces was removed with a diamond wheel and grains were exposed during dry grinding on silicon carbide sandpaper, polished using 0.25 μ m diamond in ethanol and cleaned with acetone.

238 *2.7.2 Multiple-energy x-ray fluorescence (μ XRF) mapping and spot x-ray absorption*

239 *near edge structure (μ XANES) spectroscopy data collection*

240 Synchrotron-based hard-x-ray microprobe measurements of major element distributions
241 and μ XANES were conducted at BL 2-3 of the Stanford Synchrotron Radiation
242 Lightsource (SSRL) at atmospheric temperature and pressure. Due to the limited
243 availability of synchrotron time, solid phase analyses were conducted on samples
244 collected from each experiment after only a few discrete incubation periods. Minerals
245 were sampled $\sim$ 1 month prior to the scheduled synchrotron time (which was randomly
246 assigned) to allow time for sample preparation and pre-characterization prior to
247 conducting the synchrotron analyses.

248 Data collection occurred with the SPEAR3 accelerator ring containing $\sim$ 150 to
249 300 mA at 3.0 GeV. The focused spot size was either 2x2 or 2.5x2.5 μ m with a pixel
250 step-size of 2 or 2.5 μ m, respectively. Dwell times were either 50 or 100 milliseconds
251 dependent on x-ray flux. Fe K-edge maps were generated in a continuous raster scanning
252 mode using a single-element vortex detector, and all windowed counts for each element
253 extracted from the full x-ray fluorescence spectra were normalized to the intensity of the
254 incident x-ray beam (I_0). Fe K-edge mapping was conducted at 5 or 7 discrete energies
255 (abiotic - 7123, 7126, 7128, 7130, 7133 (+/- 7127, 7129) eV; cultures - 7123, 7127, 7128,
256 7130, 7133 (+/- 7126, 7129) eV) chosen to maximize the differences in normalized
257 intensity between representative μ XANES spectra. Approximately 10-30 μ XANES
258 spectra were collected from each map area to sample the variety of Fe-phases present.
259 Each μ XANES spectrum was collected from $\sim$ 240 eV below the Fe K-edge to 300 eV
260 above the edge. All data reduction was conducted in SIXPACK (Webb, 2005) to generate

deadtime corrected, background subtracted spectra normalized to unit step edge. Maps and spectra were calibrated using the first inflection of an Fe⁰ foil at 7112 eV. The short dwell times for μ XRF mapping (50-100 ms) and μ XANES analyses (8 minutes) minimized potential radiation damage to the sample.

2.7.3 Multiple energy μ XRF maps and μ XANES data analysis

A brief description of the data analysis is given here; see Mayhew et al., 2011 for more detail. Prior to collection of μ XANES data, the XRF maps were dead time corrected and underwent Principal Components Analysis (PCA) using the MicroAnalysis Toolkit (Webb, 2011) to produce maps of the spatial distribution of unique components. These were used to guide the selection of μ XANES locations, to ensure that the μ XANES would represent the diversity of Fe-bearing phases as suggested by the unique components. PCA of the processed XANES spectra was conducted using SIXPACK (Webb, 2005) in order to identify the end-member spectra representative of the entire data set. Fe K-edge maps were fit with representative spectra to produce maps of the spatial distribution of each component throughout the map area. To understand the Fe speciation, the representative XANES spectra were fit using linear combinations of Fe model compounds.

2.7.4 XAS measurements of iron model compounds for use in least squares fitting of unknown spectra

Bulk Fe K-edge XANES were obtained for iron-bearing minerals representing a variety of different mineral groups. Materials were powdered and, if necessary, mixed

with boron nitride to achieve adequate beam transmission. Measurements were performed at SSRL on beam lines 11-2 and 4-1. Fe K-edge XANES spectra were collected from 7,000 to ~8,000 eV using a Si(220) $\phi=0$ monochromator crystal set and a slit size of $\sim 1 \times 10$ mm. The monochromator was detuned 30-50% for harmonic rejection. Two to four scans of each sample were collected either in transmission mode using an ion chamber or fluorescence mode using a Lytle detector and XANES data reduction was conducted as described above. Independent identification of mineral phases was verified by x-ray powder diffraction.

XANES spectra identified as end-member (EM) XANES spectra for any given mapped sample were fit from 7110-7150 eV with linear combinations of Fe model compounds. All fits were done using the 'Cycle Fit' function in SIXPACK (Webb, 2005). All models were fit individually as one-component fits and then the fit cycle was repeated for two-component fits using the best one-component model compound paired with the remaining model compounds. This process is repeated until adding an additional component no longer decreases the R^2 value by 10% or greater. All other XANES spectra were fit internally with the EM spectra to verify that they could be accurately described by the end-members.

2.8 Geochemical modeling

The Geochemist's Workbench (Version 5, RockWare, Inc., Golden, CO) was used to create mineral stability diagrams on the basis of the bulk aqueous and gaseous chemistry at the time of sampling for synchrotron analyses. The thermo.dat

thermodynamic database and Debye-Huckel method (appropriate for solutions with an ionic strength up to 3 molal) were used.

3. Results

3.1 Gas chemistry

Measurements of H₂ and CH₄ gas concentrations were conducted to investigate the effect of *M. thermoflexus* on the extent of H₂ generation from Fe⁰ (Reaction 1), and the concomitant biological conversion of H₂ to CH₄ (Reaction 2).

3.1.1 H₂ production and behavior in the abiotic control experiment

Due to the variation in grain size of Fe⁰ (1.7-2 mm) used in individual abiotic experiments, the concentration of H₂ in the abiotic experiments varied by ~10% between replicates. Therefore, values are only reported for a single representative; the replicates are not averaged. However, all abiotic experiments did show the same trends of increasing concentration of H₂ with time and no CH₄ production. The H₂ concentration in the abiotic experiment increased with increasing incubation time reaching a peak of ~180 μM at 4700 h (6 months) (Figure 1A). To reset the system and to determine if steady H₂ production could be reinitiated, the headspace was flushed with N₂/CO₂ at ~6000 h (Figure 1A). The H₂ concentration was drawn down to ~3 μM during flushing. Subsequently, steady H₂ production was renewed and maintained for the remainder of the experiment. Continuous measurements were ceased after ~10,000 h (14 months), but measurements made after 2 years at 55°C showed that the concentration of H₂ remained

high (Table 2). The CH₄ concentration throughout the duration of the experiment was maintained at low levels (0.01 – 0.03 μM) (Figure 1A, Table 2).

