Experimental identification of potential martian biosignatures in open- and closed-systems

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Abstract

NASA's Perseverance and ESA's Rosalind Franklin rovers have the scientific goal of searching for evidence of ancient life on Mars. Geochemical biosignatures that form because of microbe-mineral interactions could play a key role in achieving this, as they can be preserved for millions of years on Earth; and the same could be true for Mars. Previous laboratory experiments have explored the formation of biosignatures under closed systems, but these do not represent the open systems that are found in natural martian environments, such as channels and lakes. In this study, we have conducted environmental simulation experiments using a global regolith simulant (OUCM-1), a thermochemically-modelled groundwater, and an anaerobic microbial community to explore the formation of geochemical biosignatures within plausible open and closed systems on Mars. This initial investigation showed differences in the diversity of the microbial community developed after 28 days. In an open-system simulation (flow-through experiment), the acetogenic *Acetobacterium* (49% relative abundance) and the sulfate reducer *Desulfosporomusa* (43% relative abundance) were the dominant genera. Whereas in the batch experiment, the sulfate reducers *Desulfovibrio, Desulfomicrobium,* and *Desulfuromonas* (95% relative abundance in total) were dominant. We also found evidence of enhanced mineral dissolution within the flow-through experiment, but there was little evidence of secondary deposits in the presence of biota. In contrast, SiO_2 and Fe deposits formed within the batch experiment with biota but not under abiotic conditions. The results from these initial experiments indicate that different geochemical biosignatures can be generated between open and closed systems, and therefore, biosignature formation in open systems warrants further investigation.

Keywords: Biosignatures, simulation experiment, Mars, open-system, closed-system.

1. Introduction

The presence of liquid water is one of the key requirements for life as we know it, along with bio-essential elements and a source of energy. The identification of geomorphological features, such as valley networks and phyllosilicate minerals has provided compelling evidence that Mars once possessed liquid water early in its history (e.g., Carter et al., 2015, 2010; Conway and Balme, 2016; Ehlmann and Edwards, 2014; Fassett and Head, 2005; Lasue et al., 2019; Pan et al., 2018; Salese et al., 2020; Turner et al., 2016). However, since that time, changes to the climate led to a low surface pressure and cold, arid conditions, meaning the martian surface became unconducive to supporting liquid water (Wray, 2020). Over time, this would have resulted in water being restricted to transient occurrences, such as in impact-generated hydrothermal systems or metastable brines (Abramov and Kring, 2005; Osinski et al., 2013; Rivera-Valentín et al., 2020), or in the subsurface where the pressure and temperature could be more favourable (Des Marais, 2010).

In addition to the identification of aqueous environments, bio-essential elements and sources of energy for microbial metabolisms have also been reported (Gellert et al., 2004; Grotzinger et al., 2014; Ramkissoon et al., 2021; Varnes et al., 2003). This has led to two rover missions designed to explore the martian surface to search for evidence of past life: ESA's Rosalind Franklin rover (Vago et al., 2017), and NASA's Perseverance rover (which will also core and cache samples for return to Earth; Williford et al., 2018). The Perseverance rover has been exploring and sampling a diverse stratigraphy, including magmatic units (Crumpler et al., 2023; Udry et al., 2023) and a delta unit (Goudge et al., 2015b; Mangold et al., 2021, 2020). The rover has examined olivine-bearing units interpreted as being igneous (Farley et al., 2022; Liu et al., 2022; Tice et al., 2022; Wiens et al., 2022) and sedimentary rocks (Goudge et al., 2015b; Mangold et al., 2021, 2020). A range of alteration minerals, including oxides, phyllosilicates, sulfates, perchlorates, and carbonate, has also been observed by Perseverance (Farley et al., 2022; Mandon et al., 2022; Scheller et al., 2022; Simon et al., 2023; Sun et al., 2023; Wiens et al., 2022). The Rosalind Franklin rover is also set to explore clay-rich deposits at Oxia Planum (Quantin-Nataf et al., 2021). The presence of clay deposits may indicate subaqueous activity and, on Earth, such deposits can preserve signs of life (Kennedy *et al.*, 2002; Wattel-Koekkoek *et al.*, 2003). If life was able to form and have existed during more clement periods or within transient environments on Mars, it would have interacted with the local lithology and may have left behind geochemical biosignatures (Banfield et al., 2001), which may be preserved in the current rock record. In the absence of directly detecting life, identifying such biosignatures would provide persuasive evidence as to whether life ever existed on Mars.

On Earth, microbe-silicate interactions can lead to both the physical degradation of minerals (microbial weathering) and the precipitation of secondary minerals. The weathering of minerals *via* microbial activity can occur in two ways: 1) physically, whereby microorganisms penetrate the minerals causing disaggregation, or 2) chemically, for example by acidification as microorganisms excrete organic acids, $H^{+,}$ or by the release of other metabolites that increase weathering rates of minerals within the rock (Gadd, 2010). These processes enhance mineral dissolution (Banfield et al., 1999; Gadd, 2010), and alter the composition of any contemporaneous fluids involved in the weathering process. For example, Cyanobacteria have been shown to enhance the weathering of Icelandic basalt and rhyolite by one order of magnitude, which consequently influenced element release rates (Olsson-Francis et al., 2012). The presence of microbes could also directly influence the composition of the penetrating fluids *via* metabolic activity, i.e. altering pH, which could indirectly lead to the precipitation of minerals that would otherwise not form in the environment (Lin et al., 2018; Thompson and Ferris, 1990). The morphology of these secondary minerals could also be influenced by the presence of microbes, as they can act as nucleation points for minerals to form (e.g. Tobler et al., 2008). Eventually, this can lead to the encrustation of microbes, leaving behind fossilised remnants (Ferris et al., 1987; Konhauser and Ferris, 1996; Orange et al., 2013, 2009; Potter-McIntyre et al., 2017; Schopf, 2006; Schultze-Lam et al., 1995; Westall et al., 1995) or generating larger morphological structures, such as microbial mats (Chan et al., 2016; Fleming et al., 2013; Hickman-Lewis et al., 2018; Kennedy et al., 2003). This indicates that a range of evidence exists for microbe-silicate interactions that could be identified and act as potential biosignatures in martian environments.

Mineralogical biosignatures will be dependent on the types and metabolic pathways of the microbes that are present within the system. For example, certain sulfate-reducing bacteria can indirectly lead to the precipitation of $CaCO_3$ (e.g. Lin et al., 2018), whilst specific types of Fe oxidising bacteria can form sheaths/rosettes of Fe^{3+}-oxyhydroxides (e.g. Fleming et al., 2013). Localised conditions can influence the type of microbes that occupy the environment (Görres et al., 2013), either as a result of environmental conditions (e.g. pH and redox; Görres et al., 2013; Ramkissoon et al., 2021) or because of the presence of other microbes within the community (e.g. syntrophic interactions or competitive exclusion; Morris et al., 2013; Nozhevnikova et al., 2020; Prosser, 2012). One environmental factor that can influence the community dynamics, and consequently influence mineralogical biosignature formation, is whether a system is chemically open or closed. Under open-system conditions, fluids are continuously supplied and continuously removed, which could either be beneficial or harmful to the microbial community. Conversely in closed systems, by-products accumulate within the

environment. Furthermore, studies have demonstrated that within closed systems heterotrophic microbial metabolisms dominate the microbe population (Calvo-Díaz et al., 2011; Ionescu et al., 2015), and there is greater growth of opportunistic microbes, which grow on the organic carbon that builds up within the system (Baltar et al., 2012; Lee and Fuhrman, 1991). These changes in community composition between open and closed systems could, therefore subsequently impact biosignature formation.

On Mars, a mixture of open- and closed-system alteration environments has been identified. Fe-rich trioctahedral smectites from Yellowknife Bay, at Gale crater, suggest that closed-system alteration has occurred within the environment (Bristow et al., 2015). Conversely, the presence of Al-rich dioctahedral and Mg-rich trioctahedral smectites within the Stimson and Murray formations, also at Gale crater, indicate open-system alteration that experienced episodic drying (Bristow et al., 2018). In addition, Jezero crater is a valley-fed paleolake system with an outlet channel (Goudge et al., 2015b; Williford et al., 2018), which would facilitate the movement of fluids in and out of the system and suggests that open-system alteration may have occurred. Analysis of outcrops imaged by the Perseverance rover showed the lake level would have been lower (~100 m) than the level suggested for an open system, which indicates a closed system when the delta was formed (Mangold et al., 2021). The Rosalind Franklin rover will head to Oxia Planum, a clay-bearing plain on the edge of the Chryse basin and to the southwest of Mawrth Vallis (Quantin-Nataf et al., 2021; Vago et al., 2017). The proposed landing site is at the end of the Coogoon Valles fluvial system (Quantin-Nataf et al., 2021), which, if similar to Jezero crater, would lead to the movement of fluids, and would suggest open-system alteration on a regional scale. The occurrences of open-system alteration on both a regional and local scale indicate that, in the search for evidence of potential martian life, biosignatures formed in association with both open and closed systems should be explored.

