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BIOFILM ADHESION ON THE SULFIDE MINERAL BORNITE & IMPLICATIONS FOR ASTROBIOLOGY

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BIOFILM ADHESION ON THE SULFIDE MINERAL BORNITE & IMPLICATIONS
FOR ASTROBIOLOGY

BY

MARGARET M. WILSON

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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IN

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UNIVERSITY OF RHODE ISLAND

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THESIS
OF
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2019

ABSTRACT

We present research observing and documenting the model organism, *Pseudomonas fluorescens* (*P. fluorescens*), building biofilm on a natural mineral substrate composed largely of bornite (Cu_5FeS_4), a copper-iron sulfide mineral, with closely intergrown regions of covellite (CuS) and chalcopyrite (CuFeS_2). In examining biofilm establishment on sulfide minerals, we investigate a potential habitable niche for microorganisms in extraterrestrial sites. Geochemical microenvironments on Earth and in the lab can also serve as analogs for important extraterrestrial sites, such as sheltered, subsurface microenvironments on Mars. The sulfur cycle has been important to Mars's surficial and interior processes during most of its geological record (King and McLennan, 2010; McLennan and Grotzinger, 2009). By growing *P. fluorescens* on polished chips of a sulfur-rich mineral (a Martian microenvironment analog material), we asked which distinct mineral phases bacteria selected as adhesion points. We collected cell count data, applied Raman spectroscopy, and tested FTIR spectroscopy as a complementary tool, to query whether biologically derived signals increase in intensity as bacteria grow in culture, and if the character of the mineral substrate controls cell distribution to some degree. Through use of the ANOVA statistical test on Raman peak intensity data, we found significant differences between the biologically derived signal intensity (*i.e.*, bonds related to EPS, amide I, amide III) for minerals: amide presence on bornite differs from on other substrates. ANOVA tests of cell counts yielded no significant difference between mineral substrate. We report that bornite mineral surfaces are subtly preferred in early adhesion by this model biofilm former.

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PREFACE

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Biofilm adhesion on the sulfide mineral bornite and potential implications for astrobiology on Mars

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Abstract

We present research observing and documenting the model organism, *Pseudomonas fluorescens* (*P. fluorescens*), building biofilm on a natural mineral substrate composed largely of bornite (Cu_5FeS_4), a copper-iron sulfide mineral, with closely intergrown regions of covellite (CuS) and chalcopyrite (CuFeS_2). In examining biofilm establishment on sulfide minerals, we investigate a potential habitable niche for microorganisms in extraterrestrial sites. Geochemical microenvironments on Earth and in the lab can also serve as analogs for important extraterrestrial sites, such as sheltered, subsurface microenvironments on Mars. The sulfur cycle has been important to Mars's surficial and interior processes during most of its geological record (King and McLennan, 2010; McLennan and Grotzinger, 2009). By growing *P. fluorescens* on polished chips of a sulfur-rich mineral (a Martian microenvironment analog material), we visualized which distinct mineral phases bacteria selected as adhesion points. We collected cell count data and applied Raman spectroscopy to query whether biologically derived signals increase in intensity as bacteria grow in culture, and if the character of the mineral substrate controls cell distribution to some degree. Through use of the ANOVA statistical test on Raman peak intensity data, we found significant differences between the biologically derived signal intensity (*i.e.*, bonds related to EPS, amide I, amide III) tied to each mineral phase. ANOVA tests of cell counts yielded no significant difference between mineral substrate. We report that biofilm generally grows successfully on all of the minerals considered, and that there may be mineralogical preference for bornite in early cell adhesion.

1. Introduction

1.1 Scientific background

Research of the last century has illuminated the possibility of life beyond Earth. Mars is specifically of interest due to its location in the “habitable” zone of the solar system. Water once pooled across its surface, and evidence suggests the surface of early Mars had a variety of geochemical environments suitable for forming a biosphere (Martinez-Frias et al., 2007; Rull et al., 2017; Schulze-Makuch et al., 2008; Weiss et al., 2000). In particular, Mars’s geologic history is intertwined with its sulfur cycle (see 1.3). Earth’s own sulfur cycle relies heavily on microbial activities, and astrobiologists can use terrestrial sites, such as sedimentary mud rocks and massive sulfide deposits, to inform our understanding of the parameters *endoliths* require to survive. In planetary environments, *endoliths* are microorganisms that reside in rock and pores between mineral grains. Many endoliths are also extremophiles--they survive in extreme environments with living conditions such as extreme heat and cold, high acidity, intense pressure, and limited resources (Wierzchos et al., 2011). Terrestrial endoliths are of great interest to astrobiologists as they represent the possibility of Martian or other extraterrestrial micro-organisms taking refuge in similar environments (Cabrol and Grin, 1995; Wierzchos et al., 2011; Wynn-Williams, 2000).

As Mars transitioned into a colder, dryer planet, life would have had to seek refuge in the subsurface as endolithic chemolithotrophs (lifeforms able to convert inorganic reduced compounds into energy). Fe and S are the two most abundant compounds on Mars’s surface (King and McLennan, 2010; McLennan and Grotzinger, 2009; Taylor, 2013), and on Earth we see ecosystems thriving in massive iron-sulfide deposits- some of which are analogue sites for Mars (ex. Rio Tinto, Spain and ancient Meridiani

Planum, Mars) (Hurowitz et al., 2010; Martinez-Frias et al., 2007; Ohmoto et al., 2008; Schulze-Makuch et al., 2008; Tosca et al., 2005)

Benchtop experiments that simulate geochemically extreme environments can serve as analogs for critical extraterrestrial sites, such as sheltered, subsurface microenvironments (Blanco et al., 2013; Cabrol and Grin, 1995; Martinez-Frias et al., 2007; Schulze-Makuch et al., 2008; Wierzchos et al., 2012, 2011). We can observe in the lab microorganisms interacting with Mars-relevant minerals to inform our understanding of in which geochemical environments biofilms are most productive, and how biomarker signals may vary in relation to the amount of time a biofilm has to adhere to a surface.

