Habitability and biosignature formation in simulated martian aqueous environments

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Abstract

Water present on early Mars is often assumed to have been habitable. In this study, experiments were performed to investigate the habitability of well-defined putative martian fluids and to identify the accompanying potential formation of biosignatures. Simulated martian environments were developed by combining martian fluid and regolith simulants based on the chemistry of the Rocknest sand shadow at Gale Crater. The simulated chemical environment was inoculated with terrestrial anoxic sediment from the Pyefleet mudflats (United Kingdom). These enrichments were cultured for 28 days and subsequently sub-cultured seven times to ensure that the microbial community was solely grown on the defined, simulated chemistry. The impact of the simulated chemistries on the microbial community was assessed by cell counts and sequencing of 16S rRNA gene profiles. Associated changes to the fluid and precipitate chemistries were established by using ICP-OES, IC, FTIR, and NIR. The fluids were confirmed as habitable, with the enriched microbial community showing a reduction in abundance and diversity over multiple subcultures relating to the selection of specific metabolic groups. The final community comprised sulfate-reducing, acetogenic and other anaerobic and fermentative bacteria. Geochemical characterization and modeling of the simulant and fluid chemistries identified clear differences between the biotic and abiotic

experiments. These differences included the elimination of sulfur owing to the presence of sulfate-reducing bacteria and more general changes in pH associated with actively respiring cells that impacted the mineral assemblages formed. This study confirmed that a system simulating the fluid chemistry of Gale crater could support a microbial community and that variation in chemistries under biotic and abiotic conditions can be used to inform future life detection missions.

Introduction

Liquid water is central to the existence of all currently known terrestrial life. Therefore, the arid surface of modern Mars is a major barrier to habitability (Martín-Torres et al., 2015; Cockell et al., 2016). Yet, there is evidence for much wetter conditions on early Mars (4.1–3.0 Gya) (Fairén et al., 2009). The presence of geomorphological features on the martian surface provides evidence of former large-scale fluvial systems and lacustrine environments. Mineralogical evidence, from clays to salts, is also evidence of long-lived subsurface interactions between rock and groundwater-type water (ranging from cold to warm temperatures). In addition, impacts could have extended the occurrence of fluids well into the Hesperian and provided hot circulating fluids (Marzo et al., 2010; Turner et al., 2016; Carrozzo et al., 2017). Some of the dissolved species in the fluids form veins and/or, on reaching the surface, form evaporites (4.1–3.0 Gya) (Ehlmann and Edwards, 2014; Filiberto and Schwenzer, 2019). Evidence of these environments is found in the stratigraphy of lake beds, such as Gale Crater, an environment being investigated by NASA's Mars Science Laboratory *Curiosity* rover.

Mineralogy and geochemistry of the sediments suggest that Gale Crater was the former presence of a lake with episodic alternating of inflow and drying, as well as subsurface water-rock activity from temperate fluids with circumneutral pH (Grotzinger et al., 2014; Bristow et

al., 2015; Rampe et al., 2017). *In-situ* analyses of the mineralogy and atmosphere of Mars has revealed the presence of all major bio-essential elements and multiple potential electron donors and acceptors necessary for oxidation-reduction (redox) reactions (Grotzinger et al., 2014; Vaniman et al., 2014). Since Gale Crater is a target for current *in-situ* analyses by NASA's Mars Science Laboratory *Curiosity* rover, and future Mars missions are likely to target similar fluvial or lacustrine environments, characterizing microorganisms that can be supported by analogous environments on Earth is fundamentally important for investigating the habitability of these regions.

Based on the predicted temperature, redox, and pH conditions of Noachian Mars, previous studies have suggested intertidal zones as an analogue environment for studying the putative habitability of ancient lacustrine environments (Curtis-Harper et al., 2018). However, whilst analogue environments provide valuable information about hypothesis development, the proxies provided by these terrestrial environments relative to the extra-terrestrial sites to which they are analogous possess multiple issues that limit their applicability (i.e., the impact of high oxygen concentrations on geochemical cycling and the absence of a parallel abiotic environment). Closer approximations of martian chemical environments can be developed through simulation experiments, which replicate specific chemistries, as the geology and chemistry of an environment has been shown to control the diversity of microbes-(Görres et al., 2013; Schwenzer et al., 2016). Simulation of martian chemistries requires the use of appropriate mineralogies (Ramkissoon *et al.*, 2018; Ramkissoon *et al.*, 2019) and fluid chemistries (Ramkissoon *et al.*, 2019; Ramkissoon *et al.*, 2021). Previous simulation studies have used a range of fluids that were either thermochemically modeled or based on the composition of the microbial growth medium, in isolation or in combination with analogue regolith simulants to represent martian mineralogy (Schirmack et al., 2015; Fox-Powell et al.,

2016; Olsson-Francis et al., 2017; Stevens et al., 2019b). These studies have produced mixed results, with some fluid chemistries considered inhibitory or uninhabitable to specific organisms and others showing that only specific organisms could survive (Kral, Bekkum and McKay, 2004; Schirmack, Alawi and Wagner, 2015; Fox-Powell *et al.*, 2016; Schuerger and Nicholson, 2016; Stevens *et al.*, 2019; Stevens *et al.*, 2019).