Despite the evidence for both open and closed systems on Mars, work exploring martian biosignatures has focused predominantly on closed-system conditions (e.g. Olsson-Francis et al., 2017; Stevens et al., 2019; Viennet et al., 2019). The limited work that has examined the variation between open and closed systems focused on the abiotic alteration of martian environments and revealed that variations in secondary mineral assemblages would occur between the two systems (e.g. Hurowitz et al., 2005). Therefore, the presence of a biota could further affect the evolution of aqueous environments, as mineral dissolution rates would be impacted (Bray et al., 2015; Olsson-Francis et al., 2012).

The aim of this investigation is to assess how Mars-like open and closed systems could influence microbial community dynamics and the formation of potential mineralogical or morphological biosignatures. This will provide an understanding of the various types of biosignatures that could be present on Mars, aiding the analysis of data returned by the Perseverance and Rosalind Franklin rovers. To achieve this, a range of open and closed system simulation experiments was conducted under martian physicochemical conditions, in the presence and absence of life.

2. Materials and Methods

To assess the range of potential biosignatures that could form in martian aqueous environments a series of simulation experiments were conducted. A regolith simulant representative of an average global chemistry (see section 2.1.1; Ramkissoon et al., 2019) and a thermochemically-derived experimental fluid representative of martian groundwater (referred to as groundwater from hereon, see section 2.1.2), were used to simulate the martian chemical environment. The chemistry of the experimental fluids was used to monitor the evolution of the simulated open system as mineral dissolution occurred, and a range of analytical techniques was used to identify secondary minerals (see section 2.2).

Here, we define open systems as conditions that facilitate the input and output of chemical elements and compounds from the system, whereas closed systems are those where chemical elements and compounds cannot be supplied or removed from the system. Experiments were conducted in a previously described Mars sub-surface reactor (Olsson-Francis et al., 2020) in either a flow-through (representative of an open system) or batch (representative of a closed system) configuration. A microbial community was added to the biotic experiments (see section 2.1.3) and compared to the abiotic experiments to provide an understanding of how the environment would develop in the presence and absence of life.

2.1 Experimental environments and setup

2.1.1 Host rock

The OUCM-1 simulant was used to simulate the geochemistry of the global martian regolith. The simulant was created using a mixture of rocks, minerals, and a glass, to produce a chemistry within 3 wt% for each oxide to the target chemistry, and it possessed a Fe^{2+} abundance of 90% Fe_{tot} (Table 1; Ramkissoon et al., 2019). The simulant did have a high abundance of Cu (3311 ppm) when compared to martian compositions (580 ppm; Payré et al., 2019), which was unavoidable given it was a by-product of the smelting process that created the Fe^{2+}-rich Fe-silicate glass (for further details see Ramkissoon et al., 2019). The

grain size was between 0.4-0.9 mm in diameter, except for gypsum, which had an acicular crystal structure, representative of that found on the surface of Mars (0.05 to 2 mm; Cabrol et al., 2008; Minitti et al., 2013; Shorthill. et al., 1976; Weitz et al., 2018, 2006).

Prior to mixing, simulant components were washed with acetone and then deionised water using an ultrasonic bath. This process removed fine surface particulates and soluble contaminates, ensuring that the experiments were repeatable. The components were air-dried at 80 ˚C and mixed according to the proportions outlined in Ramkissoon et al. (2019). The simulant was then sterilised by autoclave at 121 °C for 20 mins.

2.1.2 Experimental groundwater

The composition of potential martian groundwaters cannot be directly measured. However, it can be assumed that the composition is reflective of the local geochemistry through water-rock reactions between fluid and surrounding host rock. Therefore, for this study, the groundwater composition was derived from the OUCM-1 simulant composition by calculating possible water-rock reactions using the thermochemical modelling programme CHIM-XPT, which determines the reaction of host rock necessary to form alteration minerals and resultant fluids through the application of mass balance and mass action equations (Reed, 1982; Reed et al., 2010).

A two-step modelling approach was used to determine a plausible groundwater composition, where a fluid was titrated with the simulant chemistry. N-bearing organic compounds have been identified in martian meteorites, e.g. ALH 84001 (Koike et al., 2020) and nitrates have also been detected in the martian regolith (Stern et al., 2015). To account for this, 0.027 wt.% of ammonia was added to the groundwater composition, which is reflective of the lower limit of N (0.1 wt.% of NO_3) reported in the martian regolith (Stern et al., 2015). The first model (run 1) consisted of titrating pure water with the simulant chemistry (and ammonia) at 25 ˚C, 1 bar and at a modelled water-rock ratio (W/R_M this reflects the amount of rock reacted within the system, see Olsson-Francis et al. (2017) for further details) of 1000. A second model (run 2) was then conducted reacting the fluid composition from run 1 and fresh simulant (and ammonia) under the same conditions used in run 1. The completion of the two runs provided an evolved fluid composition that was used as a recipe for the experimental groundwater fluid. The groundwater composition derived from run 2 and the chemical compounds used to make the experimental fluid are presented in Table 2. The experimental fluid was prepared in 1 L batches using anoxic and ultra-pure water, which was autoclaved at 121 °C for 20 mins. To this, the chemical compounds described in Table 2 were aseptically added to within one order

of magnitude of the desired concentrations in an anaerobic chamber with a CO_2, H_2 and N_2 headspace at a 90:5:5 ratio.

2.1.3 Analogue microbial community

The anaerobic microbial community used in these experiments was derived from an anoxic sediment collected from the Pyefleet mudflats, in the Colne estuary in Essex, UK (51° 48′N, 0° 22′E). The sediment was subcultured into mixtures of groundwater and OUCM-1 simulant at experimental water-rock ratios of 1 and 4. W/R_E from hereon refers to the experimental water-to-rock ratio. After 28 days, samples were sub-cultured into fresh simulant and groundwater. This process was repeated seven times over seven months to ensure that the microbial community was acclimated to the chemical environment and to minimise the amount of organic carbon transferred from the initial estuary sediment (Macey et al., 2023, 2019). The endpoint community was then used to inoculate the biotic simulation experiments.

Prior to the experiment, the microbial community was grown for 28 days in the groundwater and simulant (grain size 400-900 μm) at 25 °C and at a W/R_E of 1, as previously described in Macey et al. (2023). The community was dominated by sulfate-reducing bacteria belonging to the genus *Desulfovibrio* (representing 66% relative abundance), with acetogenic bacteria (genus *Acetobacterium, representing* 10% relative abundance) and fermentative bacteria (genus *Clostridium*, *representing* 12% relative abundance). Cells were washed with sterilised groundwater to remove any excess fluid. Ten millilitres of cells were harvested by centrifugation at 5,000 × *g*, for 5 min (Olsson-Francis et al., 2017). The cell pellet was washed three times with groundwater fluid and resuspended in 150 ml to a final cell density of 1.85 x 10^5 cells ml^{-1}. Preliminary control experiments showed that the microbial community was unable to grow in the groundwater alone, and therefore, both the simulant and groundwater were required for the simulation experiments.

2.1.4 Experimental setup and conditions

The Mars sub-surface reactor was cleaned and sterilised before each experiment using the procedure outlined in Olsson-Francis et al. (2020). An experimental W/R_E of 1 was used to represent near-subsurface conditions, i.e., a rock-dominated system such as porous sediment (see Bridges et al., 2015; Palandri and Reed, 2004). For both the flow-through and batch experiments, the regolith simulant, groundwater and microbial community (for biotic experiments) were added directly into the reaction chamber. In the flow-through experiment, fresh groundwater was fed into the reaction chamber from an external reservoir *via* an HPLC pump (Olsson-Francis et al., 2020). A flow rate of 0.15 ml min^{-1} was used to ensure that 9 ml samples of fluid would be collected per hour for analysis. The HPLC pump was turned on once

the reaction chamber settled at the target temperature, which took one to two hours. All experiments were conducted for 28 days, except for the abiotic flow-through experiment, which was stopped after 27 days owing to technical reasons. A temperature of 25 °C and a pressure of 1 bar, using a gas mixture of N_2 (50%), H_2 (40%) and CO_2 (10%), was used for all experiments.

2.2 Sampling and analysis

2.2.1 Fluid analysis

The changes in fluid chemistry resulting from the dissolution of the simulant were monitored by measuring the concentration of key rock-forming elements (specifically Al, Fe, Mg, Si, Na, Ca, S, P and Mn) in the fluid. For the flow-through experiments, fluid samples were periodically collected for one hour (to acquire 10 ml) under anaerobic conditions *via* the fluid sampling line. Periodic sampling of the fluids from batch experiments was not possible because of the sampling procedures (see Olsson-Francis *et al.*, 2020). At the end of the batch and flow-through experiments, the remaining fluid from the chamber was collected for analysis. As a control, the reservoir fluid from the flow-through experiments was analysed in parallel after 28 days.