The mechanistic process of biofilm adhesion marks the creation of a bacterial community and the various proteins used to achieve this adhesion are all indicative of life and detectable by Raman spectroscopy (Boyd et al., 2014; Kengne-Momo et al., 2012; Maquelin et al., 2002; Rygula et al., 2013; Yang et al., 2015). With sulfur and sulfides detected across Mars sites that bear astrobiological interest, it is worthwhile to investigate the effectiveness of Raman spectroscopy in biosignature detection on sulfur and iron-bearing minerals: for the purposes of this paper, amides and EPS will be the primary biomarkers investigated for evidence of surface adhesion and the established presence of microorganisms on astrobiologically relevant geological surfaces.

In this study, we develop a novel protocol for studying biofilms on Mars-relevant minerals to (a) determine the effectiveness of Raman spectroscopy in identifying characteristic peaks of adhesion proteins and other biosignatures on astrobiologically relevant minerals, (b) apply spectroscopic data in an astrobiological context, and (c)

observe whether fine-scale geochemical heterogeneity in a sulfide mineral matrix may control bacteria adhesion. This protocol uses a model organism, *Pseudomonas fluorescens* (*P. fluorescens*) to generate biofilm, establish “proof of concept,” and align spectroscopic and direct count observations to determine adhesion site selectivity in Fe/Cu/S-bearing mineral environments.

1.2 Sulfur on Mars

Mars has long been considered a sulfur-rich planet (Burns and Fisher, 1990; Greenwood et al., 2000; King and McLennan, 2010; Lorand et al., 2018; McLennan and Grotzinger, 2009). Current evidence suggests that the sulfur cycle may have dominated the planet’s surficial processes during the greater part of its geological record. A primitive mantle composition of up to twice the sulfur content of Earth’s (>400ppm), would make the differentiated interior an important S reservoir, and sulfur-bearing soil and rocks outcrop in locations of astrobiological interest (King and McLennan, 2010; Lorand et al., 2018; McLennan and Grotzinger, 2009). The Compact Reconnaissance Imaging Spectrometer for Mars (CRISM) onboard the Mars Reconnaissance Orbiter (MRO) detected Fe- and S-bearing minerals such as sulfates (Salvatore et al., 2018; Taylor, 2013; Taylor et al., 2007).

There are three major mineral forming eras in Martian history: the phyllosian (early Noachian), the theikian (late Noachian to Hesperian), and the siderikian (3.5 billion years ago to present day) (Baumgartner et al., 2017; Bibring et al., 2006; Poulet et al., 2005). These eras are derived from the major mineral deposits of hydrated

silicates, sulfates, and iron oxides that we see today. Bibring et al. (2006) and Poulet et al. (2005) used mineralogical thermal infrared mapping from the Mars Global Surveyor Thermal Emission Spectrometer (MGS TES), Mars Exploration Rover (MER) Opportunity, and the Mars Express Infrared Mineralogical Mapping Spectrometer (OMEGA) to provide a geological justification for the following climatic history of Mars. Early Mars must have had an active hydrologic system, where long-standing water could sit in contact with igneous material to generate widespread clay and phyllosilicate deposits. Distinct sulfate units on the surface today suggest Mars then transitioned from a moist and alkaline environment to an acidic one. Volcanism likely injected sulfur into the atmosphere, where it then precipitating onto the surface as sulfuric acid to generate sulfates. Eventually Mars transitioned into the cold and dry world we know today.

Habitable conditions are most likely to have occurred over 3.5 billion years ago, where there was alkaline water present in either the surface or subsurface. This habitable period would have extended partially into the theiikian, during sulfate production. While degassing SO_2 is thought to be responsible for these minerals, it is also possible that pre-existing sulfides, weathered by acidic conditions, could generate sulfates as well (Kerber et al., 2015; Lorand et al., 2018; Tian et al., 2010). Sulfides have been identified as accessory phases in shergottite meteorites (Baumgartner et al., 2017; Franz et al., 2014; Gattacceca et al., 2013; Lorand et al., 2018) and have been proposed as important constituents of some areas near the Martian surface (Bell III, 1996; Burns and Fisher, 1990).

Where potential oxidants are concerned, clays and metal oxides composing surface minerals (such as Fe_2O_3 and FeO_4^{2-}) have been detected on the surface today and were likely widely available during Mars's early clay forming phase (Lasne et al., 2016). H_2O_2 in the Martian regolith produced via the interaction of minerals and water is suggested to have been available as an oxidant during this time as well (Lasne et al., 2016).

1.3 Sample Selection

Primary sulfides disseminated in Martian crust: Sulfides are found as tiny grains in a groundmass of mafic rock. Sulfur is widely distributed on Mars, observed on the oxidized, weathered planetary surface generally as sulfates (Burns and Fisher, 1990; King and McLennan, 2010). Ancient environments could have hosted oxidative weathering of sulfides, releasing sulfur, stabilized in sulfate minerals (McLennan and Grotzinger, 2009).

Fig. 1 Environments on Mars where sulfides are anticipated. Bornite (Cu_5FeS_4) is an orthorhombic sulfide mineral that is copper-red to brown in color. Fresh exposures will quickly tarnish to an iridescent purple, which gives bornite its nickname “peacock ore.” It has a hardness of 3, poor cleavage. It belongs to the Pbca space group and the dipyramidal class. Bornite has four known crystal forms: cubic, dodecahedral, truncated octahedral, and penetration twinning on $[111]$ (Duda and Rejil, 1990; Sinkankas, 1966) (Molecular Cube by K. Joseph from the Noun Project)(Duda and Rejil, 1990; Sinkankas, 1966)(Duda and Rejil, 1990; Sinkankas, 1966).