However, the majority of these experiments used a range of pure microbial cultures, for example, methanogens (Kral et al., 2004; Schirmack et al., 2015)). Performing simulation experiments with individual strains preclude microbes that would be viable within a community-dependent context (e.g., syntrophic or necrotrophic interactions that fulfil specific growth requirements (Seth and Taga, 2014; Cruz-López and Maske, 2016; Chatzigiannidou et al., 2018; Timmers et al., 2018)). A previous community-based simulation experiment approach that used multiple environmental inocula and fluid chemistries identified that only three of eight tested fluids were habitable, which is believed to be a consequence of the ionic strength of the fluids and the presence of specific multivalent ions (Fox-Powell et al., 2016). However, only the initial inoculated fluid was tested with the potential for the environmental material used to inoculate the culture (1 % v/v) having had a significant impact on the fluid chemistry (Fox-Powell et al., 2016). In contrast, when a community-based approach was performed using multiple subculturing steps to investigate the habitability of a former martian aqueous environment, a shift in diversity was observed at each subculture step, with a loss in viability observed after three subcultures (Stevens et al., 2019), which suggests that the persistence of microbes may have been due to nutrients from the inoculum material. These studies, therefore, reflect the importance of considering environmental nutrients when attempting to identify microbes capable of growth solely in simulated fluid chemistries.

Many regolith simulants used in these previous experiments represent a “global” Mars regolith and are based on the chemistry of sites within Gale Crater (Stevens et al., 2018; Cannon et al., 2019). However, there is variation regarding the accuracy of the Fe^{2+}/Fe^{3+} ratio (Ramkissoon et al., 2019a). The most chemically accurate of the developed simulants to date is OUCM-1 (Open University-developed Contemporary Mars regolith 1) (Ramkissoon et al., 2018), which has a maximum of 3 % variation in elemental abundance compared to APXS data collected by Curiosity from Rocknest Sand shadow at Rocknest in Gale Crater (Blake et al., 2013; Ramkissoon et al., 2019a). OUCM-1 is, therefore, representative of a basaltic lithology, similar to other sites characterized across the planet by the Viking, Pathfinder, and MER missions (Clark et al., 1976; Wanke et al., 2001; Conrad, 2014; O’Connell-Cooper et al., 2017). The simulant comprises a combination of minerals, which produces the specific chemistry of Rocknest, as detailed by Ramkissoon et al., (2018). Using thermochemical models, the composition of the fluid chemistries produced from OUCM-1 simulant was used to determine the former martian aqueous environments that may have existed at Rocknest (Ramkissoon et al., 2019b, 2021).

The application of Gibbs energy calculations has previously shown that the disequilibrium generated by the weathering of martian minerals can support microbial life (Jakosky and Shock 1998; Link et al. 2005). Specifically, Gibbs energy calculations suggest that numerous anaerobic metabolisms were feasible (including sulfate reduction, iron reduction, and nitrate-dependant sulfide oxidation) based on the water chemistry from the Rocknest site (Macey et al., 2020; Ramkissoon et al., 2021)(Gellert et al., 2006)). These results support other studies that have proposed the feasibility of a range of anaerobic metabolisms in former martian aqueous environments (Kral et al., 2004; Cousins et al., 2013; Nixon et al., 2013; Price et al.,

2018). The identification of metabolisms that are theoretically viable in these proposed martian aqueous environments furthers the need for experimental testing of the environments.

In the present study, experiments were performed with the intent to validate theoretical work by identifying which microbes, if any, were capable of persistent growth under chemical conditions that simulated, by way of the OUCM-1 regolith simulant and thermochemically modeled fluids, a former aqueous environment that may have existed in Gale Crater during the Noachian. This effort included the study of community dynamics and the relative viability of distinct metabolic groups. Microbial growth would impact the fluid and rock chemistries and lead to changes that could potentially be considered biosignatures

Methodology

Developing the simulated martian groundwater chemistries

Two fluid chemistries (termed converted and modeled) were developed based on the chemistry of the Rocknest shadow sand at Gale Crater (Ramkissoon *et al.*, 2019), as shown in Tables 1 and 2. The converted fluid and simulant were used in the initial stages of the enrichment (the first three stages) to act as an intermediate chemical state to allow the community to adapt to the comparatively nutrient limited modeled fluid and simulant. The chemistry of the converted fluid also served to remove organisms that could not grow in the presence of inhibitory concentrations of elements that might be encountered through the dissolution of silicate material. The converted fluid composition was derived from the dissolution of 100 g of OUCM-1 (Ramkissoon *et al.*, 2019) in 1 kg of water. The modeled fluids, which were chemically closer to proposed martian water chemistries, were prepared by thermochemically modeling the interaction between pure water and the OUCM-1 simulant using the program CHIM-XPT (Ramkissoon et al., 2021). The values used presumed a Water/Rock ratio of 1000, modeled at 25 °C. The fluids were combined with OUCM-1 at varying Water/Rock ratios over the course of the enrichment series to provide the simulated martian groundwater chemistry, which would be expected to evolve over time due to interactions between fluid and the associated regolith (Reed, Spycher and Palandri, 2010; Ramkissoon *et al.*, 2019).

To prepare the fluids, the ions were paired to produce media with the correct elemental composition, as shown in Tables 1-2. The bioessential element hydrogen was supplied in the headspace and consistent for each enrichment stage. The concentration of sulfur (provided as sulfate and sulfide-containing compounds) and carbon was an order of magnitude higher in the converted fluids, and phosphorus was eight orders of magnitude higher relative to the modeled fluids, with CHIM-XPT allocating the phosphorus in the modeled fluids to mineral precipitates.