The elemental composition of the fluid samples was measured by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES; Agilent 5110), at the Open University. The fluid was filtered using a 0.22 µm filter, acidified using nitric acid (to a final 2% solution) and stored ready for analysis. Aliquots of 0.5 ml were taken for pH analysis before the samples were acidified.

Major and trace element concentrations were obtained from four analysis runs, which used two different element standard mixtures at 0-5 parts per million (ppm) and 0-1000 parts per billion (ppb). Calibration and check standards were prepared from certified standard element preparations, either individually mixed or as a commercially available 28-element pre-mixed standard (Fisher Chemical MS102050). Preliminary tests were conducted using simulant and fluid mixtures at a W/R_E of 1 to assist in the selection of wavelengths that showed the least interference and best repeatability of data.

A method detection limit (MDL) was obtained in addition to a standard machine detection limit (MaDL), which was derived from blanks collected during the experimental run. Potential MDLs and MaDLs were combined to ensure that the data reported were robust and reflected a true range of potential errors on values reported.

A Mettler delta 320 pH probe at room temperature was used to measure the pH. The pH probe was calibrated using commercially purchased pH buffers prior to each analysis.

2.2.3 Simulant analysis

At the end of each experiment, the simulant was removed from the reaction chamber and dried in air in a flow cabinet. The simulant was then analysed for morphological or mineral alteration that may have occurred during the experiment.

Scanning electron microscopy

Morphological analysis of simulant grains from experiments was carried out using a Zeiss Supra 55VP Field Emission Gun Scanning Electron Microscope (FEG-SEM), equipped with an Oxford Instruments energy dispersive spectroscopy (EDS) system. Air-dried grains from the experiments were mounted onto aluminium stubs and coated with gold (~20-30 nm thick) for geological analysis and no preparation was conducted for biological analysis. Images were acquired under vacuum, using an accelerating voltage of 3kV and a 20µm aperture. Chemical analysis *via* EDS was performed on surficial deposits, under vacuum or variable pressure mode (0.2 mbar) using an accelerating voltage of 20kV and a 20 or 30µm aperture.

Raman spectroscopy

Raman spectroscopy was used to determine if reduced carbon produced as a result of microbial life could be detected on the simulant. Analysis was conducted using a WITEC *Alpha 300 Confocal Raman system* at the Carnegie Institution for Science, Earth and Planetary Laboratory. Raw grains were mounted on a microscope slide using a sodium silicate binder, and randomly selected areas (0.029 mm^2) were mapped. The excitation source is a frequency-doubled solid-state yttrium aluminium garnet (YAG) laser (532 nm) operating at an output power between 0.3 and 1 mW. A 532 nm excitation source was used to replicate the laser on the Perseverance SuperCam and Rosalind Franklin's Raman Laser Spectrometer (RLS). A spot size diameter of 1.18 µm was obtained using a × 50 microscope objective, and a 600 grooves mm^{-1} diffraction grating was used. The integration time was 2s to prevent any potential damage to potential carbon deposits.

Mössbauer analysis

To identify possible changes to the bulk Fe^{2+}/Fe^{3+} ratio of the simulant, Mössbauer spectroscopy was employed. A sample of simulant (10 g) was crushed into a fine-grained powder using an agate ball mill. From this, 100 mg was used to fill circular acrylic glass holders

(~1 cm^2). Analysis was conducted using a standard Mössbauer transmission spectrometer, at the University of Stirling, with a ^{57}Co radiation source at room temperature. Calibration was conducted using the spectrum of an alpha-Fe foil with a thickness of 25μm. Analysis was completed by applying a Voight-based fitting routine (Rancourt and Ping, 1991) using the Recoil Mössabauer spectral analysis programme (Ottawa, Canada), as previously outlined in Ramkissoon et al., (2019).

2.2.2 Microbiology

Fluid samples for microbiological analysis were collected three times a day from the flow-through experiments, in the same manner as those collected for chemical analysis (see section 2.2.1). At the end of the experiment, the fluid was collected from the chamber at the end of both the flow-through and batch experiments. From these samples, 0.5 ml aliquots were used to monitor microbial growth and 5 ml of fluid was used for DNA extraction to characterise the microbial community.

Microbial growth

To monitor microbial growth, cell counts were conducted using LIVE/DEAD™ BacLight™ Bacterial Viability Kit and Lecia DMRP microscope equipped with a 490 nm fluorescent light source, as previously described (Curtis-Harper et al., 2018).

Analysis of the microbial community

DNA was extracted using a modified Griffiths technique (Griffiths et al., 2000; Macey et al., 2020) from 5 ml of fluid collected at the end of the experiments. Nuclease-free water was processed in parallel throughout the extraction as a negative extraction control. The V4-V5 region of the 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) using the primers com1 and com2 (Schwieger and Tebbe, 1998). The PCR reaction mixture contained (per 25 µL): 1 x PCRBIO Ultra Polymerase red mix (PCR BIOSYSTEMS, United Kingdom), 0.4 µM forward primer and 0.4 µM reverse primer. The PCR products were sequenced using the Illumina MiSeq Platform by the company Molecular Research LP, (Texas, USA). The data was processed by Molecular Research LP (United States) 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 also removed. The sequences were clustered at 97% similarity and phylogeny was assigned using a curated database from GreenGenes, RDPII and NCBI (DeSantis et al., 2006).

3. Results

3.1 Flow-through experiments

3.1.1 Fluid chemistry

The ICP-OES results showed no significant change in Si, K, or Na concentration throughout abiotic and biotic experiments, as presented in Tables 3 and 4. However, increases in Mg, Ca and S were observed over the first two days of the abiotic (246 μmol kg^{-1} for Mg and 1.35 × 10^4 μmol kg^{-1} for both Ca and S) and biotic (241 μmol kg^{-1} for Mg, 1.37 × 10^4 μmol kg^{-1} for Ca and 1.39 × 10^4 μmol kg^{-1} S) experiments. This initial increase in Ca and S was a result of gypsum dissolving in solution, or particles being flushed out of the reactor chamber, or a mixture of both processes, which increased their concentration in the effluent fluids. The initial increase in Mg concentration was also most likely linked to the behaviour of gypsum, which was reported to have trace quantities of Mg (0.01 wt.%; Ramkissoon *et al.*, 2019). A distinct increase in Mg, Ca, Fe and Mn concentration (55.5, 914.8, 8.1 and 4.7 μmol kg^{-1}, respectively) in the biotic experiment after day 22 was also measured. This suggests that enhanced dissolution of phono-tephrite or magnetite may have occurred due to microbial activity (Supplementary Figure 1), as this trend was not seen under abiotic conditions.

The bio-essential element P was only above the detection limit in the biotic experiment at concentrations between 1.04-3.09 μmol kg^{-1} (the concentration in the groundwater was 3.68 × 10^{-10} μmol kg^{-1}), which suggests that its occurrence in elevated concentrations within the biotic experiment was related to the presence of microorganisms. At the end of the experiment, concentrations of Ca, K, Mg, Na, S, Si, Al and Mn were within one order of magnitude of their respective initial fluid chemistry for both abiotic and biotic experiments.

The pH of the fluids decreased over the duration of the experiment, in the abiotic experiment from 8.4 (day 0) to 7.9 (day 15), and in the biotic experiment from 8.7 (day 0) to 7.7 (day 6) (Figure 1). On day 16, a pH of 7.4 was recorded for the abiotic experiment, which corresponded to the replenishment of the headspace; it is possible that an influx of CO_2 into the chamber resulted in a decrease in pH due to the CO_2 dissolving into the fluids. A similar decrease in pH after headspace replenishment occurred for the biotic experiment on day 22, where pH decreased from 7.9 to 7.2. The pH taken at the endpoint for the chambers was 8.1 and 8.4 for abiotic and biotic experiments, respectively.

3.1.2 Silicate analysis

FEG-SEM analysis of the simulant grains from the abiotic experiment revealed traces of an Fe-rich deposit on the surface of the apatite (Figure 2U). In the biotic experiments, microbial

cells were predominantly found on Fe-silicate glass, apatite, pyrite, quartz and wollastonite grains and clear evidence of dissolution was found on a feldspar crystal of the phono-tephrite (Figure 2B). Deposits were observed on Fe-silicate grains from the biotic experiments. These deposits had a lenticular crystal structure (Figure 2F) and EDS analysis showed they were rich in Cu and S and, therefore, most likely an product of the Cu in the simulant.