Ore-type unit of massive sulfide: Cu and Fe sulfide ores form through planetary processes. Hydrothermal circulation drives hot, often acidic waters through country rock, and generates predictable packages of sulfide-rich minerals when hot, chemically evolved waters vent into cooler, more oxidizing water reservoirs (e.g., black smoker systems in the seafloor on modern Earth). Magmatic processes generate igneous rocks of particular compositions: as magma cools, mineral constituents crystallize, leaving behind gradually altered residual melts (Brimblecombe, 2013; Burns and Fisher, 1993; Canfield et al., 2005); sulfides can coalesce as large ore bodies.

Sedimentary mud rocks: On Earth, ocean water containing sulfur as free SO_4^{2-} percolates through sedimentary bedding at the ocean floor. This continually feeds oxidized sulfur in the form of sulfate ions through sedimentary substrate containing Fe

and Cu, which takes up sulfur to form sulfide mineral deposits (Amend and Shock, 2001; Brimblecombe, 2013; Canfield et al., 2005; Jørgensen, 1977). On Mars, we could see similar redox fronts with stable sulfates and sulfides in ancient marine or lacustrine sedimentary systems.

The field of sulfide mineral surfaces and surface reactivity is relatively new, however it is of research interest because of sulfides' role in functionalization of organic molecules (Vaughan, 2006). Chalcopyrite (CuFeS_2) is considered the most important copper ore mineral, and during aqueous oxidation the sulfide forms a layer of Fe_2O_3 at the surface, leaving behind an unoxidized, metastable CuS_2 within the chalcopyrite crystal structure (Vaughan, 2006). This has the effect of reducing the chemical reactivity of the mineral surface. The majority of sulfides experience a similar process when reacting with water or any other chemical process; the surface is typically altered or consumed by the reaction (Vaughan, 2006).

Biochemical reactions are also responsible for the cycling of sulfides on Earth's surface. Iron sulfides are considered the most abundant and important sulfides in biosystems, because many microorganisms can heighten sulfide dissolution by oxidizing the metals in sulfide minerals (Vaughan, 2006). One such case of biologically mediated dissolution is in the case of acid mine drainage (AMD). When exposed to oxidative conditions, the sulfide minerals will dissolve and produce acidic waters. Sulfur- and iron-oxidizing bacteria will often catalyze this reaction to generate metabolic energy (Vaughan, 2006).

For this study, we selected the sulfide mineral bornite (Cu_5FeS_4) as our astrobiologically relevant substrate (Fig. 2). First, sulfides (and bornite specifically) are

known to occur in the meteorite record, and commercially available bornite specimens have patchy intergrowth of chalcopyrite and covellite—allowing simultaneous querying of related sulfides. Second, there are multiple known surface locations on Mars with sulfide-enriched basalts (Dehouch et al., 2012). Third, there is potential for concentration of sulfide ore minerals through Mars’s early sulfur cycling. Fourth, sedimentary strata that experience through-flow of sulfate-rich waters can host localized sulfidic zones, recording redox fronts in the planetary subsurface. In all of these cases, if early Mars hosted S-cycling microbial life, there is potential for localized production of biomarker compounds in/on/around sulfide particles, making them strong astrobiological targets.

1.4 Biofilm adhesion to mineral surfaces

*Fig. 2 The three major phases of biofilm growth: (I) attachment and aggregation, (II) EPS formation 3D structure development, and (III) dispersal. Green and orange cells represent live and dead *P. fluorescens* cells respectively. (I) Planktonic cells associate with a surface and form a temporary attachment. (II) Cells permanently adhere to the surface, signaling other cells to congregate and begin to excrete extracellular polymeric substances (yellow). (III) Biofilm matures, and cells near the outer surface of the biofilm can escape and build new colonies.*

Biofilm is an accumulation of microorganisms, such as bacteria that have extruded an extracellular polymeric substance (Boyd et al., 2014; Maquelin et al., 2002; Steinberg and Kolodkin-Gal, 2015). Before a dense biofilm forms, bacteria create a transient association with a surface (Boyd et al., 2014; Hinsä et al., 2003; Yang et al., 2015). When bacteria form a stable connection, bacteria produce a biofilm,

which provides a clement shelter (of particular biogeochemistry) for the microbial community to grow.

A biofilm's extracellular polymeric substance (EPS) is made from mostly polymeric sugars, proteins, lipids, and nucleic acids (Steinberg and Kolodkin-Gal, 2015; Wan Dagang, 2012). Amino acids are organic compounds indicative of biological processes. Composed of carboxyl (-COOH) and amino (-NH₂) groups, amino acids are the building blocks for proteins found in all terrestrial life and therefore make an ideal biomarker (Reece et al., 2014). Amides are a functional group within amino acids and are used by biofilms, among other things, for surficial adhesion (Maquelin et al., 2002; Reece et al., 2014). Raman spectra for amides in general have been well documented, along with complementary peaks for EPS (Edwards et al., 2005; Maquelin et al., 2002; Rygula et al., 2013).

1.5 Raman spectroscopic applications in astrobiology

Raman spectroscopy is a valuable tool for future planetary exploration endeavors and will be used for detecting biosignatures on Mars within the next two years (Beegle et al., 2015; Marshall and Marshall, 2014; Rull et al., 2017). In addition, biofilm adhesion is a critical biological process for microbial life, and leaves relatively durable amide and protein deposits on mineral surfaces, albeit at very fine scale.