3.1.2 Consumption of H₂ and production of CH₄ by *M. thermoflexus*

Hydrogen gas was generated and allowed to accumulate in the three culture experiments for different lengths of time prior to inoculation with *M. thermoflexus*. Immediately upon inoculation, H₂ is consumed due to the metabolic activity of *M. thermoflexus*. In all three cultures, *M. thermoflexus* consumed H₂ until it reached a stable aqueous concentration of ~0.1 – 0.5 μM. This steady-state concentration was reached at approximately 3500 hours after inoculation in Culture 1, at 1000 hours in Culture 1b and at 2000 hours after inoculation in Culture 2b (Figure 1B-D). This variation is likely because the cultures used as inoculum were in different stages of growth at the time of inoculation of the culture media. When the headspace was flushed the concentration of H_{2(aq)} decreased by an order of magnitude to ~0.03 μM. After flushing, the concentration of H_{2(aq)} rebounded only to the steady-state concentration maintained prior to flushing (~0.1 – 0.5 μM). *M. thermoflexus* maintained this concentration in all three cultures for as long as it was metabolically active (~10,000 hours; 14 months). After approximately 10,000 hours, the cultures were no longer amended with N₂/CO₂ and microbial activity eventually ceased. After this point, the H_{2(aq)} concentration in the cultures was higher than at any other point during the reaction progress (~15 μM in Culture 1b; 168 μM in Culture 1, a value similar to that observed in the abiotic experiment; Table 2) due to the cessation of microbial consumption of hydrogen.

351 In the three culture experiments, the production of CH₄ gas was coupled to the
352 consumption of H₂ gas. The elevated CH₄ concentration observed in Cultures 1 and 1b at
353 the first measurement was likely due to carryover during inoculation (Figure 1B, C).
354 There was no (or little) carryover of methane during inoculation of Culture 2b because
355 the culture used as inoculum had recently been repressurized with H₂/CO₂ at the time
356 Culture 2b was inoculated (Figure 1D). Prior to flushing the headspace, the concentration
357 of CH_{4(aq)} eventually plateaus at ~30 - 50 μM in all three cultures (Figure 1B-D).
358 According to calculations of the Gibbs energy, Reaction 2 remained thermodynamically
359 favorable during the time the concentration of CH_{4(aq)} remained steady. While it was
360 possible that H₂ reduced something other than CO₂, and thus caused the concentration of
361 CH₄ to plateau, we did not evaluate such a process. We did not further investigate the
362 cause of this plateau however flushing the headspace did reinitiate CH₄ production. The
363 time it took for the concentration of CH_{4(aq)} to plateau varied between cultures, taking
364 approximately 2500 hours in Cultures 1 and 2b but only 360 hours in Culture 1b. The
365 increase in the concentration of CH₄ in Culture 2b appears to have been more gradual,
366 perhaps because no CH₄ was transferred with the inoculum. This was in contrast to
367 Culture 1, which exhibited an initial decrease in the concentration of CH_{4(aq)} before
368 increasing. Observable CH₄ production was delayed for ~800 hours (~ 30 days after
369 inoculation) despite a decrease in the H₂ concentration beginning from the first
370 measurement (taken at 264 hours (~8 days) after inoculation). Notably, the concentration
371 of H_{2(aq)} reached the relatively steady background concentration (~0.25 μM) as CH₄
372 production was just beginning in Culture 1 (Figure 1B). Flushing the headspace reduced
373 the concentration of CH_{4(aq)} to <1 μM in all cultures. *M. thermoflexus* continued to be

metabolically active and steady CH₄ production was reinitiated with no delay in all cultures. Methane production persisted in all three cultures at least until the cessation of regular measurements at ~10,000 hours.

3.2 Aqueous geochemistry

3.2.1 pH, DIC, and CO_{2(aq)}

The bulk pH of the abiotic and culture experiments fluctuated between 7-8 and was directly affected by both the pH increase associated with water-Fe⁰ reaction progress and the continual replacement of media with fresh, pH 7 media. The pH of the active culture experiments was slightly lower (~ ½ pH unit) than in the abiotic experiment during the period of sampling, however the coarse scale measurements conducted colorimetrically with pH 5-10 paper likely obscured finer scale fluctuations.

An overall decrease in CO₂ was observed in all experiments until CO₂ was replenished when the headspace was flushed, after which CO₂ again decreased (Figure S1). The concentration of aqueous CO₂ was calculated from the concentration of CO_{2(g)} in the headspace assuming equilibrium between CO_{2(g)} and CO_{2(aq)} (e.g. Zeebe et al., 1999). The speciation of CO_{2(aq)} into HCO₃⁻ and CO₃²⁻ and the total dissolved inorganic carbon (DIC) content of the experiments was calculated from the concentration of CO_{2(aq)} and the fluid pH. The concentration of CO_{2(aq)}, relative to the concentration of the other aqueous species of carbon, in the active culture experiments was higher than in the abiotic experiment presumably the result of slightly lower pH values maintained in these experiments (whereas at higher pH values a greater fraction of carbon is present as

HCO₃⁻). Fluctuations in the carbon content (as represented by the CO_{2(aq)} concentration; Figure S1) were a result of the consumption of CO_{2(aq)} by water-rock reactions, methanogenesis, and possible carbonate mineral precipitation. Given the continuous replenishment of CO₂ when aqueous media gassed with N₂/CO₂ was replaced during sampling, it was not possible to calculate a net CO₂ loss to test for mass balance between CO₂ depletion and CH₄ increase.

3.2.2 Iron

The total concentration of dissolved Fe (Fe_(aq)) is considered to be approximately equal to the total dissolved Fe(II)_(aq) under the experimental geochemical conditions (pH 7-8; Eh ~ -0.4 to -0.2 V). We did not account for sorbed or complexed aqueous Fe. In the abiotic experiment, the concentration of Fe_(aq) first increased to ~900 µM and then decreased to ~50 µM (Figure S2). Flushing the headspace initiated a rise in the concentration to ~480 µM which again decreased to ~80 µM after 2 years (Table 2).