Grains of gypsum were difficult to locate post-experiment. Estimations using the concentration of S and Ca in the fluid suggested that approximately half of the gypsum in the simulant was removed from the chamber over the initial 15 days of the experiment. The crystal structure of gypsum meant that only one dimension of the grains had the desired 0.4-0.9 mm in length, which allowed them to be flushed out of the system.

Although microbial cells were detected on grain surfaces by FEG-SEM, no disordered carbon signatures were detectable by Raman spectroscopy from either abiotic or biotic experiments. It is possible, however, that the randomly selected maps did not cover areas with microbial cells present.

The results from the Mössbauer analyses showed an increase in Fe^{3+} in both abiotic and biotic experiments when compared to the proportions determined for the unaltered simulant (Ramkissoon et al., 2019). A variation in Fe^{3+}/ Fe_{tot} between abiotic and biotic experiments was also observed (Table 5). The biotic experiment resulted in 21.9 % ±2% of Fe^{3+}, which was greater than the 16.2% ±2% measured in the abiotic experiment and 12.5% reported for the unaltered simulant (Ramkissoon et al., 2019). Analysis of simulant exposed to biotic conditions also showed evidence of haematite, which was not a part of the initial simulant mixture or identified in the simulant analysed from the abiotic experiment. This would indicate that, within the flow-through setup the microbial community induced iron oxidation. However, surface deposits were not detected by SEM-EDS analysis.

3.1.3 Microbial growth

During the flow-through experiment, microbial growth steadily increased from 1.85×10^5 cells ml^{-1} to 1.34×10^6 cells ml^{-1} (day 13) and remained relatively constant until the end of the experiment, indicating that steady-state growth was achieved (Figure 3).

3.1.4 Microbial Community

Analysis of the endpoint community showed that sulfate-reducing and acetogenic bacteria dominated the microbial community (representing 98% relative abundance; Figure 4). The

sulfate-reducing *Desulfosporomusa* (43 % relative abundance) and the acetogenic *Acetobacterium* (49 % relative abundance) were the dominant genera. The sulfate-reducing genera *Desulfovibrio* and *Desulfomicrobium* were also present at 3 % and 2 % relative abundance respectively. However, their abundances were greater in the initial microbial community (Figure 4). For example, *Desulfovibrio* represented 66% of the relative abundance in the initial microbial community.

3.2 Batch experiments

3.2.1 Fluids

The fluid was analysed at the end of both the abiotic and biotic batch experiments. The pH of the abiotic sample had not changed significantly, increasing from an initial pH of 7.5 to 7.6 after 28 days (compared with an increase from 6.6 to 7.7 under biotic conditions). Figure 5 demonstrates an overall increase in the concentration of Ca, K, Mg, S, Si and Mn in both the abiotic and biotic experiments, with a greater increase in the biotic experiment. For example, the concentration of Mg at the end of the biotic experiment was 1342.8±0.11 μmol kg^{-1}, more than double the concentration measured in the abiotic experiment (591.0±0.05 μmol kg^{-1}). Fe was only detected in the biotic experiment and therefore, resulted from microbial activity, which enhanced the dissolution of the simulant. Molybdenum was not detected in the initial fluid but was measured at the end of the experiment in the biotic experiment (0.09±0.02 μmol kg^{-1}) and in the abiotic experiment (0.26±0.021 μmol kg^{-1}). The results for Al showed a decrease in concentration between the initial and end point samples in the biotic experiment, which differs from what was observed for other elemental concentrations. This reduction in concentration could be an indication of mineral precipitation because Al is not a key bio-essential element and is unlikely to have been utilised by the microbes.

3.2.2 Silicates

The biotic batch experiment resulted in a range of morphological features, and microbial cells were located on the surfaces of olivine, pyrite, apatite and quartz grains. Wollastonite grains from both abiotic and biotic experiments exhibited surfaces covered with rod-shaped crystals (Figure 2O and 2P), which EDS showed were composed of Ca and C. Needle-shaped crystal clusters, again composed of Ca and some C, were also identified on grains of anorthosite in the biotic experiment and quartz grains in the abiotic experiment. These Ca and C phases were thought to be secondary phases of $CaCO_3$, based on the chemical composition and the crystal structures (e.g. Cheng et al., 2014; Blue et al., 2017). Spherical particles (<1 μm) were also identified on the surfaces of gypsum grains from the biotic experiments and were associated with fractures. EDS analysis indicated that these particles were Si rich, however,

owing to the size of these particles, it was difficult to obtain chemical data without that of the background host grain. These types of particles and associated fractures were not observed in the abiotic experiment. Orthorhombic crystals rich in Ca and S were also observed on quartz grains from the abiotic experiments that were not seen in the biotic experiments. Deposits rich in Fe that had a hollow rod shape were identified on the surface of felspar crystals within phono-tephrite grains, but were not observed in abiotic samples.

Mössbauer analysis of the simulant from both abiotic and biotic batch experiments showed an increase in Fe^{3+} abundance when compared to the initial simulant. However, abiotic experiments resulted in a 26 % ± 2% increase in Fe^{3+}, which was greater than the 20.8 % ± 2% observed for biotic experiments.

The presence of disordered carbon, reflected by peaks at ~ 1350 and ~1590 cm^{-1}, was observed by Raman spectroscopy on simulant material obtained from the abiotic experiments (Figure 6); disordered carbon was not observed in the simulant obtained from the biotic experiments. However, this could be due to a sampling error, a more detailed statistically relevant analysis would be needed to verify the distinction between of the presence or absence of disordered carbon in the abiotic and biotic experiments.

3.2.3 Microbial growth

After 28 days, cell numbers were 3.29 $\times 10^5$ cells ml^{-1} (standard deviation 3.56 $\times 10^4$) in the fluid. This was almost one order of magnitude lower than cell densities recorded from the fluid collected in the biotic flow-through experiments. However, these values do not reflect cells attached to the simulant or those possibly attached to the reactor wall.

3.2.4 Microbial Community

Analysis of the microbial community showed an increase in the abundance of sulfate-reducing bacteria from 75% relative abundance in the initial inoculum to 95% relative abundance in the biotic batch experiment. In the inoculum, *Desulfovibrio* accounted for 66% relative abundance, and *Desulfomicrobium* and *Desulfuromonas* both accounted for > 10 % relative abundance. Acetogenic bacteria (*Acetobacterium*) were detected at 4 % relative abundance in the batch experiment, lower than the level detected in the initial microbial community (10 %). This differed from the flow-through experiments, where sulfate-reducing and acetogenic bacteria accounted for 50% and 49 % of the relative abundance of the community, respectively.

4. Discussion

Traditionally, investigations into the identification of potential martian biosignatures utilised simulated martian environments in batch culture, which represent a closed-system environment (e.g. Olsson-Francis et al., 2017; Stevens et al., 2019). However, mineralogical evidence has revealed that a mixture of open- and closed-system environments existed on Mars (e.g. Achilles et al., 2020; Bristow et al., 2018, 2015; Dehouck et al., 2020; Ehlmann et al., 2011), which has not been accounted for in previous investigations. Given the dynamic nature of open-system environments and the variations in secondary mineral formation identified on Mars, it is possible that these systems may also lead to the formation of different mineral and morphological biosignatures. In this work, open and closed systems (flow-through and batch, respectively) under abiotic and biotic conditions were simulated to examine the variation in biosignatures that would arise from these different systems.

4.1 Microbial community dynamics

The open and closed systems mimicked in both flow-through and batch experiments, respectively, were able to support the growth of an anaerobic microbial community, and different endpoint communities evolved in the two experiments. Sulfate-reducing bacteria were the main genera in the biotic batch experiment (75 % relative abundance) with acetogenic bacteria representing 4 %; whereas in the biotic flow-through experiment, sulfate-reducing and acetogenic bacteria represented 43 % and 49 % relative abundance, respectively.

The dominance of these two genera in these simulated martian environments supports previous work that suggested sulfate reduction and acetogenesis as viable metabolisms in martian chemical conditions (Cousins, 2015; Macey et al., 2023, 2020). Whilst it is not possible to identify the exact cause of the differential enrichment of specific genera between these experiments, there are several possibilities. The continuous removal of nutrients produced *via* mineral dissolution in the flow-through experiments compared to the batch experiments would be expected to play a significant role in microbial community dynamics. Also, stochastic variation from a random variable, for example, minor variation in the microbial community, or deterministic processes driven by selection, such as some genera being able to competitively exclude others by growing more efficiently in a specific fluid chemistry or the enriched genera being able to tolerate toxicities from the distinct fluid chemistries that evolved over the course of the experiments. Given the variation between open and closed systems, and the established impact that this can have on fluid chemistry and community composition (e.g.

Calvo-Díaz et al., 2011; Ionescu et al., 2015), the environmental conditions would be expected to play a major role in the evolution of the two enriched communities.