Previous studies have created libraries of Raman spectra for identifying proteins, bacterial species, and minerals (Beegle et al., 2015; Haskin et al., 1997; Jorge Villar et al., 2006; Maquelin et al., 2002; Rygula et al., 2013). Efforts to characterize the

mineralogy of Jezero Crater, the landing site for Mars 2020, via Raman spectroscopy are underway (Beegle et al., 2015; Salvatore et al., 2018). Also, ExoMars, a joint mission between the European and Russian space agencies, plans to bring a Raman spectrometer to Mars (Marshall and Marshall, 2014; Rull et al., 2017). A library of Raman spectra for astrobiologically important minerals is in development. We offer data that reflect the resolution power of Raman spectroscopy in detecting markers of biological activity and consider the rigor of the technique through comparison with a classic direct count approach.

2. Materials and Methods

2.1 Sample preparation

Bornite samples, provided by Ward Science, were first analyzed using powder x-ray diffraction (XRD) and confirmed to contain bornite along with traces of covellite (CuS). Bornite samples were subsampled into approximately 1-cm-wide chips before being secured in a polishing ring with orthodontic resin. Samples were polished first by hand using tap water to wet 8" silicon carbide grinding disks (grits 400, 600, and 1200), followed by monocrystalline diamond suspension (grits 3 μm , 1 μm , 0.25 μm) on 8" TexMet C polishing cloths; all polishing cloths (sku # 40-1108, 36-08-400, 36-08-0600, & 30-08-1200) and suspension fluid (sku # 40-6531, 40-6530, 406629) purchased from Buehler. Once polished, a shallow acetone bath was used to remove the samples from resin. One sample was reserved for electron probe micro-analysis (EMPA). Polished chips were broken carefully into smaller chips and affixed to glass squares for

submersion in culture broth. Samples were given one final polish with the 0.25 μm diamond suspension, rinsed with DI water, and left to dry.

2.2 EMPA analysis

Chemical analyses and x-ray element mapping for one polished bornite sample was completed (Electron Microprobe Facility, Brown University, Providence, RI). Signals used included *S Ka*, *Fe Ka*, and *Cu ka*. Spectrometers conditions were *Sp2 PET*, *Sp3 LLIF*, *Sp5 LIF*, *Sp3 LLIF*, *Sp4 LLIF*, *Sp5 LIF*, *Sp4 LLIF*, *Sp4 LLIF*, *Sp3 LLIF*, *Sp2 PET*, with column conditions at 20 keV and 20nA. The only elements that needed overlap correction were *Mn Ka* and *Co Ka*. At least 12 points of analysis were taken for each of the three distinctly visible mineral phases observed within the sample. The three mineral phases were determined to be bornite, chalcopyrite, and covellite.

2.3 *Pseudomonas fluorescens*

Chemosynthesis is defined by Merriam-Webster as “the synthesis of organic compounds by energy derived from inorganic chemical reactions.” Chemosynthetic organisms rely on these inorganic chemical reactions for their metabolism, whereas **photosynthetic** organisms need light to generate organic compounds. Chemosynthesis should not be confused with **chemotaxis**, which refers to how a bacterial cell will move in relation to chemical resources. **Obligate aerobic** organisms require oxygen to metabolize carbohydrates and grow.

P. fluorescens is an obligate aerobe that can exhibit chemotaxis when necessary, however flagella are not required for biofilm formation (de Weert et al., 2007; Smith & Doetsch, 1968; Wan Dagang, 2012). Instead, *P. fluorescens*' biofilm formation is influenced by surface hydrophobicity, electrostatic, and van der Waals interactions. Steric effects (nonbinding interactions that can dictate molecule reactivity) can also influence biofilm adhesion for *P. fluorescens*. Flagella help propel the bacteria cell in near-surface swimming while pili assist in walking or crawling along the surface (Conrad et al., 2011; Wan Dagang, 2012).

2.4 Culture preparation and incubation

We dissolved a *P. fluorescens* culture wand in 1 liter of sterile BD Difco™ Dehydrated Culture Media. The broth was

Fig. 3 Optical image of *P. fluorescens* cells on polished bornite, 100 micron scale (left) and 10 micron scale (right). The purple matrix is bornite, gold is chalcopyrite, and blue is covellite. Individual cells can be seen clustering on the covellite and bornite phases.

left to incubate in a fume hood, and later experiments used this primary culture as their source.

A few drops of the primary culture were added to a smaller bottle of LB Broth, 12 to 24 hours before initiating experiments; cells were transferred to smaller bottle of broth during exponential growth.

Triplicate mineral chips were incubated in the bottles for 2, 6, 12, and 24 hours. After sterilizing with ethanol, one mineral chip was placed in each well of a 6-well plate (Thermo Scientific, item # 130184) before filling the wells with ~9 ml of LB broth and

~1 ml liquid culture and leaving the samples to incubate. When incubations completed, I transferred the samples into a clean 6-well plate where they were able to dry still in the fume hood. A transfer pipette moved left over liquid media and biofilm into a UV cuvette so that a NanoDrop ND 1000 UV/VIS spectrophotometer (courtesy of URI's INBRE laboratory facilities) could measure the fluid's optical density and generate a growth curve for *P. fluorescens* which aligned with established literature.

2.5 Raman spectroscopy analysis

Samples were rinsed in triplicate with filtered tap water and allowed to dry. We used a WiTech confocal Raman spectrometer with a 758 nm laser at ~2.00 mW power. For each sample, three readings were collected at 100x magnification (each reading is a total of 100 five second accumulations) for each mineral phase (bornite, covellite, and chalcopyrite). Spectra underwent cosmic ray removal and background correction. R studio was used to generate spectral plots. Individual *P. fluorescens* cells were visible and counted using the spectrometer's optical microscope. Samples incubated for 24 hours showed the most promise for detecting biofilm adhesion deposits. 10 images were captured from 10 different locations on each 24h sample surface to be used for later cell counts.