An initial rise in the concentration of Fe_(aq) was not documented in Cultures 1 and 1b prior to decreasing to an average background concentration of ~70 µM that remained steady until the headspace was flushed. The concentration was significantly higher in Culture 1 versus the abiotic experiment (P<0.01 according to Analysis of Variance (ANOVA)). A rise in the concentration of Fe_(aq) was documented in Culture 2b before it decreased to an average background concentration of ~40 µM. As in the abiotic control, flushing the headspace initiated a rise in the concentration in all culture experiments (Figure S2). After flushing, the concentration of Fe_(aq) decreased more gradually. After the cultures were no longer amended with CO₂, and methanogenesis ceased/decreased

(after ~10,000 hours), the concentration of $\text{Fe}_{(\text{aq})}$ in Cultures 1 and 1b (no data for Culture 2b), was at the same level as in the abiotic experiment (~ 80 μM ; Table 2).

3.2.3 Si

Significant trends in the concentration of $\text{Si}_{(\text{aq})}$ were more difficult to discern due to the input of aqueous Si from the dissolution of the borosilicate glass vials used as the reaction vessels (data not shown). However, the concentration of $\text{Si}_{(\text{aq})}$ was higher in the culture experiments than in the abiotic experiment during the first approximately 10,000 h of reaction progress. Two years after inoculation (when *M. thermoflexus* was no longer metabolically active) the concentration of $\text{Si}_{(\text{aq})}$ in the culture experiments decreased to values similar to those in the abiotic experiment (Table 2).

3.3 Solid phase Fe chemistry

The speciation of Fe in the solid phase was investigated in the abiotic and culture experiments at four time points using synchrotron radiation based x-ray absorption spectroscopy. Multiple energy x-ray fluorescence mapping was coupled with micro Fe K-edge x-ray absorption near edge spectroscopy to identify Fe-bearing mineral phases and their distribution at the microscale through representative areas within samples. The secondary phases were intermixed on the microscale and because of the penetration depth of the x-rays many spectra (including the “end-members” of the data sets) represent fluorescence signals from multiple closely associated phases. Therefore, even the end-members of the datasets were often mixtures of phases and were fit by multiple model compounds.

3.3.1 *Fe speciation and distribution in the abiotic experiment*

The abiotic experiment was investigated after 48 hours, 2 months, 10 months, 13 months, and 2 years of incubation. At all time points, it was possible to identify end-member spectra within the XANES data set which represented the experimental starting materials (basalt; Fe(III)-oxides; +/- olivine, which occurs as distinct phenocrysts within the basalt; Figure 2A). The occurrence of secondary phases changed with reaction time (Table 3; Figure 2B). After 48 hours, the earliest time point analyzed, an altered olivine associated with Fe-substituted brucite ($\text{Mg}_x\text{Fe}_{1-x}(\text{OH})_2$), an incipient reaction product, was identified as an end-member (Figure 3A; also see Mayhew et al., 2011). Two months after incubation, an altered olivine associated with a Fe-phyllsilicate was detected but it was not possible to determine the extent and distribution of the phases with this dataset.

After approximately 10 months of incubation, a unique end-member spectrum representative of an Fe-phyllsilicate was identified (Table 3; Figure 3B). While it can be difficult to distinguish between species within a single mineral group using XANES, the phyllsilicate appeared to be more similar to a chlorite or smectite as opposed to a serpentine (Table 3). Micro-XRF maps fit with end-member spectra revealed identifiable particles of the starting materials (basalt, Fe(III)-oxides, olivines) at both 48 h and 10 months of reaction time. However, basalt particles dominate the 48 h data while secondary Fe-phyllsilicates were the dominant phase at 10 months (Figure 3A, B). Similarly, both an altered olivine associated with Fe-substituted brucite and a Fe-phyllsilicate were detected in data collected 3 months later. Due to problems with XRF map data collection, the extent and distribution of these phases could not be assessed for the data collected at 13 months. After approximately 2 years of incubation, it was still

possible to identify an Fe-bearing phyllosilicate spectrum as the fourth end-member (Table 3). The map of Fe speciation and distribution after two years of reaction is very similar to the map after one year of reaction (Figure 3B, C). Distinct particles of basalt and Fe(III)-oxides remain apparent and Fe-bearing talc is pervasive and widely distributed.

3.3.2 Fe speciation and distribution within the culture experiments

Due to limited availability of synchrotron time, it was not possible to investigate all cultures at all time points. Culture 1 was investigated at 2 months, 10 months, 13 months, and 2 years after inoculation. Culture 2b was investigated at 8.5 months after inoculation, and Culture 1b was investigated at 7 months and 20 months after inoculation. Data collected at 20 months and later represent time points when methanogenesis was decreasing or had ceased because the cultures were not amended with CO₂ after 16 months of incubation time.

3.3.2.1 Culture 1

At 2, 10, 13, and 24 months after inoculation, basalt and Fe(III)-oxides were identified as end-member spectra in the Culture 1 experiment. An altered olivine associated with Fe-substituted brucite was the only other phase identified at 2 months and was also detected at 10 months post-inoculation. The altered olivine is the same secondary phase identified in the abiotic experiment at 48 hours (Table 3). At 10 months, a fourth end-member spectrum was identified that was best fit by an Fe-bearing pyroxene (Figure S3) (e.g. (Ca(Fe,Mg)₂Si₂O₆), a phase not detected in earlier abiotic or culture data (Figure 4C; Table 3). XANES fitting of the multiple energy maps with the end-member

spectra revealed that 2 months post-inoculation the majority of material within the mapped area is basalt whereas basalt particles appear less prevalent after 10 months of reaction (Figure 4A, B). The distribution of the unique Fe-bearing pyroxene spectrum within the map area at 10 months was limited to a single large particle and some scattered pixels associated with basalt surfaces. Notably, we did not detect an end-member spectrum dominated by Fe-phyllsilicate such as was identified in the abiotic experiment. However, when data was collected 3 months later, a Fe-phyllsilicate bearing end-member (~80% Fe-phyllsilicate) was detected but its distribution was limited in extent and intensity as compared to the occurrence of Fe-phyllsilicate within the abiotic experiment (compare Figures 4C and 3B). In contrast, at 24 months, an Fe-phyllsilicate was the only alteration phase identified. It was pervasive and occurred as large, areally extensive sheets similar to the occurrence of Fe-phyllsilicates in the abiotic experiment at 10 months and 2 years (compare Figures 4D and 3B, C).

3.3.2.2 *Culture 2b*

Basalt and Fe(III)-oxide end-member spectra were identified in Culture 2b at 8.5 months after inoculation. An altered olivine associated with Fe-substituted brucite and a second, slightly oxidized olivine were also identified as end-member spectra (Table 3). We did not detect either an end-member spectrum dominated by Fe-phyllsilicates, as observed in the abiotic experiment, or an end-member spectrum best fit by an Fe-bearing pyroxene, as observed in Culture 1.