The difficulty in defining the relative importance of stochastic versus determinative processes in microbial community assembly is a well-established issue in ecology (Antwis et al., 2017; Stegen et al., 2012; Zhou and Ning, 2017). If the variation between open and closed systems was a consequence of stochastic processes, then the composition of the enriched community would not be expected to be replicated in further experiments. However, if selective processes played a dominant role in determining community composition, then conservation of the enriched genera would be expected (Yao et al., 2019; Yuan et al., 2019). Microbial community composition in sulfur-rich environments has previously been shown to be greatly influenced by environmental factors, such as nutrient availability that drive selection (LeBrun et al., 2018; Yao et al., 2019), but this has not been universally observed (Baptista et al., 2008).

4.2 Effects on fluid compositions

The increase in element concentrations in both the abiotic and biotic batch experiments was consistent with the results from previous investigations, which found that elemental concentrations increased with time under similar temperatures and abiotic conditions (e.g., Bullock et al., 2004). Conversely, flow-through experiments resulted in fluid chemistries that remained relatively consistent, with variations within one order of magnitude (except for Ca and S concentrations) throughout the duration of the experiment, indicating that the incoming fluid was a dominant control of fluid chemistry in the experiment. This result differs from previous work (e.g. Dove and Crerar, 1990; Hodson, 2006; Hurowitz et al., 2005), where increased elemental concentrations were observed. However, most previous studies used temperatures greater than 100 ˚C or acidic solutions that increase the rate of mineral dissolution (e.g. Gudbrandsson et al., 2011; Hänchen et al., 2006), instead of a groundwater composition or pure water that reflects the near neutral pHs suggested by the detection of phyllosilicate minerals (Ehlmann and Edwards, 2014; Poulet et al., 2005). .

In a ternary plot of dissolved Si, Na + K and Ca + Mg + Fe (Figure 7), there is a clear separation between the endpoint fluids resulting from the batch and flow-through experiments. It also shows that the endpoint flow-through fluid (both from the biotic and abiotic experiments) is akin to terrestrial basaltic fluids, which plot towards Na + K, and away from Ca + Mg + Fe. The opposite was true for endpoint fluids from the batch experiments (biotic and abiotic), which lay between terrestrial basaltic fluids and the martian fluids derived from thermochemical modelling and experimentation reported in the literature (e.g. Bullock et al., 2004; Filiberto and Schwenzer, 2013; Moore and Bullock, 1999; Schwenzer et al., 2016). The variation in

composition between flow-through and batch experiments was related to the concentration of Ca in the batch experiments, which was over one order of magnitude greater than the initial fluid composition. This increase in Ca resulted from differing dissolution rates of minerals within the simulant, specifically of gypsum. In the flow-through experiments, Ca and S ions from gypsum dissolution were removed from the system. Whereas Ca and S ions within the batch experiments remained within the system and led to the increases in concentrations found in the fluid (Figure 5).

The high Ca + Mg + Fe concentrations reported in martian fluids are predominantly formed *via* reactions at either low $W/R_M s$ (1000 or less), using acidic fluids or single host rock compositions (e.g. Baker et al., 2000; Bullock and Moore, 2004; Schwenzer et al., 2016). This either leads to increased dissolution or does not factor in the dissolution rates of individual minerals. These factors would influence the dissolution of the rock/mineral and the types of minerals that would precipitate as the solution becomes saturated with various ions, which in turn would impact the fluid composition.

In the case of the batch experiments reported here, the concentration of Ca and S ions increased as gypsum dissolution occurred, which led to the precipitation of Ca-based minerals ($CaCO_3$) that would eventually lead to lower Ca concentrations. This was not the case for the flow-through experiments, as these ions were removed before the fluid became oversaturated and minerals could precipitate. However, the flow rate will also influence the dissolution and precipitation of minerals and microbial growth rates (e.g. Gijs Kuenen, 2019; Savage et al., 1992), and therefore, the fluid composition within the system. It is possible that a lower flow rate in the flow-through experiments would have also led to increased ion concentrations, which would have affected the fluid composition and eventually led to the precipitation of minerals. The composition of the fluid, and the formation of secondary minerals, would be partially controlled by a system that is either open or closed. This highlights the importance of clearly defining the environmental context of the system when exploring biosignature formation or when identifying potential martian fluid compositions.

4.3 Secondary deposits

Analysis of the simulant after the experiments revealed secondary alteration features that were distinctly different between the biotic flow-through and batch experiments (Table 6). The biotic flow-through experiment exhibited evidence of enhanced mineral dissolution, Mössbauer analysis indicated the formation of haematite (see section 3.1.4), and only trace amounts of Fe-rich deposits were observed under flow-through abiotic conditions. However, the biotic batch experiment resulted in the precipitation of a Cu-S deposit, $CaCO_3$, silica and a Fe-rich

deposit (see section 3.2.4); only $CaCO_3$ was identified in the abiotic batch experiment. The formation of $CaCO_3$ and silica is in accordance with secondary phases identified in alteration experiments using martian analogue basalts, however, these experiments were conducted using abiotic flow-through systems with enhanced dissolution kinetics, such as high temperatures or an acid pH (Baker et al., 2000; Hurowitz *et al.*, 2005).

Morphological analysis using SEM-EDS revealed silica nanoparticles formed on the surfaces of gypsum grains from the biotic batch experiment (Figure 2AJ), but no particles were identified on grains from the abiotic batch experiment (Figure 2AI). Although these silica nanoparticles were not associated with microbial structures, they may be a result of microbial activity because silica concentrations would increase as microbes scavenged bio-essential elements, such as Fe from silicate material, not found in sufficient quantities in the fluids. Previous investigations that explored the formation of silica deposits in terrestrial geothermal environments have proposed amorphous silica deposits formed abiotically and not as a result of metabolic activity (Mountain et al., 2003; Lalonde *et al.*, 2005).

Although these silica nanoparticles are abiotically synthesised, they could play a role in the preservation of microbes and biomarkers. Experimental investigations on the fossilisation of microbes by silica have shown that organic compounds associated with microbial surfaces (e.g. extracellular polymeric substances or lipopolysaccharides) provide reactive surfaces that facilitate silica nucleation, which lead to the silicification and preservation of the microbes (Hugo et al., 2011; Orange et al., 2013, 2009; Tobler et al., 2008; Toporski et al., 2002; Westall et al., 1995). However, chemical (e.g. pH and redox) and physical (e.g. temperature) properties will also play a significant role in silica precipitation (Yee et al., 2003). Microbes can also play a role in silica mobilisation that leads to the formation of amorphous silica deposits, recording enrichments of bio-essential elements and metals, and fossilised microbes (Sauro et al., 2018), which could be used as evidence for life. Silica nanoparticles found in our experiments were commonly associated with fractures and, in some instances, nanoparticles appeared embedded within the grain surfaces (Figure 2AJ). The fractures observed on the grains could have provided nucleation points for the silica nanoparticles as surface roughness has previously been shown to facilitate silica precipitation (van den Heuvel et al., 2020). It is possible that, given enough time, silica particles could eventually begin to adhere to the surfaces of microbes and ultimately encrust them in silica. Over time, these could grow to larger structures, such as silica sinters or stromatolites (Mountain et al., 2003; Aubrecht *et al.*, 2008), which could also record geochemical enrichments of bio-essential elements (Gangidine et al., 2020; Sauro et al., 2018). This would support previous suggestions that silica deposits on Mars would be prime sampling targets for martian rovers (Cady et al., 2018; Farmer and