Table 1. Raman band assignments used for analysis, references provided. Note that bornite has a weak Raman scattering due to its metallic character, thermal sensitivity, and lack of covalently bonded sulfur atoms. References: [1] Chao and Zhang, 2012; [2] Guicheteau et al., 2008; [3] Kahraman et al., 2009; [4] Maquelin et al., 2002; [5] Mernagh and Trudu., 1993; [6] Parker et al., 2003; [7] Crundwell, 1988; [8] Goble and Scott, 1985; [9] Ishii et al., 1993.

Organics		Minerals	
Frequency (cm ⁻¹)	Assignment	Frequency (cm ⁻¹)	Assignment
1650-1680 ^[1,2,4]	Proteins (amide I)	378 ^[5]	Chalcopyrite
1571-1572 ^[1]	Proteins (amide II)	352 ^[5]	Chalcopyrite

1440-1455 ^[1,3,4]	Lipids	322 ^[5]	Chalcopyrite
1230-1295 ^[1,4]	Proteins (amide III)	293 ^[5-7]	Chalcopyrite (lattice vibrations)
1000-1010 ^[1-3]	Proteins (phenylalanine)	300 ^[5,6,8]	Bornite
720-734 ^[1,4]	Nucleic acids	474 ^[6,9]	Covellite (S-S stretch)
637-695 ^[1,3]	Proteins (tyrosine)	267 ^[6,9]	Covellite (other lattice modes)
565-582 ^[1,2]	Carbohydrates	263 ^[5]	Covellite
479-495 ^[1,3]	Carbohydrates	139 ^[5]	Covellite
408-423 ^[1,3]	Carbohydrates	116 ^[5]	Covellite

2.6 Cell counts via plating

P. fluorescens was inoculated in liquid culture (LB broth) and left to incubate at 27 °C for 24 hours. At time 0 hours, 2 hours, 12 hours, and 24 hours, a 100 µm aliquot of culture was diluted with 900 µm of PBS and suspended (10^{-1} concentration). 100 µm of the 10^{-1} dilution was then diluted in another 900 µm of PBS, and so on until a dilution of 10^{-7} was reached. 3x10 µm of each dilution was placed onto an LB media plate and incubated at 27 °C until viable colonies could be counted. The results of that cell count and the corresponding growth curve are presented below.

Table 2 Cell count using standard plating technique. Colonies counted (C_n) were totaled, then used to calculate CFU (colony forming unit) per µl.

Time step (hours)	Dilution	C_1	C_2	C_3	CFU/µl
0	10^{-4}	57	58	80	1.95×10^5
2	10^{-5}	14	16	27	5.70×10^5
6	10^{-6}	74	115	97	2.86×10^7
12	10^{-7}	55	63	67	1.85×10^8
24	10^{-5}	80	94	80	2.54×10^6

Fig. 4 *P. fluorescens* growth curve, showing how CFU/μl increases over time. The lag phase is approximately from 0 to 2 hours, exponential phase is 6 to 24 hours. Data were not collected beyond 24 hours since later experiments will not exceed 24 hours of incubation time.

2.7 Statistics

The ANOVA two-factor with replication is a statistical technique that proves differences among group means in a sample and is appropriate for use in this study because we aim to see what effect, if any, slight differences in geochemistry have on biosignature strength (Kim, 2014; Mehrens et al., 2005). Using ANOVA two-factor with replication, we compared the average intensities of the three mineral phases at each timestep (t at 0, 2, 6, 12, and 24 hours) for three peaks of interest: amide I ($1650-1680\text{ cm}^{-1}$), amide III (1294 cm^{-1}), and nucleic acids ($720-734\text{ cm}^{-1}$). These specific wavenumbers were selected within published ranges for proteins and carbohydrates indicative of DNA (Chao and Zhang, 2012; Crundwell, 1988; Goble and Scott, 1985; Guicheteau et al., 2008; Ishii et al., 1993; Kahraman et al., 2009; Maquelin et al., 2002; Mernagh and Trudu, 1993; Parker et al., 2003). The ANOVA addresses the null hypothesis (H_0) that there is no change in organic compound surface loading, as observed in related peak intensity values, across mineral phases; the corresponding

hypothesis (H_a) is that the peak intensity values vary significantly across mineral phases, supporting mineralogical control over organic loading.

3. Results and Discussion

3.1 EMPA analysis

Fig. 5 X-ray element maps generated by EPMA. Scale bar is 1000 microns. Left: Cu. Middle: Fe. Right: S. Covellite (cov), chalcopyrite (chalc), bornite (bor). The brighter the color, the higher the element concentration. Bornite has fine-scale heterogeneity, and the three mineral phases are identifiable in these images. Images were rendered false color using Photoshop.

Microprobe data confirmed visual observations that the sample had three distinct sulfide mineral phases: bornite (Cu_5FeS_4), covellite (CuS), and chalcopyrite (CuFeS_2). The electron microprobe scanning electron microscope (SEM) accessory showed that covellite formed along defects and narrow fissures, whereas chalcopyrite crystals were embedded in the bornite matrix.

Table 3. Major elemental abundances of polished bornite and accessory minerals (ppm), and standard deviations ($n=12$). Typical composition (in ppm) of bornite is 111300 Fe, 633100 Cu, and 255600 S; covellite is 664600 Cu, 335400 S; chalcopyrite is 304300 Fe, 346300 Cu, and 349400 S (Duda and Rejil, 1990; Sinkankas, 1966).