The converted and modeled fluids were prepared under anaerobic conditions by using an anaerobic chamber (Coy, United Kingdom) with a headspace of $CO_2/H_2/N_2$ (90:5:5). Stock solutions of each component were prepared, autoclaved, and mixed to produce the fluid chemistries stated in Table 1.

Collection of estuarine material and establishing of enrichment regime

The microbial community from the Pyefleet mudflats, Colne Estuary, East Mersea (UK) (51° 48′N, 0° 22′E) was used as an analogue (Mckew et al., 2013). Sediment cores were aseptically collected from the surface of the estuary sediment using 50 cm × 15 cm cores in May 2018. These cores were transported to the Open University and stored at 4 ºC for two days before processing.

Using an anaerobic chamber (Coy, United Kingdom), we diluded 80 g of anaerobic sediment (collected from the bottom of the core, representing anoxic sediment) 1: 3 in the converted fluid and homogenized by stirring. A total of 10 ml of the slurry was used to inoculate (a 1:4 dilution) a 100 ml glass serum vial containing 35 ml of converted fluid and 5 g of OUCM-1 simulant (**Figure 1**). Prior to inoculation, the simulant material and fluids were autoclaved and combined aseptically in a Microbiology Safety Cabinet.

Cultures were incubated at 25 °C, within the range of previously published models that estimate the conditions of the Noachian as warm and wet (Tian et al., 2009; Carter et al., 2015; Ramirez and Craddock, 2018); however, this does introduce a limitation as it does not account for diurnal or seasonal variation. After 28 days, 100 µl of culture was aseptically removed and washed in fresh converted fluid. For this, cells were harvested (centrifugation at 4000 × *g*, for 5 min), washed three times, and re-suspended in 100 µl of converted fluid. A total of 45 ml of

converted fluid containing 5 g of OUCM-1 simulant (stage 2) was inoculated with the washed cells (representing 0.2 % inoculum). For stage 3, this process was repeated with the same fluid. For stage 4, the converted fluid was replaced with the modeled fluid, which was more dilute (see Table 1). For the subsequent stages, the Water/Rock ratio was shifted from 9 to 1, with 100 µl of the enrichment transferred to 10 ml of the modeled fluid and 10 g of OUCM-1 (stage 5). This was repeated two additional times (stages 6-7). The shift in Water/Rock ratios was implemented to select a community capable of growth under limited nutrient availability. All stages of the enrichment were performed in triplicate. The headspace gas (H_2/CO_2 (80:20)) was supplied at 1 bar, and the vials were incubated at 25 °C.

For each enrichment, the total cell numbers were measured after 28 days. The samples were stained with the LIVE/DEAD BacLight bacterial viability kit (Invitrogen) and analyzed with a Leica DMRB microscope equipped with epifluorescence (Leica Microsystem, Bensheim, Germany), as described above. All enumerations were conducted with 50 fields of view counted per sample (Curtis-Harper et al., 2018), and only the live cells were counted. Due to the high Water/Rock ratio in stages 5-7, continual cell counts were not feasible.

Characterization of the microbial community

DNA was extracted by using a modified Griffiths technique (Griffiths et al., 2000) from 5 ml of enrichment ($n=3$). The extraction was modified with three additional bead beating steps (six m/s for thirty secs) and the addition of co-precipitant pink in the precipitation step (Bioline, United Kingdom). Nuclease-free water was processed through the extraction as a negative extraction control. The V4-V5 region of the microbial 16S rRNA gene was amplified by Polymerase Chain Reaction using the universal 16S rRNA gene primers com1 and com2 (CAGCAGCCGCGGTAATAC (Schwieger and Tebbe, 1998) and

CCGTCAATTCCTTTGAGTTT (Stach *et al.*, 2001). The PCR reaction mixture contained (per 25 µL) 1 × PCRBIO Ultra Polymerase red mix (PCR BIOSYSTEMS, United Kingdom), 0.4 µM forward and reverse primers, and 5 ng of DNA. The PCR products were sequenced using the Illumina Miseq Platform by Molecular Research LP (Texas, USA) and processed using a customized pipeline (Dowd et al., 2008a, 2008b). All pair-end sequences were merged, chimeras removed, and sequences less than 150 bp and/or with ambiguous base calls were removed. The sequences were clustered to OTUs at 97% similarity, and phylogeny was assigned by using a curated database from GreenGenes, RDPII and NCBI (DeSantis et al., 2006). Contaminant sequences identified in the negative controls were eliminated from the datasets (Adams et al., 2015). Principal component analysis was performed with the program ClustVis (Metsalu and Vilo, 2015).

Chemical analysis of the simulated martian groundwater chemistries

After 28 days of incubation, 5 ml of enrichment was aseptically removed from the serum vials and filtered (0.2 µm) for chemical analyses. Individual elements in the fluids were measured by Inductively Couple Plasma Optical Emission Spectroscopy (ICP–OES) using an Agilent 5110 model instrument at the Open University (UK) as previously described (Macey et al., 2020). The elemental composition of the sediment used as inoculum was also analyzed by ICP-OES after digestion in hydrofluoric acid for forty-eight hours.