649 Des Marais, 1999; McMahon et al., 2018; Ruff and Farmer, 2016). Indeed, SiO_2 has been
650 identified at rover landing sites, from the ground by the NASA MER rover, Spirit, in volcanic
651 hydrothermal environments in Gusev crater (Ruff and Farmer, 2016), NASA Curiosity rover in
652 fracture-associated haloes in Gale crater (Frydenvang et al., 2017), and from orbit in the
653 basement unit and near the delta at Jezero Crater, the NASA Perseverance rover's landing
654 site (Tarnas et al., 2019), and in sediment fan layers at Oxia Planum, the ESA Rosalind
655 Franklin rover landing site (Quantin-Nataf et al., 2021).
656
657 The generation of $CaCO_3$ crystals was observed in both abiotic and biotic batch experiments;
658 however, qualitative assessment of crystal sizes showed they were larger in the biotic
659 experiments (Figure 2O and Figure 2P). The formation of relatively larger crystal sizes
660 identified in biotic batch experiments could be indirectly linked to sulfate-reducing microbial
661 activity. Sulfate reducers have also been shown to produce HCO_3^- as a metabolic by-product
662 of sulfate reduction and increased pH, which leads to fluids becoming oversaturated with
663 $CaCO_3$ and eventually to mineral precipitation (Lin et al., 2018). Such a situation was proposed
664 to explain the *in situ* calcification of microbial mats found in the Barberton greenstone belt,
665 South Africa (Westall et al., 2011). While no $CaCO_3$ deposits were identified in the biotic flow-
666 through experiment, sulfate reducers did account for 48 % of the endpoint community, and
667 therefore, sulfate reduction and the production of HCO_3^- could have occurred, albeit to a lesser
668 extent. The open nature of the system meant fluids did not oversaturate as ions were
669 continuously being removed, and so precipitation of $CaCO_3$ was prevented. Whilst the
670 production of $CaCO_3$ could be linked to sulfate reduction, the formation of $CaCO_3$ deposits
671 under abiotic conditions in the batch experiment makes it difficult to use $CaCO_3$ as a
672 mineralogical biosignature without additional characteristics, such as morphology of the
673 minerals, the presence of biomarkers or chemical enrichments that also point towards a
674 biological origin (e.g. Djokic et al., 2017; Gangidine et al., 2021). However, its formation in
675 higher quantities in the biotic experiments conducted in this project is suggestive of an
676 interesting biomediated mechanism of formation. The carbonate deposits detected at Jezero
677 crater (Clavé et al., 2022; Salvatore et al., 2018; Wiens et al., 2022) may have an evaporitic
678 or hydrothermal origin but may also have a significant potential for biosignature preservation
679 (Clavé et al., 2022; Tarnas et al., 2021).
680
681 SEM-EDS analysis showed a Fe-rich deposit with a tube-like morphology on the surface of
682 phono-tephritic feldspars in the biotic batch experiment (Figure 2D). Such deposits had not
683 been identified in the abiotic batch or flow-through experiments and are therefore considered
684 to be the fossilised remnants of microbial activity. The deposit was made of individual
685 structures (<1 μm in diameter) with a morphological resemblance to microbial sheaths

(Fleming et al., 2013), which form as a result of biomineralization (Chan et al., 2016). Microbial sheaths are normally microns in diameter, form filaments (Preston *et al.*, 2011; Fleming *et al.*, 2013; Chan *et al.*, 2016) and are composed of nanometre fibrils or particles (Banfield et al., 2000; Emersont and Ghiorse, 1993). These structures are often associated with Fe^{2+}-oxidising bacteria (e.g. Chan et al., 2016; Fleming et al., 2013), although no known Fe^{2+}-oxidising bacteria were identified in the analogue community (Figure 4). Given the duration of these experiments, the Fe-rich deposits may represent the early stages of sheath mineralisation and, if given more time, could have formed filaments and eventually biofilms. The fact that these morphological features were observed only in the batch experiment may be a result of from the amount of biomass or the nature of the system (i.e. compounds were not continuously being removed as in the flow-through system). Therefore, such features, if they exist, might be restricted to areas on Mars where closed-system conditions enabled the accumulation of Fe over time to form such structures.

Raman spectroscopy identified disordered carbon only on silicates from the abiotic batch experiment and not in the abiotic flow-through, biotic batch or biotic flow-through experiments. The presence of disordered carbon has been used to support the identification of fossilised ancient life in terrestrial environments (e.g. Schopf et al., 2005); however, organic material can be abiotically synthesised in aqueous systems *via* serpentinisation (Sforna et al., 2018; Steele et al., 2022). Given the absence of organic carbon in the simulant (Ramkissoon et al., 2019), water-rock interactions occurring within the experiments conducted here could have led to the serpentinisation of mafic minerals, such as olivine and augite, found within the simulant (Ramkissoon et al., 2019), potentially generating organic carbon in the process. Therefore, the presence of disordered carbon alone cannot be used as an indicator of life on Mars.

The results from Raman spectroscopy also showed the absence of disordered carbon in biotic samples from flow-through and batch experiments, although microbial structures were found on the surfaces of silicate samples. A previous study conducted by Stevens et al. (2019) used Raman spectroscopy to identify the presence of microbes on a rock substrate, however, that study also highlighted the difficulties that were encountered in identifying signatures that were known to have been associated with biota. It is possible that the absence of disordered carbon found on samples taken from the biotic experiments conducted here was a result of sample heterogeneity or the analysis not covering regions where microbial structures were present, particularly considering the fact that, due to the oligotrophic conditions, microbial biomass was limited. Thus, the lack of disordered carbon does not necessarily mean the absence of life, given the presence of microbes in the experiments presented here. Therefore, in the search for life on Mars, other biosignatures in addition to disordered carbon need to be identified.

Mössbauer analysis revealed differences in Fe^{2+} and Fe^{3+} abundances between abiotic and biotic conditions for both flow-through and batch experiments. However, these differences varied between the types of experiments. Biotic flow-through experiments revealed increases in Fe^{3+} compared to abiotic flow-through experiments, although no deposit was identified *via* SEM-EDS analysis, whereas increases in Fe^{2+} were observed in the biotic batch experiments compared to abiotic conditions. The difference in Fe^{2+} and Fe^{3+} abundances can be explained by the observed differences in the endpoint community between the batch and flow-through experiments. Variations between the chemical environments of the batch and flow-through experiments may have promoted different primary metabolisms (i.e. sulfate reduction potentially being favoured over iron reduction under batch conditions). Members of the genera *Desulfuromonas, Desulforsporomusa* and *Desulfovibrio,* which were the most abundant genera in the batch experiments, are capable of iron reduction. These microbes use Fe^{3+} as the terminal electron acceptor (producing Fe^{2+}), in addition to sulfate (Berg et al., 2019; Lovley et al., 1993), and therefore, would have increased the Fe^{2+} within the environment. Although, *Acetobacterium* and *Sporomusa* are capable of Fe^{0} oxidation to produce Fe^{2+} (Philips et al., 2019), the absence of Fe^{0} within the simulant means it is more likely that the higher abundance of Fe^{2+} observed in the batch experiment was a result of sulfate-reducing activity, which emphasises the importance of exploring the influence that different environmental systems can have on the formation of geochemical biosignatures.

On Earth, the oldest evidence of life has been preserved as mineralogical biosignatures in Archean rocks (e.g., Djokic et al., 2017; Hassenkam, 2017; Hickman-Lewis et al., 2018; Noffke et al., 2013; Walsh, 1992; Westall et al., 2001), which indicates mineralogical biosignatures can be preserved over geological timescales. This is supported by estimations of crustal residence times, which indicate that phosphates and silica survive up to 3.8×10^8 yrs, marine carbonates, metallic oxides, and clays can survive up to 3.5×10^8 yrs, and metallic sulfides can survive up to 2×10^8 yrs within the Earth's crust (Farmer and Des Marais, 1999). However, ancient biosignature-bearing mineral deposits on Earth have experienced subsequent geological processing, for example, metamorphism (e.g. Kranendonk et al., 2008; Rollinson, 2002), which could lead to the destruction or modification of these minerals and any preserved biosignatures. Owing to the absence of plate tectonics, Mars is relatively less geologically active than Earth (McSween, 2015), but evidence of activity, such as diagenesis and aeolian abrasion (e.g. Achilles et al., 2020; Bridges et al., 2014), has been observed, which would also lead to biosignature modification and destruction. If life did exist on Mars, biosignatures, such as those reported in this study, have the potential to survive for millions of years in deposits but they would have been modified by geological processes. Therefore, to help interpret the

data returned from current and future life detection missions, the work conducted in this study should be expanded to constrain how biosignatures, such as those identified in our experiments, would be altered by a range of geological processes.

The simulant used in this preliminary study is representative of the global martian dust composition (Ramkissoon et al., 2019). However, Mars has a range of lithologies, such as sulfur-rich deposits, e.g. the Paso Robles soil (Lane et al., 2008; Yen et al., 2008), the sedimentary sequences at Gale crater (Rapin et al., 2019), and haematite enrichments, as found e.g., at ”Hema2” a Fe^{3+}-rich deposit at Meridiani Planum (Rieder et al., 2004) or in the ”Stoer” drill sample on Vera Rubin Ridge in Gale crater (Rampe et al., 2020). These different geochemical environments could also influence microbial community dynamics and the types of biosignatures that could form, which may differ from those reported here. Therefore, additional studies that simulate a range of geochemistries would complement our study and ensure the full range of possible biosignatures could be identified.