Bornite						
Element	Bornite	σ_{bornite}	Chalcopyrite	$\sigma_{\text{chalcopyrite}}$	Covellite	$\sigma_{\text{covellite}}$
S	255400	0.8846	339300	1.223	310300	1.075
Fe	110800	0.3837	285400	1.029	8758	0.03034
Cu	623900	2.161	358100	1.291	662800	2.296

Table 3 shows the three phases identified in geochemical analysis. Note in B, there are dark veins within the mineral (low iron). These are veins of covellite (CuS). Bornite, with a higher weight percent of Cu compared to chalcopryrite or covellite, dominates A, leaving chalcopryrite as the brightest regions in B and C.

3.2 Raman spectroscopy

Four plots were generated for each mineral in the amide III range to show the Raman spectra for each mineral phase (bornite, chalcopryrite, covellite) within the sample and the glass control; spectra collected for covellite are presented in Fig. 5, remaining spectra are in the appendices. Well-defined peaks from proteins, like amides, that are directly involved with attaching bacterial cells to mineral surfaces correspond to 1250 cm^{-1} , 1550 cm^{-1} , and 1670 cm^{-1} .

B. Covellite

C. Chalcopyrite

D. Glass

Fig. 6 Raman spectra for bornite (A), covellite (B), chalcopyrite (C), and glass (D) in the amide III region, at 24 hours. Data has undergone SNV normalization. Covellite and glass have the most well-defined trends, with covellite having a higher intensity in the lower range. Bornite and chalcopyrite have less defined peaks in this range and likely have a lower biomass content compared to covellite and glass. This shows that there could be more biomass loading on glass and covellite. Glass, being more transparent than bornite, likely allowed for a better signal to be resolved, whereas with covellite it could be a potential adhesion preference for the biofilm.

Raman spectra were processed using standard normal variate (SNV)

normalization followed by min/max normalization for each wavenumber range of interest, as justified in the next section. (Gautam et al., 2015; Liland et al., 2015). For ANOVA tests, a continuity correction factor is used to correct p-values, since we are looking for a binomial distribution (probability of successfully detecting a Raman shift in biosignature regions).

Samples incubated for 24 hours were analyzed within 1 week of being harvested. 12 hours were analyzed within 1 to 4 weeks, 6 hours within 3 to 4 weeks, 2 hours within 1 month. Longer wait times for Raman analysis led to a deteriorated intensity, which background processing should correct for.

3.3 Key changes in biosignature intensity over time

As discussed in section 1.4, as a biofilm evolves, cells excrete increased amounts of EPS and proteins. High concentrations of amides are indicative of increased biofilm production, as they are a major building block of proteins and suggest the presence or former presence of amino acids. Simply put, based on these results, the longer a biofilm grows, the higher the concentration of amino acids and EPS. As a result, biomarker intensity will increase over time.

In all samples, amide III intensity varies statistically significantly between substrate, whereas other biosignatures don't show enough change to be considered significantly different. Amide I and amide III both have strong Raman signals, however, amide I tends to have overlapping peaks with different secondary structures, as does EPS (Rygula et al., 2013; Singh, 2009). Secondary protein structures have far more resolved peaks in the amide III region (Singh, 2009). As in Fig. 6, we can see that the amide I peak ($\sim 1650\text{-}1680\text{ cm}^{-1}$) is obscured by neighboring signals. The same tends true for all wavenumbers greater than 1000 cm^{-1} .

Table. 4 Raman peak values used in calculating ANOVA tables for covellite, bornite, chalcopyrite, and glass (control). Data have undergone SNV normalization & cosmic ray removal.

Tyrosine/Guanine ($637\text{-}695\text{ cm}^{-1}$)					
Mineral Phase	0h	2h	6h	12h	24h
Covellite	1.47	1.28	1.69	1.08	1.361.36
	1.64	1.54	1.22	1.79	1.49
	1.23	1.17	1.50	1.13	1.50
	1.40	1.68	1.23	1.72	1.40
	1.64	1.45	1.36	1.10	1.28
	1.30	1.28	1.48	1.85	1.43
	1.23	1.28	1.57	1.48	1.21
	1.38	1.43	1.77	1.60	1.51
	1.48	1.63	1.43	1.40	1.36
Bornite	1.27	1.55	2.09	2.00	1.47
	1.59	1.90	2.46	1.83	1.78

	1.45	1.66	1.39	3.44	1.49
	1.52	1.62	1.06	1.08	1.52
	1.37	1.31	1.43	1.59	1.82
	1.53	1.42	1.63	2.19	2.07
	1.67	1.55	1.82	1.20	0.96
	2.31	1.61	1.28	1.70	1.41
	1.42	1.25	1.62	1.88	3.75
Chalcopyrite	2.061.50	1.56	0.93	2.31	1.56
	1.50	1.82	1.22	1.68	2.32
	2.09	1.85	2.41	2.27	1.93
	2.49	1.73	1.85	1.96	1.74
	2.69	1.63	1.76	2.15	2.18
	2.76	2.12	1.63	1.92	2.43
	1.74	1.68	2.22	1.62	1.69
	1.82	1.60	1.50	2.39	1.30
	1.90	2.07	1.45	1.55	2.30
Glass	1.37	1.25	1.20	2.17	1.57
	1.30	2.10	1.54	2.00	2.22
	2.21	2.06	1.37	1.79	3.05
	1.86	1.46	2.82	1.69	2.51
	1.35	2.31	1.74	2.46	2.48
	1.71	1.86	1.62	3.42	2.10
	1.64	1.65	2.45	1.53	1.36
	1.52	1.86	2.74	1.48	2.05
	1.47	1.58	2.12	2.06	1.57

Nucleic Acids (720-734 cm⁻¹)