The ionic composition of fluids was measured by Ion Chromatography (IC) using a Dionex ICS3000 with a Dionex AS-DV autosampler. Ions were quantified relative to a dilution series of standards (1 – 1000 ppm). Samples were acidified by using nitric acid (1 % final volume) prior to analysis by IC. The pH of the fluids was measured with an Orion 3-Star Thermo Scientific benchtop pH meter with an uncertainty of 0.01 pH unit at room temperature. To

assess the possible impact of the enriched anaerobic microbial community on the fluid, the significance of changes in pH and fluid chemistry was tested by using a 2-tailed paired Student's *t*-test. The impact of the simulant on the fluid chemistry is extrapolated from the analysis of the simulant and fluid combined and the differences to the input chemical composition of the fluid chemistry.

Chemical analysis of the simulant material

The simulant material (from the biotic and abiotic experiments) was removed from the final enrichment (stage 7) after 28 days, placed in a petri dish, and dried at room temperature in an anaerobic chamber ($CO_2/H_2/N_2$ (90:5:5) headspace) for three days. The simulant material was analyzed by FTIR (Fourier-transform infrared spectroscopy) and NIR (Near-infrared spectroscopy). FTIR spectra were obtained by a Thermo Scientific Nicolet iS5 FTIR Spectrometer and an iD5 Single-Bounce Attenuated Total Reflectance (ATR) attachment equipped with a diamond crystal, housed in the Planetary Spectroscopy Laboratory at Birkbeck, University of London. Reflectance measurements, published as absorbance units, were obtained over a spectral range of 500 to 4000 cm^{-1} at a resolution of 4 cm^{-1}. A diamond calibration standard was used, and atmospheric water (H_2O) and carbon dioxide (CO_2) subtractions were made. No further processing of data (e.g., smoothing or Fourier self-deconvolution) was used to avoid introducing artefacts. Band positions were reported simply as the observed maxima rather than maxima obtained from second derivations or from curve fits. Six analyses were taken of each powdered sample, which was re-homogenized after each spectral acquisition to obtain an accurate spectral profile of the material and its associated organic functional groups. Three NIR reflectance analyses of each sample were performed using a RxSpec® 700Z Spectrometer housed at the University of Westminster, London UK. NIR reflectance spectra were obtained over a range of 0.35 to 2.5 μm.

Results

Characterization of the Microbial Community

Abundance of cells across the enrichment

The abundance of cells from the inoculation to the end of the first stage of the enrichment series increased (8.27×10^7 to 1.25×10^8 cells/ml). Yet, over the entire enrichment series, the number of cells decreased from 1.25×10^8 (stage 1) to 2.10×10^7 cells/ml (stage 7) (**Figure 2**), with a significant decrease following the transition of the enrichment series from the concentrated fluids to the modeled fluid at stage 4 (from 1.05×10^8 to 7.80×10^7 cells/ml) ($P = 0.049$). A significant reduction in cell numbers occurred again with the change in Water/Rock ratio from 9 (45 ml / 5 g) to 1 (10 ml / 10 g) (cell numbers decreased from 7.80×10^7 (stage 4) to 3.60×10^7 cells/ml (stage 5)) ($P = 0.014$). Cell abundance was more consistent within the 1:1 Water/Rock ratio, decreasing at a lower and non-significant amount between subcultures (**Supplementary Table 1**).

Diversity of the microbial community over the enrichment

Microbial diversity was monitored throughout the experiment by using the 16S rRNA gene. Overall, diversity initially increased from T0 (inoculum) to the end of stage 3 but then steadily decreased in subsequent enrichment stages. This shift in diversity is supported by both PCA analysis and a range of ecological statistics, including the Shannon index, Simpson index, Berger-Parker index, and Margalef index (**Supplementary Figures 1, 2** and **Supplementary Table 2**). The initial inoculum was dominated by *Clostridiaceae* (33 %), *Desulfobacteraceae* (12 %), and *Desulfovibrionaceae* (12 %). Multiple families known to be present in the nascent estuarine sediment were not detected in the enrichment from stage 1 onwards (*Nitrosophaeraceae, Spirochaetaceae, Rhodobacteraceae* and *Halothiobacillaceae*). Some bacterial families, such as *Desulfobacteraceae*, were relatively abundant in the initial sediment

community (12 %) but were only present at low relative abundance (< 1 %) during the enrichment series.

The most abundant phyla throughout the enrichment series were Thermodesulfobacteriota and Bacilliota (**Supplementary Figure 3**). Their abundance varied between 17 – 80 % and 12 – 49 % throughout the experiment (**Supplementary Figure 3**). The phylum Euryarchaeota was detected in the initial enrichments with the converted fluid chemistry and increased from 19 % to 39 %, from stages 1 to 3. On transition to the modeled fluid chemistry and reduced Water/Rock ratio (stage 4 and stage 5, respectively), members of the Euryarchaeota phylum were detected at less than 5 % relative abundance (**Figure 3**). The same pattern was observed at the family level, with some families enriched between stages 1 and 3 (*Halobacteriaceae* and *Halanaerobiaceae)* that then declined in relative abundance following the transition to the modeled fluids (stage 4). Following the transition to modeled fluids, *Desulfovibrionaceae* (10 - 32 %), *Desulfomicrobiaceae* (10 - 60 %), *Eubacteriaceae* (5 - 50 %), and *Clostridiaceae* (2 - 6 %) increased in relative abundance at stage 4. The enriched communities at stages 5, 6, and 7 predominantly comprised of sulfate-reducing bacteria (*Desulfovibrio*, *Desulfobulbus*, *Desulfomicrobium*) (20 - 70 %) and acetogenic bacteria (*Acetobacterium*) (21 - 42 %). The endpoint community at stage 7 was specifically dominated by *Eubacteriaceae* (2 - 30 %), *Clostridiaceae* (1 - 25 %), and *Desulfovibrionaceae* (35 - 77 %), with variation in relative abundances of these three families between the replicates. In summary, the abundance and diversity of the microbes decreased over the course of the enrichment. The enriched community was increasingly dominated by sulfate-reducing and acetogenic bacteria after each subculture, and it remained at a stable taxa relative abundance following the transition to the 1:1 Water/Rock ratio.