3.3.2.3 *Culture 1b*

In Culture 1b at 7 months after inoculation, basalt and an altered olivine

associated with Fe-substituted brucite were identified (Table 3). Interestingly, an Fe(III)-oxide end-member spectrum was also identified despite the fact that Fe(III)-oxides were not included as an initial reactant in this experiment, however it was not abundant. Similar to Culture 1, end-member spectra dominated by Fe-phyllsilicates or Fe-bearing pyroxene were not observed at this early time point. At 20-months, basalt, altered olivine associated with Fe-substituted brucite, Fe-bearing pyroxene, Fe-bearing phyllsilicate, and Fe-bearing carbonate were detected. Interestingly, this single dataset contained within it the entire variety of Fe-bearing secondary phases observed in earlier data as well as a unique Fe-bearing carbonate (Table 3). The Fe-phyllsilicate is widely distributed while the other secondary phases are limited to only a few distinct particles (Figure S4).

4. Discussion

4.1 Growth of *Methanothermobacter thermoflexus*

The culture experiments described herein were designed to test the long-term effects of microbial methanogenesis on the gaseous, aqueous, and solid phase geochemistry of water-basalt-Fe⁰ reaction systems. Laboratory culturing environments mimicked the production and slow release of H₂ gas from water-rock reactions, a process more relevant to subsurface environments than typical culture experiments over-pressured with H₂ gas. Values of H_{2(aq)} in the abiotic experiment reached ~150 μM (0.15 mmol/kg; Figure 1A) a value lower than those reported for the Lost City hydrothermal vent field (<1-15 mmol/kg) and the Rainbow hydrothermal vent field (16 mmol/kg) which support H₂-fueled microbial communities (Schrenk et al., 2004; Kelley et al.,

2005; Proskurowski, et al., 2006). Thus, the first test in this study was whether a H₂-dependent microorganism such as *M. thermoflexus*, a thermophilic, alkaliphilic, methanogenic *Archaeon* (Kotelnikova et al., 1993), could be sustained over experimentally long time scales by the evolution of hydrogen gas from water-Fe⁰ interaction. *M. thermoflexus* was sustained in the cultures for over 1 year and drew the concentration of H_{2(aq)} down to 0.1-0.5 μM. Quantitative cell counts were not obtained, however microscopic examination of fluid samples did allow for first-order assessments, of any decrease or increase of cell abundance. Fluorescence microscopy revealed an initial increase in cell density during the first ~4 days after inoculation after which time the cell density appeared to remain constant to 10,000 h.

While the cells may not have been multiplying, the metabolic activity of *M. thermoflexus* changed the geochemistry of the reaction system relative to the abiotic experiments. Microbial methanogenesis consumed H₂ gas as it was produced, maintaining H₂ concentrations at levels far below those in comparable abiotic experiments. Methane gas was produced in the culture experiments but was absent in the abiotic experiments. The reinitiation of CH₄ production after the headspace was flushed in all 3 of the culture experiments and the continued production of CH₄ throughout the duration of the experiment (>10,000 h) was further evidence for the persistent metabolic activity of *M. thermoflexus*. Growth of *M. thermoflexus* in these experiments is consistent with the previously observed short-term growth of other methanogens using Fe⁰ as an energy source (e.g. Daniels et al., 1987; Belay and Daniels, 1990; Lorowitz et al., 1992; Karri et al., 2005). These researchers demonstrated growth of methanogens using Fe⁰ as an electron donor but because the goals of the experiments were different than the work

presented here, the previous experiments were conducted on the order of days to weeks and did not characterize the chemistry to the extent presented in the current work.

There was a significant decrease in the concentration of CH₄ in Culture 1 at ~2000 hours (Figure 1). Control experiments to test for diffusion of gas across the butyl rubber stopper over similar time scales and sampling frequency did not result in a significant loss of gas with time. Furthermore, after flushing the headspace of all experiments we observed a steady increase in CH₄ concentration with time and no evidence for CH₄ loss. We were not able to definitively determine the cause of the intermittent decreases in CH₄ concentration.

4.2 Fe(III) reduction by *M. thermoflexus*

Twenty-four hours after inoculation of Culture 1 with *M. thermoflexus*, a distinct color change from red to green, which can commonly indicate a transition from oxidized to reduced Fe, was apparent especially in contrast to the abiotic experiment (Figure S5). This observation coincided with an earlier rise in the concentration of Fe_(aq) in Culture 1 versus the abiotic experiment (Figure S2). Furthermore, the decrease in the concentration of Fe_(aq) was slower in Culture 1 and the steady background concentration higher than in the abiotic experiment, suggesting an additional source of Fe(II) to solution in Culture 1 (Figure S2). Microbially-mediated reduction of Fe(III)-oxides and release of Fe(II) from this solid fraction according to reaction 3, may explain these observations.

We also observed a decrease in the concentration of H₂ from 264 hours, before vigorous CH₄ production begins at ~800 hours and even while the concentration of CH₄ decreased

(Figure 1B), supporting the hypothesis that reducing equivalents were consumed in a process other than CH₄ production.

Fe(III)-oxides were also present in Culture 2b but a color change was never observed. However, further physiological studies in our lab replicated H₂-driven Fe(III) reduction by *M. thermoflexus* under similar geochemical conditions (E. Ellison, unpublished results). Although Fe(III)-reduction by *M. thermoflexus* was unexpected and had not been previously discussed, methanogen-catalyzed Fe(III)-oxide reduction has been demonstrated in many previous studies and has important implications for understanding Fe redox processes and methane production in anoxic environments (e.g. Bond and Lovley, 2002; van Bodegom et al., 2004; Liu et al., 2011).

4.3 Distinct secondary Fe-bearing mineral phases in active cultures vs. abiotic and inactive culture experiments

The precipitation of secondary Fe-bearing mineral phases was comparable in the culture and abiotic experiments at early time points but diverged as the experiments progressed. An altered olivine associated with a Fe-substituted brucite was the first alteration phase detected in the experiments. This phase was not predicted in the mineral stability model calculated using the bulk aqueous geochemistry of the experiments (Figure 5) due to the high bulk SiO_{2(aq)} concentration resulting from the dissolution of basalt. The borosilicate reaction vessels, as indicated from control experiments conducted in the absence of mineral particles (data not shown), also undergo dissolution and contribute to the high SiO_{2(aq)} content of the experiments. Thus, while Fe-substituted brucite was detected in both the abiotic and culture experiments it was limited in

distribution to association with olivine surfaces. After 13 months, this alteration phase was no longer detected in either the abiotic or culture experiments, perhaps due to conversion to a Si-bearing phase (e.g. Kelemen et al., 2011). The dissolution of the reaction vessels is a significant drawback to conducting low temperature mineral alteration experiments in borosilicate vials as is common experimental practice (e.g. Mayhew et al., 2011, 2013; Neubeck et al., 2011, 2014; Okland et al. 2014) and one that we plan to address in future experimental work.