Mineral Phase	0h	2h	6h	12h	24h
Covellite	1.06	1.08	1.30	1.72	0.82
	1.29	1.12	1.25	1.22	0.94
	1.08	1.29	1.24	1.35	1.42
	1.32	1.46	1.23	1.08	1.16
	2.18	1.18	1.86	1.70	1.58
	0.69	1.03	1.66	1.71	1.19
	1.75	1.54	0.93	0.85	1.42
	1.35	1.71	1.49	1.43	1.23
	1.09	1.42	1.36	1.22	1.29
Bornite	1.56	1.34	1.43	1.10	1.45
	1.54	2.05	0.95	1.67	1.09
	1.38	1.41	1.24	1.44	1.44
	1.30	1.48	1.86	1.03	1.62
	1.37	1.21	1.50	1.46	1.74
	1.53	1.71	1.38	1.53	1.57
	1.07	1.27	0.97	2.34	0.90
	0.85	1.28	1.18	1.27	1.43
	1.70	1.51	1.43	1.91	1.21

Chalcopyrite	1.39	1.80	1.41	1.60	1.09
	1.19	1.22	0.91	2.11	1.53
	2.11	1.37	1.19	1.72	1.49
	1.68	1.13	1.51	1.52	1.19
	2.11	2.22	1.32	0.95	1.57
	1.06	1.58	1.36	1.17	1.38
	1.19	1.22	1.10	0.75	1.39
	1.32	1.29	1.02	0.94	1.31
	1.29	1.69	1.38	1.23	1.69
Glass	1.97	1.57	0.71	1.01	0.52
	0.79	1.14	1.90	1.16	1.47
	1.11	2.37	1.49	1.55	1.26
	1.54	1.45	1.19	1.27	1.62
	1.50	1.30	1.52	1.22	1.39
	1.54	1.02	0.95	1.43	1.32
	1.09	1.18	1.78	1.87	1.11
	1.26	0.69	1.44	1.01	1.51
	1.32	1.44	1.01	1.46	1.41

Amide III (1230-1295 cm⁻¹)

Mineral Phase	0h	2h	6h	12h	24h
Covellite	2.06	2.43	2.49	1.35	2.89
	3.06	2.10	2.19	2.20	2.29
	2.32	2.78	2.34	2.31	2.12
	2.18	2.51	2.41	2.18	2.31
	2.14	2.25	2.04	3.04	2.48
	2.41	2.22	2.31	2.84	2.39
	2.24	2.03	2.22	2.42	2.18
	2.34	2.28	2.03	2.98	2.27
	2.12	2.30	2.83	3.01	2.69
Bornite	2.27	2.45	2.14	2.87	2.73
	2.62	1.59	2.82	1.85	2.83
	1.68	2.43	2.59	1.54	1.87
	2.38	2.61	2.55	2.40	2.81
	2.03	2.12	1.65	1.70	2.09
	2.76	1.88	2.70	2.73	2.01
	2.27	1.96	1.92	3.12	2.36
	1.91	2.17	2.33	2.74	2.93
	3.01	2.00	2.19	2.13	1.88
Chalcopyrite	1.91	2.29	1.76	1.74	1.31
	1.50	2.17	1.30	1.89	1.49
	2.70	1.94	2.01	2.33	1.86
	1.85	1.33	2.02	1.64	1.98
	2.16	2.03	1.66	1.88	1.72
	2.57	1.45	1.59	2.20	2.02
	1.96	1.79	1.75	1.83	1.65

	1.81	2.48	2.02	1.67	2.09
	1.58	2.07	3.05	1.75	1.80
Glass	2.58	2.41	2.25	2.62	1.96
	2.55	2.44	2.41	2.27	2.47
	2.53	2.24	2.72	2.31	1.97
	2.37	2.65	2.60	2.58	2.52
	2.47	1.95	2.53	2.78	1.96
	2.78	2.58	2.45	2.26	2.34
	2.37	2.37	2.50	2.38	2.28
	2.60	2.04	2.65	2.95	2.47
	2.47	2.71	2.45	2.76	2.18

Amide I (1650-1680 cm⁻¹)

Mineral Phase	0h	2h	6h	12h	24h
Covellite	1.02	0.92	1.24	2.09	1.42
	1.54	2.25	2.02	2.11	1.45
	1.54	2.11	1.25	2.28	1.99
	1.91	0.91	1.68	1.90	1.60
	2.24	0.87	1.66	1.29	1.76
	1.92	1.80	1.93	1.75	1.51
	1.72	2.01	2.07	1.74	1.67
	1.72	1.02	2.39	1.60	0.96
	1.93	1.86	1.51	1.97	2.31
Bornite	2.34	1.79	1.20	1.62	1.81
	2.44	1.24	1.09	1.45	1.31
	1.15	1.23	1.22	1.74	2.17
	1.03	1.73	1.46	1.66	1.63
	1.91	1.88	1.44	1.13	1.16
	1.77	2.13	1.37	2.04	1.66
	1.89	1.85	1.09	1.78	1.74
	1.42	2.54	1.35	1.78	1.40
	2.14	2.09	1.77	1.49	1.36
Chalcopyrite	1.50	1.44	1.54	2.03	1.35
	1.10	1.57	1.66	1.19	2.39
	1.44	2.19	1.41	1.57	2.03
	1.53	2.32	0.79	1.73	1.59
	2.07	0.99	1.54	1.52	1.68
	1.63	1.39	1.79	1.24	1.03
	1.76	1.70	1.25	1.49	2.10
	2.17	1.58	2.70	1.80	1.83
	1.52	1.59	1.29	1.18	1.76
Glass	1.30	1.87	1.71	1.66	1.56
	1.25	1.96	1.45	1.30	1.65
	2.07	2.12	1.45	1.44	1.63
	1.63	1.40	1.75	1.85	1.61

1.78	1.80	2.12	1.78	1.18
1.58	2.49	1.32	1.12	1.63
1.42	1.91	1.38	1.39	1.47
1.39	1.67	1.31	2.34	1.37
1.44	1.70	1.45	1.54	1.54

The ANOVA test of background-corrected spectral intensity rejects the null hypothesis that they are the same. Over time, there is a significant difference between the biosignature intensities.