4.5 Relevance to martian systems

In the experiments presented here, microbial activity was shown to produce distinct mineralogical and morphological biosignatures in chemically open and closed systems under martian conditions. On Mars, a mixture of regionally-sized open and closed systems have been identified, such as closed and open basins like Gale and Jezero craters (Fassett and Head, 2008; Goudge et al., 2015a). However, within these regionally scaled systems, localised open and closed systems can develop and persist. For example, Gale crater exhibits a mixture of mineral deposits that were formed from either chemical leaching (open system; Achilles et al., 2020; Bristow et al., 2018) or isochemical alteration (closed system; Bristow et al., 2015). Therefore, based on the results of this initial study, if microbial life existed on Mars at Gale crater, the presence of both systems would have led to a mixture of geochemical biosignatures that may be preserved. These could include microbially-induced dissolution textures in areas that facilitated open-system alteration, such as the Stimson and Murray formations that have clays rich in Mg and Al (Bristow et al., 2018) or the Glen Torridon region that is dominated by clays rich in Mg and K (Dehouck et al., 2020). Microbially-induced mineral precipitation would be present in settings that restrict or limit the mobilisation of fluids (i.e. closed-system alteration), such as under the conditions that created clay deposits at Yellowknife bay (McLennan et al., 2013; Vaniman et al., 2014).

Given that the chemistry of the OUCM-1 simulant was based on a global regolith composition (Ramkissoon et al., 2019; Yen et al., 2005), similar geochemical biosignatures as those observed in this initial study may also be preserved elsewhere on Mars, for example at Jezero

crater and Oxia Planum, if life had been present at some point in Mars' history. At Jezero crater, the presence of outflow and inlet channels indicate an open system (Goudge et al., 2015b) and *in situ* analysis of outcrops suggests that some depositional units may have been formed under a closed system (Mangold et al., 2021). Even though Oxia Planum could be considered as an open system on a regional scale, it is conceivable that localised occurrences of both open and closed systems may also have operated. Therefore, secondary minerals formed in these experiments can guide sampling at Jezero crater and Oxia Planum.

Silica deposits have been identified by orbiting spacecraft at both Jezero crater and Oxia Planum (Quantin-Nataf et al., 2021; Tarnas et al., 2019), and carbonates have been observed by *in situ* analysis of Jezero crater's floor (Tice et al., 2022). These deposits may preserve indicators of life, similar to terrestrial settings (Gangidine et al., 2020; Sauro et al., 2018; Williams et al., 2021), which could then be detected by the rovers. However, the presence of oxidisers such as oxychlorines in the martian regolith (e.g. Glavin et al., 2013; Hecht et al., 2009; Scheller et al., 2022) could impact the preservation and detection of potential martian biosignatures. Laboratory experiments have shown that the decomposition products of oxychlorines from heating can alter organic compounds preserved in mineral matrices (e.g. Lewis et al., 2021; Steininger et al., 2012) and lead to the decomposition of calcium carbonate (Cannon et al., 2012). It has also been shown that the presence of oxychlorides in solutions can lead to the oxidation of Fe^{2+} (Mitra et al., 2020; Mitra and Catalano, 2019). Although minerals, such as those observed in this study, can potentially preserve biosignatures further investigations are required to determine how these biosignatures could be affected by the martian environment.

5. Conclusion

In this study, a novel approach was undertaken to explore the types of possible biosignatures that could form within different martian aqueous environments. The results reported here suggest that geochemical biosignatures that can form in closed and open systems are different (batch and flow-through experiments, respectively). Geochemical and morphological analyses showed the formation of $CaCO_3$ crystals, silica nanoparticles, and Fe-rich tube-like deposits under biotic closed-system conditions, whereas there was little evidence of secondary mineral deposits within a biotic open system. Instead, evidence of enhanced mineral dissolution and a change in the Fe^{2+}/Fe^{3+} ratio was observed. The difference is also reflected in the microbial diversity. Sulfate-reducing bacteria dominated the closed-system experiment, whereas a mixture of sulfate-reducing bacteria and acetogens was prevalent in the open-system experiment.

This preliminary investigation highlights the importance of simulating both open and closed systems when studying the formation of geochemical biosignatures under laboratory simulation conditions. This is of particular importance when investigating potential biosignatures that may have formed in more transient habitable environments. While further work is required to explore the development of biosignatures in a wider range of geochemical environments, it is clear that the currently active rovers have encountered and will continue to encounter examples of both types of potentially habitable systems. Therefore, it is important to consider the role of selective dissolution and mineral precipitation in the search for biosignatures.

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Data availability

Amplicon sequence data generated in this study were deposited to sequence read archives (SRA) - SRR23009032 – SRR23009033.

Author Contributions

N. K. Ramkissoon: Conceptualization, investigation, project administration, methodology, formal analysis, writing – original draft. M. C. Macey: Investigation, formal analysis, writing – review & editing. E. Kucukkilic-Stephens: Investigation. T. Barton: Investigation, writing – review & editing. A. Steele: Investigation, writing – review & editing. D. N. Johnson: Methodology, writing – review & editing. B. P. Stephens: Investigation. S. P. Schwenzer: Conceptualization, funding acquisition, writing – review & editing. V. K. Pearson: Conceptualization, funding acquisition, writing – review & editing. K. Olsson-Francis: Conceptualization, funding acquisition, methodology, writing – review & editing.

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Tables

Table 1. OUCM-1 Simulant chemistry and component proportions (Ramkissoon et al., 2019).

Chemistry (wt.%)		Component proportion (wt.%)	
SiO_2	41.87	Fe-Silicate glass	30
TiO_2	0.81	Phono-tephrite	40
Al_2O_3	10.80	Quartz	3
Cr_2O_3	0.12	Olivine	8
FeO	17.43	Anorthosite	7
Fe_2O_3	2.77	Wollastonite	3
MnO	0.21	Pyrite	4
MgO	6.76	Magnetite	3
CaO	7.82	Apatite	1
Na_2O	2.91	Gypsum	1
K_2O	2.14	**Total**	**100**
SO_3	5.65		
P_2O_5	0.72		
Cl	0.00		
Total	**100.00**		

Table 2. Composition of thermochemically derived initial groundwaters used in these experiments and the list of components used to make the experimental fluid.

Initial groundwater composition (mol L^{-1})		Components used in experimental fluid (mol L^{-1})	
Cl^-	1.91E-03	$NaHSiO_3$	9.87E-04
HCO_3^-	2.20E-05	$NH_4CH_3CO_2$	1.11E-05
SO_4^{2-}	8.51E-05	NaHS	5.92E-06
HPO_4^{2-}	3.68E-13	NaCl	1.18E-03
HS^-	5.92E-06	$AlKSO_4$	8.92E-11
K^+	2.30E-04	$MgSO_4$	2.48E-09
NH_4^+	1.10E-05	$FeSO_4$	2.59E-09
Na^{2+}	2.17E-03	K_2HPO_4	3.68E-13
Mg^{2+}	2.48E-09	$MnSO_4$	7.40E-08
Ca^{2+}	6.08E-04	KOH	2.30E-04
Mn^{2+}	7.40E-08	$CaCl_2$	7.23E-04
Fe^{2+}	2.59E-09	$CaSO_4$	8.50E-05
Al^{3+}	8.92E-11	$Ca(OH)_2$	1.61E-04
SiO_2	9.87E-04		
$TiOH_4$	1.89E-05		

1429 Table 3. ICP-OES of sampled fluid chemistries taken from abiotic flow-through experiments.

		Fluid chemistry (μmol kg^{-1})								
Sampling days	pH	S	K	Na	Mg	Ca	Mn	Mo	Al	Si
Initial Fluid	8.4	118.14	173.33	2260.34	1.46	860.13	0.22	0.017	0.68	1346.40
1	8.1	13660.82	582.96	2799.27	246.86	14319.23	0.87	0.059	0.94	1341.92
2	8.0	5632.19	336.76	2850.42	60.27	6625.09	0.25	0.165	0.82	1134.27
3	7.8	2706.45	211.08	2039.72	32.18	2707.48	0.12	B.D.	0.52	789.32
4	7.9	1242.61	187.24	1911.92	12.13	1715.91	0.06	B.D.	0.44	721.17
5	7.9	919.64	173.17	2124.88	9.22	1589.21	0.05	B.D.	0.41	818.73
6	7.9	667.22	172.56	2191.17	9.99	1384.06	0.04	B.D.	0.29	847.28
7	7.8	582.26	150.36	1889.82	8.52	1100.41	0.03	0.01	0.30	801.28
8	7.8	478.35	135.37	1853.11	7.67	962.43	0.46	B.D.	0.33	780.77
9	7.9	324.97	142.28	1874.25	7.28	797.75	0.03	B.D.	0.34	802.85
10	7.9	175.59	138.29	1797.43	5.08	636.36	0.02	B.D.	0.11	763.82
11	7.8	B.D.	136.34	1817.27	6.22	538.80	0.03	B.D.	0.43	767.81
12	8.0	B.D.	148.57	2040.41	3.99	547.44	0.03	B.D.	0.38	836.53
13	8.3	55.96	139.57	1942.72	14.07	535.21	0.03	B.D.	0.41	809.97
14	7.9	79.10	176.81	2343.59	4.87	558.62	0.03	B.D.	0.27	881.54
15	8.1	72.80	158.54	2111.84	4.92	501.08	0.03	B.D.	0.73	806.27
16	7.4	100.12	187.65	2449.20	32.28	844.81	0.38	B.D.	0.28	1039.84
17	7.3	93.94	169.34	2294.00	19.20	668.55	0.19	B.D.	0.28	950.68
20	7.4	98.37	162.69	2280.43	6.81	556.22	0.05	B.D.	0.42	870.00
21	7.6	100.37	159.87	2256.68	10.10	544.79	0.04	B.D.	0.40	862.45
22	7.6	109.41	161.87	2289.31	18.56	553.38	0.05	B.D.	0.34	866.08
23	7.8	102.49	166.27	2379.08	6.26	571.14	0.05	B.D.	0.37	888.02
24	7.9	95.19	161.46	2353.42	5.88	555.12	0.06	B.D.	0.25	870.36
27	7.7	105.86	167.80	2254.77	6.30	556.82	0.15	B.D.	0.30	861.31
28	-	-	-	-	-	-	-	-	-	-
End exp. chamber solution	8.1	103.11	157.98	2348.55	7.24	564.06	0.09	B.D.	0.33	864.23
Left over stock solution	8.4	115.0	152.9	2369.3	5.70E+00	562.4	1.82E-01	B.D.	0.54	853.3
Method detection limit		29.008	34.005	86.104	0.062	75.563	0.003	0.007	0.065	98.738
Machine detection limit		1.535	0.593	1.416	0.084	0.986	0.010	0.010	0.087	1.658