As the reaction progressed, distinct Fe-bearing secondary phases were detected in the active cultures vs. abiotic experiments. An Fe-phyllsilicate was predicted to form in experiments characterized by relatively higher pH and relatively high H_2 concentrations (lower Eh; Figure 5). In fact, an Fe-phyllsilicate was the dominant secondary Fe-bearing phase in the abiotic experiment at 10 months, 13 months, and 2 years, times at which the continued production of H_2 in the absence of a sink led to the accumulation of aqueous H_2 . An Fe-phyllsilicate was detected in Culture 1 at 13 months but was limited in distribution within the map area (Figure 4C). An Fe-phyllsilicate was also detected in Culture 1 after 2 years and in Culture 1b at 20 months of reaction (Table 3) when these experiments had accumulated higher concentrations of H_2 due to the decrease/cessation of microbial methanogenesis after ~10,000 hours (Table 2). A ternary diagram depicting the relative percentages of aqueous CH_4 and H_2 and CO_2 gas for each experiment at the time of sampling for synchrotron investigations illustrates the relationship between $CO_{2(aq)}$ and the existence of a secondary Fe-phyllsilicate phase (Figure 6). Two distinct populations of samples are apparent: samples with relatively high concentrations of H_2 and low concentrations of CO_2 define a field characterized by the presence of Fe-bearing

628 phyllosilicates (blue) while samples with high concentrations of CO₂ and low
629 concentrations of H₂ define a field characterized by the absence of Fe-bearing
630 phyllosilicates (red).

631 The chemical composition of the phyllosilicate is unknown, however if we
632 assume an equal proportion of Mg and Fe and a relatively simple talc mineral formula,
633 such a phase may have precipitated according to Reaction 4.

636
637 An Fe-carbonate was predicted to be stable in experiments characterized by
638 relatively lower pH and lower H₂ concentrations (higher Eh; Figure 5). The abiotic
639 experiment after only 48 hours of reaction, before large amounts of H₂ accumulated, had
640 a similar chemistry to that of the active culture experiments (Figure 6). In the active
641 culture experiments (Culture 1 at 2 and 10 months, Culture 1b at 7 months, and Culture
642 2b at 8.5 months), *M. thermoflexus* held the concentration of H₂ to a minimum steady-
643 state value of 0.1-0.5 μM. Carbonate was detected using QEMSCAN (a technique that
644 combines backscatter electron signal and energy-dispersive x-ray spectra to identify
645 mineral phases) as a small fraction (1%) of the area of the sample from Culture 1 at 10
646 months. Carbonate mineral precipitation may have occurred according to Reaction 5.

However, we were unable to verify the detection of the carbonate phase using synchrotron radiation based spectroscopy, Raman spectroscopy, and XRD which may have been due to the small size ($\sim 5 \mu\text{m}^2$) of the carbonate grains or the sample preparation procedure (washing minerals to inhibit precipitation of salts; see section 2.7) causing dissolution of carbonates. Alternatively, extensive precipitation of carbonate phases may not have occurred due to being kinetically hindered by the energy required for dehydration of the Mg^{2+} ion at low temperatures ($<60\text{-}80^\circ\text{C}$; e.g. Gautier et al., 2014).

An Fe-bearing pyroxene was detected in Culture 1 at 10 months and Culture 1b at 20 months of reaction. A XANES spectrum dominated by Fe-bearing pyroxene was not detected in the abiotic experiment or primary materials. Further, we were not able to find or identify pyroxenes as distinct phenocrysts within a subsample of the unreacted basalt (373 grains analyzed) using electron microprobe, SEM, and optical microscopy. While synchrotron-based spectroscopic techniques facilitated detection and identification of this secondary phase (Figure S3), the detection and identification of this phase was subsequently definitively corroborated by Raman spectroscopy (Figure 7). A single, stand-alone Fe-bearing pyroxene crystal was detected in each culture but the phase was predominantly associated with, and distributed across, basalt particles in one to four pixel groups ($\sim 2.5 - 10 \mu\text{m}^2$ and not analyzable by Raman spectroscopy; Figure 4B), much smaller and spatially localized than the Fe-bearing phyllosilicate in the abiotic experiment that formed sheets up to $50 \mu\text{m}$ across (Figure 3B, C). The size and distribution of the pyroxene was suggestive of precipitation in association with cells attached to basalt surfaces. However, we were unable to replicate this result as Culture 1 was the only active culture in which Fe-bearing pyroxene was detected. The observation

of Fe-bearing pyroxene in Culture 1b was after the cultures were no longer amended with $\text{CO}_{2(g)}$.

Hedenbergite, the Fe end-member of the Fe-Mg clinopyroxene solid solution series, is found in highly reducing conditions, across a range of temperatures, and often in the presence of native metals (Burton et al., 1982; Krot et al., 2000; Hu et al., 2011 and references therein). In meteorites it may precipitate directly from high Fe(II), Ca, and Si bearing fluids at relatively low temperatures ($\sim 150^\circ\text{C}$; Krot et al., 2000). While such conditions are relatively rare on Earth today, they are similar to those in our experiments. Had an Fe-bearing pyroxene precipitated according to Reaction 6, it would have caused a decrease in pH greater than that associated with the precipitation of an Fe-phyllosilicate (see Reaction 4).

There are multiple possible explanations for detection of the Fe-bearing pyroxene: 1) it was present within the starting basalt, but rare, and thus only detected in a subset of experiments, 2) it was present within the starting basalt only as small crystallites, but was made more apparent by bacterially mediated selective leaching and/or dissolution of the amorphous basalt or 3) it precipitated preferentially in Cultures 1 and 1b. While it is not possible to definitively rule out options one and two, it is difficult to invoke low-temperature pyroxene nucleation and precipitation as there is no known mechanism. However, mineral stability models of our experimental system predicted that an Fe-pyroxene would be stable under pH conditions only ~ 1 -2 pH units higher than those

measured in the experiments (Figure 5). Taking into account the pH measurements conducted at the bulk scale, as well as the dynamic nature of the water-Fe⁰ reaction system, it is necessary to consider the possibility that experimental conditions may have been, at least temporarily and/or locally, favorable for the precipitation of a Mg/Fe-pyroxene phase. This possibility is discussed in greater detail in section 4.4.