Geochemical characterization

Changes in fluid chemistry

ICP-OES analysis of the fluid (**Table 3**) was used to measure the shifts in chemistries over the course of the entire enrichment series **(initial fluid chemistries are detailed in Tables 1-2)**. Ca decreased steadily from stages 1 to 3, which indicates that the estuarine-derived nutrients supplied by the inoculum had depleted. There were significant increases in the concentration of Mg (P = 0.010), Ca (P = 0.016), K (P = 0.012), and W (P = 0.020) following the transition from the converted to the modeled fluids (stage 4), increasing further with the transition to the 1:1 Water/Rock ratio (stage 5). These increases in concentration after 28 days of incubation were not expected to accompany the shift in fluid chemistry since these elements were present at lower concentrations in the modeled fluid relative to the converted fluid. The specific changes in chemistry are, therefore, presumed to be related to the dissolution of silicate material.

IC also identified significant differences in fluid chemistry between the biotic and abiotic test groups after 28 days (stage 7), with a higher concentration of K (58-73 ppm abiotic to 442 - 486 ppm biotic) (P = 0.001) and a lower concentration of SO_4^{2-} (1023-1100 ppm abiotic to 140-187 biotic) (P = 0.014) in the biotic test group relative to the abiotic test group (**Supplementary Table 3**). The pH decreased significantly from the starting values for each enrichment stage; pH decreased from 8.1 to 6.6-6.9 for the converted fluids (P = 0.0410) and from 9.2 to 7.9-7.2 for the modeled fluids (P = 0.0145). In stage 7, the pH of the fluid chemistry also decreased significantly in the abiotic test group from the starting pH over 28 days (pH 8.5-8.7) (P = 0.009).

Changes in simulant chemistry

The simulant material from the final enrichment (stage 7) was analyzed using FTIR and NIR (**Supplementary Figures 4 and 5**). Exclusively in the biotic sample, minor peaks located at 1360 cm^{-1}, 1454 cm^{-1}, and 1520 cm^{-1} were observed superimposed on top of the 1460 cm^{-1} C-O carbonate band. These could be assigned to N-H bending or C-N stretching, symmetric bending of CH_2 or asymmetric bending of CH_3, and CH_3 asymmetric bending vibrations, respectively. NIR detected a ferric absorption edge at 0.8 µm, which implies the presence of secondary nanophase oxides and/or crystalline ferric oxides. In the biotic spectra, a broad absorption centered at 6 µm is probably due to electronic iron transition. In summary, the IC, FTIR, and NIR analysis techniques of the fluids or simulant material revealed the presence of changes that were exclusive to the biotic test group.

Discussion

The habitability of a simulated martian chemical environment was investigated by enriching for a microbial consortium capable of growth solely on the chemistry of simulant and associated fluids. This study characterized the community dynamics and relative viabilities of distinct taxonomic groups associated with specific metabolisms that were feasible within this simulated environment. The enrichment regime was performed by using two fluid chemistries, a converted fluid and a modelled fluid chemistry (Ramkissoon et al., 2021). By diluting the estuarine nutrients of the subcultures in the enrichment series, we attempted to ensure that the community's nutritional requirements were met by either the chemical environment or other community members (e.g., exuded organic carbon (Christie-Oleza et al., 2017)). The two fluid chemistries used in this enrichment series were chosen to transition the community gradually from the nutrients supplied by the initial environmental material to the comparatively nutrient-limited modeled fluid and simulant. Twenty eight days was selected as the incubation time between transfers to attempt to prevent cross feeding organisms dominating the enrichment that were not directly surviving on the supplied chemical environment (Wright et al., 2019).

Shifts in microbial diversity over the enrichment series

The final stage of the enrichment series was diverse and contained microbes capable of a range of different metabolisms. The community was dominated by sulfate reducing bacteria, acetogens, and other generalist, anaerobic, and fermentative bacteria. The dominant sulfate reducers varied over the course of the enrichment series, with *Desulfovibrio, Desulfobulbaceae*, and *Desulfomicrobiaceae* persisting in stages 6 and 7 of the modeled fluids and regolith simulant, and *Desulfosporomusa* and *Desulfovibrio* the most relatively abundant sulfate reducing bacteria in the converted fluids and regolith simulant (stages 1 to 3). Many species of sulfate reducing bacteria are metabolically diverse and capable of both autotrophic