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Table 4. ICP-OES of sampled fluid chemistries taken from biotic flow-through experiments.

Sampling Days	pH	Fluid chemistry (μmol kg^{-1}) S	P	K	Na	Mg	Ca	Mn	Fe	Mo	Al	Si
Initial Fluid	8.7	80.35	B.D.	85.36	839.81	1.29	515.58	B.D.	B.D.	B.D.	0.54	934.15
1	8.0	14014.74	1.64	418.45	980.83	241.72	14198.58	0.71	B.D.	B.D.	0.57	1060.91
2	7.4	7825.15	1.76	259.36	873.43	67.18	8032.10	0.18	B.D.	B.D.	0.57	899.61
3	7.9	4077.36	1.92	186.39	780.96	36.03	4323.58	0.04	B.D.	B.D.	0.83	711.97
4	7.3	2409.50	B.D.	171.12	842.16	77.45	3060.30	0.35	B.D.	B.D.	0.97	899.61
5	7.4	1854.06	B.D.	169.36	950.38	47.57	2417.83	0.28	B.D.	B.D.	1.14	1144.47
6	7.1	1675.21	3.35	181.04	1083.79	28.91	2228.87	0.22	B.D.	B.D.	1.12	1252.93
7	7.2	1116.00	1.11	166.36	1039.42	17.42	1637.92	0.15	B.D.	B.D.	1.14	1156.58
8	7.2	666.23	2.30	163.75	1063.60	13.01	1217.87	0.08	B.D.	B.D.	0.86	1181.04
9	7.5	344.10	1.48	130.58	873.91	8.71	816.02	0.07	B.D.	B.D.	1.00	951.45
10	8.1	122.49	2.62	120.50	826.32	6.95	598.52	0.03	B.D.	B.D.	0.92	923.15
11	8.2	55.37	B.D.	132.73	932.68	5.82	587.97	0.03	B.D.	B.D.	0.90	1035.34
12	8.1	50.72	1.93	122.27	877.00	7.69	557.68	0.07	B.D.	0.11	0.96	978.12
13	8.1	57.20	B.D.	120.27	868.30	7.73	558.18	0.19	B.D.	0.10	0.71	956.51
14	7.9	56.63	3.09	127.54	936.11	6.87	594.76	0.13	B.D.	0.10	0.64	1043.71
15	7.7	57.69	B.D.	129.00	957.51	8.23	610.05	0.13	B.D.	0.10	0.71	1066.96
16	8.2	58.45	1.77	109.99	813.32	7.13	506.25	0.14	B.D.	0.11	0.77	906.27
17	7.8	65.10	1.04	100.91	742.24	7.74	458.60	0.15	B.D.	0.11	0.78	802.16
20	7.5	69.01	1.16	96.51	721.84	6.88	439.63	0.17	B.D.	0.10	0.69	779.41
21	7.9	76.75	2.03	112.53	866.21	13.73	523.17	0.17	B.D.	0.10	0.78	923.43
22	7.2	21.68	1.98	128.97	934.37	55.47	914.78	4.70	8.13	0.34	0.48	1150.38
23	7.6	18.93	1.26	105.85	797.75	39.09	618.41	4.59	7.05	0.32	0.49	982.07
24	7.5	19.38	2.64	132.83	1049.12	23.60	670.33	3.09	1.46	0.27	0.55	1208.56
27	7.7	30.89	2.10	105.82	833.28	11.01	467.30	0.86	0.02	0.30	0.63	887.54
28	8.1	24.74	1.34	101.09	795.40	9.79	461.89	0.61	0.00	0.32	0.58	846.42
End exp. chamber solution	8.4	76.27	B.D.	100.73	949.95	2.26	564.49	B.D.	B.D.	B.D.	0.58	1041.71
Left over stock solution	7.7	82.18	B.D.	89.25	842.20	2.25	483.10	B.D.	B.D.	B.D.	1.47	923.33
Method detection limit		4.7601	0.7166	0.0610	0.4751	0.0000	0.0053	0.0030	0.0100	0.0805	0.05	0.4532
Machine detection limit		0.4886	0.1965	0.0232	0.1790	0.0411	0.0143	0.0010	0.0045	0.0036	0.01	0.0962

Table 5. Mössbauer analysis of simulant material taken from flow-through and batch experiments, and OUCM-1 simulant taken from (Ramkissoon et al., 2019). Measurements have a ±2% detection limit error.

	Site population (%)									
	Fe-Silicate Glass	Phono-tephrite	Olivine	Pyrite	Fe^{3+}	Magnetite ($Fe^{2.5+}$)	Magnetite (Fe^{3+})	Hematite	Total	Fe^{3+}/Fe_{Tot}
OUCM-1	30	31	12	11	4	7	5	0	100	0.13
Abiotic, flow-through	33	34	5	8.1	6.5	7.2	6.1	0	99.9	0.16
Biotic, flow-through	33	26	6.2	9.4	6.4	8.2	5.3	6.1	100.6	0.22
Abiotic, batch	30	18	8.4	12.7	6	10.6	8.7	6	100.4	0.26
Biotic, batch	38	23	5.8	8.9	5.2	7.9	7.2	4.4	100.4	0.21

Table 6. Summary of alteration to silicate material.

	Flow-through Abiotic	Flow-through Biotic	Batch Abiotic	Batch Biotic
Secondary mineral	Fe deposit	Cu-S deposit	$CaCO_3$ crystals	$CaCO_3$ crystals SiO_2 spheres Fe-deposit Cu-S deposit
Dissolution textures	No	Yes	No	No
Fe partitioning*	Fe_{tot}^{3+} - 16.2% Fe_{tot}^{2+} - 83.7%	Fe_{tot}^{3+} - 21.9 Fe_{tot}^{2+} - 78.7	Fe_{tot}^{3+} - 26.0 Fe_{tot}^{2+} - 74.4	Fe_{tot}^{3+} - 20.8 Fe_{tot}^{2+} - 79.7
Raman	No carbon	No carbon	Disorder carbon	No carbon

*Initial Fe portioning for unaltered simulant was 12.5 % and 87.5% for Fe_{tot}^{3+} and Fe_{tot}^{2+-}, respectively

Figure Captions

Figure 1. The pH taken from effluent fluids collected from abiotic (orange) and biotic (blue) flow-through experiments.

Figure 2. SEM secondary electron images taken of each simulant component showing the different surface morphologies and deposits identified from the simulation experiments.

Figure 3. Cell counts taken from biotic simulation experiments. Black dashed line represents the cell count for the initial inoculum. The headspace was refreshed on day 3, 11 and 21. The errors were determined by calculating the standard deviation from the three samples taken daily.

Figure 4. 16S rRNA gene profiles of microbial communities enriched in simulated martian fluids under flow-through of batch conditions after 28 days. The 16S rRNA gene profile of the

inoculum is from Macey et al., 2020. All genera pictured are present at >1 % relative abundance.

Figure 5. Graphs of fluid chemistry form abiotic (a) and biotic (b) batch experiments.

Figure 6. Raman spectrum of disordered carbon observed on simulant taken from the abiotic batch experiment.

Figure 7. Ternary plot showing the relationship of fluid compositions between fluids derived in this study, modelled martian fluids, experimental martian fluids and naturally occurring terrestrial fluids.