The occurrence of different secondary mineral phases also resulted in distinct concentrations of aqueous constituents in the active culture vs. abiotic and inactive culture experiments. Experiments in which an Fe-phyllsilicate was detected have concentrations of Si_(aq) < 700 μM while those experiments where Fe-phyllsilicates were absent have concentrations of Si_(aq) > 700 μM (Table 2). The drop in the concentration of Si_(aq) in the culture experiments coinciding with the detection of the Fe-phyllsilicate suggests that the precipitation of the Fe-phyllsilicate was directly responsible for the decrease in Si_(aq) concentration. In fact, twice as much Si is consumed per mole of Fe-phyllsilicate than per mole of Fe-pyroxene precipitated (see reactions 4 and 6). A similar, though not as robust, pattern was observed with the concentrations of Fe_(aq). The concentration of Fe_(aq) was generally higher in the culture experiments at earlier time points when the Fe-bearing phyllsilicate was absent (Table 2; Figure S2). It then decreased to values similar to that in the abiotic experiment coincident with the detection of the Fe-bearing phyllsilicate, again suggesting that precipitation of this phase in part controlled the concentration of Fe_(aq). Similar to Si, more Fe is consumed per mole of Fe-phyllsilicate than per mole of Fe-pyroxene precipitated (see reactions 4 and 6). Thus, there appears to be a set of complex controls on the geochemical parameters that likely

include the activity of the methanogen and the related presence vs. absence of secondary Fe-bearing phases.

4.4 The effect of microbial methanogenesis on the reaction products

Understanding the behavior of H_2 , CO_2 , and pH during continuous *in situ* microbial methanogenesis is important because of the influence of these species on the nature of secondary mineral phases. The concentration of H_2 dictates the redox state (e.g. Frost, 1991) and can also effect mineral precipitation and dissolution. For instance, geochemical models of peridotite-water reactions show that when H_2 is removed from the system, Fe-brucite is predicted to convert to magnetite, forming Fe-poor brucite, and producing H_2 to maintain equilibrium (McCollom and Bach, 2009). Carbon dioxide availability and pH are intimately linked, where an influx of CO_2 buffers the pH of the experimental system despite H^+ consumption during progressive water/rock reactions and, in turn, pH controls the speciation of dissolved inorganic carbon. Furthermore, the stability of different mineral phases is often uniquely tied to pH conditions, and the abundance of CO_2 and HCO_3^- directly influence the occurrence of carbonate mineral phases.

We suspected that *M. thermoflexus* would influence the secondary mineralogy in our water-basalt- Fe^0 system because microbial methanogenesis was an important control on the concentration of H_2 and CO_2 in the experimental system. Specifically, we predicted that microbial methanogenesis, a sink for both H_2 and CO_2 , would result in lower concentrations of H_2 and CO_2 in the culture experiments relative to abiotic experiments. Hydrogen concentrations were lower in the active culture experiments as

741 predicted. However, contrary to our prediction, the concentration of $\text{CO}_{2(\text{aq})}$ was lower in
742 the abiotic and inactive culture experiments than in the active culture experiments
743 presumably the result of a slightly lower pH maintained in the active culture experiments
744 leading to a greater fraction of dissolved inorganic carbon present as $\text{CO}_{2(\text{aq})}$.

745 The pH of fluids in abiotic water-rock reaction systems increases with reaction
746 progress and we hypothesized that this would occur in our culture experiments. However,
747 we did not document an increase in pH in the culture experiments until after ~10,000
748 hours, when microbial methanogenesis had ceased. This correlation suggests that *M.*
749 *thermoflexus* inhibited the expected rise in pH. Methanogens, including species of the
750 genus *Methanothermobacter*, are able to maintain a cell-environment pH gradient
751 through either the release or uptake of H^+ (e.g. through the use of a Na^+/H^+ antiporter)
752 depending on the specific organism and/or environmental conditions (Daniels et al.,
753 1984; Schoneit et al., 1984). While we did not measure a cell-environment pH gradient,
754 such a process has been shown to result in acidification of the bulk culture medium
755 (Schoneit et al., 1984). However, the mechanism in the culture experiments remains
756 undetermined. It is possible that the higher concentrations of $\text{CO}_{2(\text{aq})}$ suppressed Fe-
757 phyllosilicate precipitation (e.g. Tosca et al., 2011) and led to the occurrence of distinct
758 secondary mineral phases in the active culture vs. abiotic and inactive culture
759 experiments.

760 It is also possible that the active presence of *M. thermoflexus* either directly or
761 indirectly resulted in the detection of pyroxene in Culture 1. Fe(III)-hydroxides were
762 included in the initial reactants in Cultures 1 and 2b and, while Fe(III)-(hydr)oxides are
763 common secondary phases in natural serpentinizing systems, it is important to note that

they were not predicted to be stable under the highly reducing conditions of these experiments (Figure 5). We did observe the reduction of Fe(III)-hydroxides in Culture 1. This process consumes protons and produces Fe^{2+} (Reaction 3), thus causing a local increase in pH and $\text{Fe}^{2+}_{(\text{aq})}$, and would have made conditions favorable for precipitation of the Fe-bearing pyroxene, particularly in Culture 1 (Figure 5). It is well established that microbes can create microenvironments on mineral surfaces by limiting the diffusion of ions (e.g. Fe, Mg, Si) away from the mineral surface (e.g. Ullman et al., 1996; Benzerara et al., 2004) and can also act as a reactive surface for nucleation and initiation of mineral precipitation (e.g. Urrutia and Beveridge, 1994; Fortin et al., 1997; Fortin et al., 1998). It may be possible that one such mechanism made conditions favorable for precipitation of an Fe-bearing pyroxene. That the presence and activity of *M. thermoflexus* may have played a role in the precipitation of the Fe-bearing pyroxene is further supported by the absence of the pyroxene in the culture experiment after two years of reaction (when *M. thermoflexus* was no longer active) as evidenced by the accumulation of H_2 to concentrations similar to those in the abiotic experiment.