and heterotrophic growth – this includes members of the genera *Desulfovibrio*, *Desulfobacterium*, *Desulfosporomusa*, and *Desulfomicrobium* (Sass et al., 2004; Plugge et al., 2011; Sánchez-Andrea et al., 2020) (**Supplementary Table 4**). As a by-product of autotrophic growth, these genera members produce hydrogen sulfide, which can result in the formation of sulfide precipitates with metal ions in solution (Sheoran et al., 2010). Certain sulfate reducers are also capable of iron reduction (including *Desulfovibrio* (Lovley et al., 1993; Li et al., 2006) and *Desulfosporomusa* (Sass *et al.*, 2004)), which enhances the formation of siderite ($FeCO_3$)(Coleman et al., 1993). Gibbs free energy calculations have shown that iron reduction and sulfate reduction are both thermodynamically viable in martian fluid chemistries modeled from the geology of Gale Crater (Macey et al., 2020). Given the presence of microbes capable of both metabolic processes, these results suggest the importance of considering phenotypic plasticity with regard to viability in simulation experiments, with microbes that are capable of multiple reduction-oxidation reactions able to exploit the available electron donors and acceptors (Comte et al., 2013; Abinandan et al., 2020; Kundu et al., 2020).

Sulfate reducing bacteria can also excrete organic compounds when grown autotrophically (Londry and Des Marais, 2003). In addition to the low levels of organic carbon present within the regolith simulant (0.23 w.t. %) (Ramkissoon et al., 2019a) and supplied in the modeled fluid through the addition of ammonium acetate as one of the key components (0.01 mM), there is also the potential for the sulfate-reducing bacteria to provide additional organic carbon to the microbial community, supporting heterotrophic growth of other microbes (Frank et al., 2013). Heterotrophy is a valid metabolic strategy with regard to putative viability under martian chemical conditions, as organic carbon at Gale Crater has been predicted to be between 800 and 2,400 ppm, and this has been argued to be sufficient to support a chemoorganoheterotrophic community of 10^5 cells/g of sediment (Sutter et al., 2016).

Furthermore, simulation experiments that test individual microbial strains (Bauermeister et al., 2014; Billi et al., 2019; Serrano et al., 2019), precluding the viability of organisms with dependencies on synergetic co-feeding (Timmers et al., 2018; Park et al., 2019). Non-autotrophic members of the community are important for considering the chemistry of the simulated environment, as the production of organic acids and excess protons during heterotrophic or fermentative growth will further lower the pH and affect the dissolution of the silicate material (Hiebert and Bennett, 1992; Bennett et al., 2001). During the experiment, the variations observed between the replicates and between the stages of the enrichment is potentially a result of the succession of cross-feeding bacteria, which results in the reduction in abundance of the other members of the community (Wright et al., 2019). Variation will also have been introduced as a result of heterogeneity in the inoculum and by stochastic differences between the populations (Antwis et al., 2017).

The change in chemistry following the transition to the modeled fluids may explain the reduction in diversity between stages 1 and 7. The dilution of nutrients from the estuarine material may have impacted the diversity, with many metal ions being fundamental for microbial metabolism (Abbas and Edwards, 1990). Conversely, it is possible that the increase in concentration of specific elements resulted in toxicity (i.e., higher concentrations of copper and aluminium (Flemming and Trevors, 1989)). The enrichment regime will have played a role in the reduction of diversity, with the length of each stage selecting against slower growing organisms with thermodynamically viable metabolisms and, therefore, excluding them from the enriched community (Ramkissoon *et al.*, 2019) (e.g., anaerobic ammonium oxidizing archaea (Lehtovirta-Morley, 2018)). Decrease in diversity and abundance through the transition may also be a result of the elimination of estuarine derived elements or enhanced competition for bioessential elements. Based on the continual reduction in taxonomic diversity

observed over the seven stages, coupled with the steady number of cells observed from stage 5-7, it is possible that a reduction in diversity may have continued with subsequent subculturing, with taxa putatively identified as "cross feeding" potentially being eliminated. However, as the aim of the experiment was to establish whether microbes could survive under this defined chemical environment and identify which microbes, this question is answered by the enrichment performed at this stage, and stage seven is therefore considered as the endpoint of the experiment.

Martian chemistry and the identification of biosignatures

Changes to the simulant and fluid chemistry because of the enriched microbial community could have utility as chemical biosignatures. In the present study, we screened for changes in fluid chemistry and simulant material exclusive to the biotic test group, with these differences potentially resulting from the enriched microbial community. FTIR and NIR spectroscopy have previously been used in remote sensing payloads (Bibring et al., 2005), and the detection of microscopic biological structures (Preston et al., 2015 and references therein) confirmed the occurrence of changes in chemistry specific to the biotic test group. The higher concentration of K observed in the biotic test group could be due to enhanced dissolution of simulant material, whereas the significantly reduced concentration of sulfate (below the supplied chemistry of the fluid) could be due to the production of hydrogen sulfide. Hydrogen sulfide is an ambiguous biosignature, however, as there are biotic and abiotic production mechanisms (Brimblecombe, 2003). Therefore, the shifts in chemistry detected when using the geochemical techniques (FTIR, NIR, ICP-OES, IC) are ambiguous biosignatures. However, they could potentially contribute toward a collective set of evidence to indicate the presence of a biogenic signal that could then be studied by further analysis to discount contamination and abiotic origins, in line with the progressive scale of evidence proposed by Green *et al.*, 2021. This, and the persistent

lack of a single conclusive biosignature identified by other simulation studies (Stevens et al., 2019b) is important as guidance for the current sampling activities by the Perseverance rover and for planning future life detection missions.