5. Conclusions

Methanothermobacter thermoflexus was sustained by the constant geochemical evolution of H_2 gas from water-basalt- Fe^0 reactions for over 1 year, during which the metabolic activity of *M. thermoflexus* appears to have changed the geochemistry of the reaction system relative to an abiotic experiment. Lower pH conditions (~0.5 pH unit) were maintained in the active cultures relative to the abiotic and inactive culture experiments. Due to the lower pH maintained in the culture experiments during active

microbial methanogenesis the speciation of dissolved inorganic carbon differed from that in the abiotic and inactive culture experiments.

Thus, the predicted and observed secondary mineral assemblage in the active culture experiments was distinct from that in the abiotic experiment (i.e. an Fe-carbonate versus an Fe-phyllsilicate). However, the predicted carbonate phase was not detected in the active culture experiments; instead an Fe-bearing pyroxene was detected in Culture 1 at one year of reaction. An Fe-phyllsilicate was predicted and observed in the culture experiments when *M. thermoflexus* was no longer actively metabolizing. If preservation of a distinct mineral assemblage that formed coevally with the activity of the microorganisms is possible, it may record and preserve information that would aid in unraveling its origin (e.g. Fe and Si isotopic signatures). While the potential for silicate minerals to be biosignatures is high, the origin of the pyroxene in this study was not definitively determined and it appeared to be transient. Thus, further work is required to understand its relevance as a biosignature and its potential for preservation in the geologic record.

Detection of distinct mineral assemblages representative of active cultures vs. abiotic systems is a promising observation in these proof-of-concept experiments. The demonstration of a microbial effect on the secondary mineral assemblage in this experimental system has important implications for understanding the effect of microbial activity on natural water-rock reaction systems. This work suggests that H₂-utilizing, CO₂-driven methanogenesis can affect the geochemistry of *in situ* secondary mineral assemblages in detectable and identifiable ways. With this knowledge and in combination with previous work on abiotic peridotite-, olivine-, and pyroxene-water reactions at low

temperatures (Mayhew et al., 2013), we are poised to shift future experimental work to investigate the effects of microbial metabolism on the alteration of mafic and ultramafic rocks. In these systems, understanding the potential for the precipitation and detection of biologically-influenced secondary minerals over both short and long time scales (e.g. preservation in the rock record) will have important implications for the potential of these minerals to act as biosignatures and indicators of endolithic activity.

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Figure Legend

Figure 1. Aqueous concentrations of H₂ and CH₄ in abiotic and culture experiments documented through reaction time.

Figure 2. Fe K-edge XANES spectra of **A.)** starting experimental materials and **B.)** secondary Fe-bearing phases in the abiotic and culture experiments. Vertical black lines indicate 7130 eV. Generally, spectra with peaks at lower energies represent Fe(II)-bearing phases and spectra with peaks at higher energies represent Fe(III)-bearing phases. The secondary phases appear more oxidized than the starting reaction materials.

Figure 3. Maps of the distribution of end-member XANES in the abiotic experiment at different time points. In all maps, green represents basalt and red represents Fe(III)-oxides. **A.)** 48 hours of reaction, blue represents olivine + brucite. Scale bar = 40 µm. **B.)** 10 months of reaction, blue represents the Fe-bearing phyllosilicate. Scale bar = 50 µm. **C.)** 2 years of reaction, blue represents the Fe-bearing phyllosilicate. Scale bar = 60 µm.

Figure 4. Maps of the distribution of end-member XANES in Culture 1 at different time points. Green represents basalt and red represents Fe(III)-oxides at all time points. **A.)** 2 months of reaction, blue represents olivine + brucite. Scale bar = 40 µm. **B.)** 10 months of reaction, blue represents a Fe-bearing pyroxene. Scale bar = 30 µm. **C.)** 13 months of reaction, blue represents a Fe-bearing phyllosilicate, grey represents a self-adsorbed magnetite. Scale bar = 50 µm. **D.)** 2 years of reaction, blue represents the Fe-bearing phyllosilicate. Scale bar = 60 µm.

Figure 5. Eh/pH diagram showing the Fe-bearing mineral phases predicted to be stable in the culture and abiotic experiments at time points when the experiments were sampled for synchrotron analyses. The $f\text{CO}_2$ was varied, from the lowest to highest values represented by the experiments, and allowed to speciate across pH and Eh and the only stability field affected was that of the carbonate mineral siderite. The siderite field outlined in gray occurs at lower $f\text{CO}_2$ ($=10^{-1.7}$) and thus is smaller than the field outlined in red representative of higher $f\text{CO}_2$ ($=10^{-0.55}$). Average activities of other key aqueous constituents were used because the average was found to be representative of the range measured at the time points of interest. Black boxes indicate conditions specific to the labeled experiments and the width accounts for the uncertainty in pH measurements (± 0.5). $T=55^\circ\text{C}$; $P=1$ atm; activities $\text{Fe}=10^{-3.88}$ M, $\text{Si}=10^{-3.2}$ M, $\text{Mg}=10^{-1.5}$ M, $\text{Ca}=10^{-1.9}$ M. Values for Na ($=10^{-0.3}$ M) and K ($=10^{-2}$ M) were not measured, the starting concentration in the media was used. Aluminum was included because it is a constituent of basalt and an important component of many phyllosilicate minerals but the activity of Al was not measured so was assumed to be buffered by $\text{Al}(\text{OH})_{3(\text{s})}$ at 10^{-8} M.

Figure 6. Ternary diagram depicting the relative percentages of aqueous CH₄ and H₂ and CO₂ gas for each experiment at the time of sampling for synchrotron investigations. CH₄ and H₂ are scaled to the maximum concentration of CO₂. Two distinct populations of samples characterized by the presence versus absence of Fe-bearing phyllosilicates are apparent.

1106 Figure 7. Raman spectrum of the Fe-bearing pyroxene detected in Culture 1 after 1 year
1107 of reaction overlain with a reference spectrum of augite, a clinopyroxene. The close
1108 match of diagnostic pyroxene peaks between the reference and experimental spectrum is
1109 evidence of a robust pyroxene identification.
1110

Figure 1

Figure 1. Aqueous concentrations of H_2 and CH_4 in abiotic and culture experiments documented through reaction time. Time = 0 hours is the point at which each culture experiment was inoculated.

Figure 2

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Figure 7

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* Lafuente B, Downs R T, Yang H, Stone N (2015) The power of databases: the RRUFF project. In: Highlights in Mineralogical Crystallography, T Armbruster and R M Danisi, eds. Berlin, Germany, W. De Gruyter, pp 1-30

Table 1. Parameters of individual culture and abiotic experiments. (*sulfate free; ¹withheld in order to observe the evolution of the experimental system in the absence of Fe(III)-oxides)

Experiment	Date inoculated	seawater media*	headspace gas	basalt	Fe ⁰	ferrihydrite	sulfate
Abiotic	NA	35 ml	80% N ₂ , 20% CO ₂	1.25 g	0.1 g	0.4 mM	3 mM
Culture 1	4/10/09	35 ml	80% N ₂ , 20% CO ₂	1.25 g	0.1 g	0.4 mM	3 mM
Culture 1b	7/16/09	35 ml	80% N ₂ , 20% CO ₂	1.25 g	0.1 g	None ¹	3 mM
Culture 2b	4/17/09	35 ml	80% N ₂ , 20% CO ₂	1.25 g	0.1 g	0.4 mM	3 mM

Table 2

Table 2. Aqueous concentrations (μM) of key geochemical species in the aqueous phase at the time of sampling for synchrotron analyses.

Experiment/hours*	pH	$\text{H}_{2(\text{aq})}$	$\text{CH}_{4(\text{aq})}$	$\text{CO}_{2(\text{aq})}$ **	Fe	Si	Mg	Ca
<i>Culture 1 - 1584</i>	7	0.53	61.4	4610	278	770	25800	10000
<i>Culture 1b - 5472</i>	7	0.01	0.92	4420	144	720	34400	12000
<i>Culture 2b - 7632</i>	7	0.19	18.7	2660	438	730	34100	12000
<i>Culture 1 - 7800</i>	7	0.40	17.6	2140	241	780	34400	12000
<i>Culture 1 - 9648</i>	7.5	0.19	31.6	1540	151	660	32900	11000
<i>Culture 1b - 15408</i>	7.5	15.2	83.7	743	76.6	470	29500	11000
<i>Culture 1 - 17736</i>	8	168	40.3	848	74.9	550	28900	13000
<i>Abiotic - 48</i>	7	5.58	0.03	4658	101.5	460	36000	12000
<i>Abiotic - 1416</i>	7	92.9	0.01	3300	556	680	26200	11000
<i>Abiotic - 7632</i>	7.5	119	0.01	1110	95.9	540	33200	14000
<i>Abiotic - 9480</i>	7.5	155	0.02	977	66.3	530	32600	13000
<i>Abiotic - 17568</i>	8	244	0.03	1060	79.0	560	28100	9000

* Hours is time of sampling for synchrotron analyses; aqueous and gaseous measurements were made within +/- 48 hours

** this is solely $\text{CO}_{2(\text{aq})}$, not total inorganic carbon

Table 3

Table 3. Non-negative least square fits of Fe model compounds to end-member XANES spectra that represent alteration, and/or unique, phases.

Fe model Compound	Normalized Proportion*	R ²	eV shift
Abiotic control @ 48 hours			
Brucite	0.38	8.6e ⁻³	0.26
olivine	0.62		
Abiotic control @ 2 months (1416 hours)			
Olivine	0.72	2.1e ⁻⁴	0.21
Greenalite	0.28		
Abiotic control @ 10 months (7632 hours)			
Phase 1			
San Carlos Olivine	0.67	4.6e ⁻⁴	0.52
Brucite	0.33		
Phase 2			
Fe-phyllsilicates	1.00	3.7e ⁻⁴	0.23
Abiotic control @ 13 months (9480 hours)			
Phase 1			
Fe-phyllsilicates	1.00	1.1e ⁻⁴	-0.05
Phase 2			
olivine	0.56	1.5e ⁻⁴	0.45
Fe-bearing pyroxene	0.44		
Abiotic control @ 2 years (17568 hours)			
Phase 1			
San Carlos olivine	0.66	1.5e ⁻³	0.48
Fe-bearing pyroxene	0.22		
Ankerite	0.12		
Phase 2			
Fe-phyllsilicates	0.89	1.1e ⁻⁴	0.11
Fe-bearing pyroxene	0.11		
Culture 1 @ 2 months (1584 hours)			
Phase 1			
San Carlos olivine	0.73	6.7e ⁻⁴	0.54
Brucite	0.19		
Ankerite	0.08		
Phase 2			
Brucite	0.44	1.4e ⁻³	0.15
San Carlos olivine	0.34		
Fe-phyllsilicate	0.12		
Hercynite	0.09		
Culture 1 @ 10 months (7800 hours)			
Phase 1			
San Carlos olivine	0.65	4.9e ⁻⁴	0.23
Brucite	0.35		
Phase 2			
Fe-bearing pyroxene	0.59	3.2e ⁻⁴	0.08
Chromite	0.16		
Siderite	0.14		
Basalt	0.11		
Culture 1 @ 13 months (9648 hours)			
Phase 1			

Self-adsorbed magnetite	NA	NA	NA
<i>Phase 2</i>			
Fe-phylosilicates	0.81	9.2e^{-5}	-0.03
olivine	0.11		
chromite	0.08		
<i>Culture 1 @ 2 years (17736 hours)</i>			
Fe-phylosilicates	0.87	9.0e^{-5}	0.07
Magnetite	0.13		
<i>Culture 1b @ 7 months (5472 hours)</i>			
<i>Culture 1b @ 20 months (15408 hours)</i>			
<i>Phase 1</i>			
Fe-phylosilicates	0.74	1.5e^{-3}	0.48
basalt	0.26		
<i>Phase 2</i>			
Fe-bearing pyroxene	0.52	4.8e^{-4}	-0.11
brucite	0.24		
olivine	0.22		
<i>Phase 3</i>			
Fe-bearing carbonate	0.67	2.2e^{-4}	-0.34
Fe-bearing phosphate	0.33		
<i>Phase 4</i>			
Fe-bearing pyroxene	0.53	3.1e^{-3}	0.24
Fe-bearing carbonate	0.28		
olivine	0.19		
<i>Culture 2b @ 8.5 months (7632 hours)</i>			
<i>Phase 1</i>			
olivine	0.65	2.6e^{-4}	0.12
Fe-phylosilicates	0.21		
Fe(III)-oxide	0.14		
<i>Phase 2</i>			
brucite	0.46	1.3e^{-3}	-0.08
olivine	0.30		
hercynite	0.14		
Fe-phylosilicates	0.10		

*Identification of phases that occur close to, or below, ~10% of the Fe fluorescence, is uncertain due to the quantitative detection limit of the technique as determined from empirical mixtures (O'Day et al., 2004; Ostergren et al., 1999).