Conclusion

The simulated chemical conditions provided by the martian simulant OUCM-1 and the modeled fluid constitute a habitable chemical environment for multiple anaerobic organisms with distinct metabolic pathways. The continuous subculturing of an anaerobic community from a terrestrial environmental inoculum on the defined martian chemical environment resulted in the reduction in diversity, producing an endpoint community that comprised sulfate-reducing bacteria, acetogenic bacteria, and other generalists, anaerobic bacteria capable of growth across multiple sub-culturing steps. The combination of FTIR, VIS-NIR, and IC allowed for identification of chemical changes that occurred exclusively in the biotic test group, and these could be considered potential biosignatures. However, the extent to which these changes in chemistry could be identified *in-situ* by future life detection missions is also unclear, given the consortium of equipment that would be required to identify a collective set of possible biosignatures.

Contributions

Conceptualization, MCM, NKR, SPS, VKP, SPS.; methodology and investigation, MCM, NKR, LP, MTR, BPS, EKS, SC; data curation, MCM, LP, NKR and SC.; writing—original draft preparation, all authors.; writing—review and editing, all authors.; All authors have read and agreed to the published version of the manuscript.

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Author Disclosure Statement

No competing financial interests exist.

Data Availability

Amplicon sequence data generated in this study were deposited to sequence read archives (SRA).

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Table 1. Molar concentration of compounds in the thermochemically modelled and simulant derived (converted) martian fluid chemistries

	Molar concentration (M)	
	Modelled fluid	Converted fluid
$NaHSiO_3$	9.87E-04	0
$C_2H_7NO_2$	1.11E-05	0
NaHS	5.92E-06	8.65E-05
NaCl	1.18E-03	0
$AlKSO_4$	8.92E-11	0
$MgSO_4.7H_2O$	2.48E-09	0
$FeSO_4.7H_2O$	2.59E-09	0
K_2HPO_4	3.68E-13	3.97E-05
$MnSO_4.H_2O$	7.40E-08	1.90E-05
KOH	2.30E-04	1.74E-04
$CaCl_2$	7.23E-04	0
$CaSO_4$	8.50E-05	0
$Ca(OH)_2$	1.61E-04	1.38E-03
$(NH_4)_2SO_4$	0	5.00E-04
$Fe(III)SO_4$	0	1.10E-04
$MnCl_2$	0	2.94E-05
NaOH	0	3.69E-04
FeO	0	2.62E-03
$3Al_2O_3.2SiO_2$	0	9.89E-04
$3MgO.4SiO_2$	0	1.52E-03
SiO_2	0	4.67E-03
TiOH	0	9.23E-05

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Table 2. Molar concentration of ions in the thermochemically modelled and simulant derived (converted) martian fluid chemistries

	Molar concentration (M)	
	Modelled (Ramkissoon et al., 2018)	Converted
Cl^-	1.91E-03	2.94E-05
SO_4^{2-}	8.51E-05	4.08E-04
HS^-	5.92E-06	5.80E-05
SiO_2	9.87E-04	7.35E-03
Al^{3+}	8.92E-11	9.89E-04
Ca^{2+}	6.08E-04	1.38E-03
Mg^{2+}	2.48E-09	1.52E-03
Fe^{2+}	2.59E-09	2.62E-03
K^+	2.30E-04	2.53E-04
Na^+	2.16E-03	5.42E-04
Mn^{2+}	7.40E-08	3.38E-05
HPO_4^{2-}	3.68E-13	3.97E-05
$Ti(OH)_4$	1.89E-04	0
NH_4	1.11E-05	5.00E-04
Fe^{3+}	0	1.10E-04

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Table 3. Concentration of elements in the inoculum material and the fluid chemistries in combination with OUCM-1 simulant and inoculum in the biotic test group at the end of each stage. Concentrations are in M.

Stage	Chemistry	Liquid(ml) :simulant(g)	pH	Al	Ba	Ca	Cu	Fe	K	Mg	Mn	Mo	Na	S	Si	Sn	Sr	W
0	**Slurry**	**5 g slurry**	7.2	1.24E-05	9.77E-06	5.73E-03	2.04E-06	3.84E-03	3.84E-03	2.85E-03	3.48E-05	3.28E-07	2.76E-03	9.55E-04	0	1.68E-06	1.18E-05	1.34E-07
1	**Converted**	**45 g 5 ml**	6.91	0	2.63E-07	7.25E-03	0	3.39E-04	1.38E-04	1.20E-04	2.80E-05	0	7.40E-04	0	3.10E-03	6.07E-05	4.79E-07	7.07E-06
2	**Converted**	**45 g 5 ml**	6.74	0	1.46E-07	4.25E-03	0	1.50E-05	1.36E-04	1.02E-04	2.65E-05	0	6.09E-04	0	3.42E-03	3.96E-05	3.88E-07	2.72E-06
3	**Converted**	**45 g 5 ml**	6.6	0	2.63E-07	3.25E-03	0	3.93E-04	1.41E-04	1.28E-04	2.82E-05	0	7.83E-04	0	3.20E-03	9.27E-05	4.91E-07	1.09E-05
4	**Modelled**	**45 g 5 ml**	8	0	7.30E-12	1.68E-02	5.51E-06	9.64E-04	1.05E-03	2.63E-03	1.44E-05	3.34E-06	2.74E-03	1.12E-05	2.28E-03	1.43E-04	1.37E-11	3.26E-05
5	**Modelled**	**10 ml 10 g**	7.3	2.30E-03	2.19E-11	6.18E-02	2.68E-05	5.00E-04	3.33E-03	1.28E-02	1.69E-05	2.61E-05	6.09E-03	3.06E-05	1.96E-03	1.77E-04	5.14E-11	1.52E-04
6	**Modelled**	**10 ml 10 g**	7.2	1.65E-02	1.46E-11	3.13E-02	3.46E-04	4.64E-04	3.08E-03	1.81E-02	6.73E-06	1.02E-03	6.09E-03	7.17E-03	1.92E-03	1.43E-04	3.42E-11	1.36E-04
7	**Modelled**	**10 ml 10 g**	7	2.59E-03	1.77E-06	7.00E-03	5.35E-05	0	1.17E-02	3.78E-03	4.91E-07	6.15E-05	4.18E-03	2.37E-03	1.78E-03	2.78E-04	9.13E-06	5.98E-04

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Supplementary Table 1. Abundance of cells (cells/ml) at the start and end of each enrichment stage

	Stage 1 start	Stage 1 end	Stage 2 start	Stage 2 end	Stage 3 start	Stage 3 end	Stage 4 start	Stage 4 end	Stage 5 start	Stage 5 end	Stage 6 start	Stage 6 end	Stage 7 start	Stage 7 end
Replicate 1	1.09E+05	1.10E+08	1.32E+06	9.74E+07	1.17E+06	9.24E+07	1.11E+06	7.76E+07	9.32E+05	4.44E+07	5.32E+05	2.09E+07	2.51E+05	2.34E+07
Replicate 2	1.24E+05	1.26E+08	1.13E+06	1.07E+08	9.62E+05	9.98E+07	8.95E+05	6.65E+07	5.97E+05	3.08E+07	2.76E+05	2.59E+07	2.32E+05	1.97E+07
Replicate 3	1.13E+05	1.15E+08	1.07E+06	8.63E+07	8.06E+05	1.02E+08	9.56E+05	8.01E+07	7.49E+05	2.96E+07	2.76E+05	3.20E+07	2.99E+05	2.46E+07

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Supplementary Table 2. Diversity statistics of the averaged 16S rRNA gene community profiles of the enrichments. SD represents standard deviation.

Enrichment stage	Shannon index	SD	Simpson index	SD	Berger-Parker index	SD	Margalef index	SD
T0	0.59	ND	0.34	ND	1.27	ND	2.11	ND
1	1.19	0.65	0.55	0.24	2.03	0.84	3.14	0.32
2	1.82	0.11	0.78	0.03	2.85	0.46	2.99	0.59
3	1.91	0.27	0.78	0.07	2.80	0.69	2.97	0.24
4	1.59	0.51	0.66	0.19	2.23	0.89	2.86	0.11
5	1.29	0.51	0.56	0.19	2.19	0.90	2.14	0.27
6	1.38	0.39	0.57	0.18	2.29	0.85	2.22	0.14
7	1.33	0.33	0.59	0.19	1.99	0.69	2.03	0.42

Supplementary Table 3. Concentration of elements in the fluid chemistries in combination with OUCM-1 simulant from Stage 7 measured from Abiotic and Biotic Test groups. Concentrations are in M.

Experiment	Sample name	Replicate	Na^{+}	NH_4^{+}	K^{+}	Mg^{2+}	Ca^{2+}	Cl^{2-}	NO_2^{-}	NO_3^{2-}	SO_4^{2-}
Abiotic	Stage 7	1	0.0026	0.00013	0.0014	0.0019	0.04	0.0018	0.00026	0.0038	0.011
Abiotic	Stage 7	2	0.0023	0.00014	0.0019	0.0021	0.037	0.0016	0.00026	0.0034	0.011
Abiotic	Stage 7	3	0.0022	0.00012	0.0017	0.0019	0.039	0.0016	0.00027	0.0035	0.011
Biotic	Stage 7	1	0.0026	0.0003	0.012	0.002	0.079	0.0018	0.00026	0.0037	0.0017
Biotic	Stage 7	2	0.0023	0.00031	0.011	0.0021	0.08	0.0018	0.00026	0.0033	0.002
Biotic	Stage 7	3	0.0023	0.00027	0.013	0.002	0.072	0.0016	0.00027	0.0035	0.0019

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Supplementary Table 4. Identification of genera with metabolisms previously identified in the literature

Genus	Family	Metabolisms detected within the genus	Carbon acquisition strategies	Literature
Acetobacterium	*Eubacteriaceae*	Acetogenesis	Organotroph	(Simankova and Kotsyurbenko, 2015; Groher and Weuster-Botz, 2016)
Clostridium	*Clostridiaceae*	Fermentative	Autotrophic and Heterotrophic	(Keis et al., 2001; Liu et al., 2008; Kaur et al., 2014)
Thauera	*Rhodocyclales*	Denitrification	Heterotrophic	(Mechichi et al., 2002; Wang and He, 2020)
Desulfovibrio	*Desulfovibrionaceae*	Sulfate reduction	Autotrophic and Heterotrophic	(Postgate and Campbell, 1966; Stanley and Southam, 2018; Sánchez-Andrea et al., 2020)
Desulfosporomusa	*Veillonellaceae*	Sulfate reduction	Autotrophic and Heterotrophic	(Sass et al., 2004)
Desulfobulbus	*Desulfobulbaceae*	Sulfate reduction	Heterotrophic	(Suzuki et al., 2007; El Houari et al., 2017)

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