Table 5. ANOVA single factor test of Raman intensity over time at target regions. Alpha value of 0.05. Tests show that changes in amide III intensities on the glass control & amide I intensities on glass and bornite were significant.

Tyrosine/Guanine (637-695 cm ⁻¹)						
Mineral Phase	SS	df	MS	F	P-value	F critical
Covellite	0.039	4	0.252	0.252	0.907	2.61
Bornite	0.797	4	0.199	0.705	0.593	2.61
Chalcopyrite	1.12	4	0.280	2.00	0.114	2.61
Glass	1.53	4	0.383	1.58	0.200	2.61

Amide III (1650-1680 cm ⁻¹)						
Mineral Phase	SS	df	MS	F	P-value	F critical
Covellite	0.191	4	0.0477	0.418	0.794	2.61
Bornite	0.340	4	0.0850	0.453	0.769	2.61
Chalcopyrite	0.280	4	0.0699	0.521	0.721	2.61
Glass	0.599	4	0.150	0.150	0.0182	2.61

Nucleic Acids (720-734 cm ⁻¹)						
Mineral Phase	SS	df	MS	F	P-value	F critical
Covellite	0.114	4	0.0285	0.306	0.872	2.61
Bornite	0.248	4	0.0619	0.675	0.613	2.61
Chalcopyrite	0.418	4	0.104	0.944	0.450	2.61
Glass	0.0217	4	0.00543	0.405	0.997	2.61

Amide I (1230-1295 cm ⁻¹)						
Mineral Phase	SS	df	MS	F	P-value	F critical
Covellite	0.527	4	0.132	0.761	0.557	
Bornite	1.42	4	0.356	2.82	0.0377	2.61

Chalcopyrite	0.280	4	0.0699	0.444	0.776	2.61
Glass	0.814	4	0.204	2.64	0.0481	2.61
						2.61

Table 5 shows the ANOVA analysis of peak intensity at target wavenumber regions as a function of time. For all but amide I (bornite, glass control) & amide III (glass control) regions, signals do not vary significantly from time 0h to time 24h. Overall, growth seems to be fairly uniform throughout the incubation series, with the control exhibiting the most change over time. This is expected, as SNV normalization of Raman spectra scales the data so samples are comparable.

Table. 6 Calculated ANOVA p-values for mineral phase influencing peak intensities of tyrosine/guanine, nucleic acids, amide III, and amide I regions. Alpha value of 0.05.

P-values for statistical significance of mineral phase impacting peak intensity					
	0h	2h	6h	12h	24h
Tyrosine/Guanine	0.000302	0.001239	0.219	0.0744	0.0426
Amide III	0.0221	0.0210	0.00630	0.0110	0.000261
Nucleic Acids	0.779	0.590	0.833	0.613	0.449
Amide I	0.518	0.300	0.133	0.136	0.492

In Table 6, we see the ANOVA results comparing the intensity of each peak between mineral phases at each time step. As was the case in Table 4, amide III intensity seems to be influenced by our variable—in this case, the substrate. The tyrosine/guanine regions also show statistically significant differences between the mineral phases during the lag phase (0-2 hours), where bacteria adapt to their growth conditions, and at 24 hours (see appendix 3, fig. 1) (Steinberg and Kolodkin-Gal, 2015; Wan Dagang, 2012). The glass control Total cell numbers are fairly diluted in the lag phase and may not be evenly distributed. At 24 hours, biofilm growth has entered its exponential phase; many cells have adhered to the surface, increasing biomass, and in turn increasing the signal from tyrosine/ guanine regions. These statistical results suggest that fine-scale

differences in geochemistry are an influencing factor in biofilm growth and adhesion. Selection of specific mineral phases for adhesion points happens at a critical stage of early growth, and the mineral-specific distribution of adhesion points becomes visible later in the evolution of the biofilm-mineral system, when organic material has accumulated more densely on the mineral surface.

Averaged cell count results suggest that biofilm preferentially associated with covellite (depleted in Fe, higher in Cu and S relative to bornite and chalcopyrite), although cell counts are not statistically significant. Close examination of the polished sample surface via scanning electron microscopy (SEM) revealed that covellite grew along narrow fissures in the mineral. Bacteria have increased success in growth on uneven and rugged surfaces (Branda et al., 2005), which may explain why *P. fluorescens* seemed to congregate along these covellite veins. Fe makes up a smaller weight percent of bornite than chalcopyrite (11.13% and 30.43% respectively) (Duda and Rejil, 1990; Sinkankas, 1966). Geochemistry may be an important controlling factor in this study. These results indicate *P. fluorescens* had higher populations of bacterial cells on phases with depleted Fe and enriched in S.

3.4 Cell counts

To determine directly the effect geochemistry has on cell adhesion, analysis of variance tests were also performed on cell counts of samples that had been incubated for 24 hours. Twenty-one $5\ \mu\text{m}^2$ regions of interest on covellite, bornite, and chalcopyrite were used. The following statistics were calculated using ANOVA single factor.

Table. 7 Number of cells counted over $21 \times 5\ \mu\text{m}^2$ regions of interest on a 24h sample, and ANOVA single factor test (alpha factor = 0.05). ANOVA tests presented here were conducted on just the mineral samples, not glass, as glass had very low cell count and generated a p-value of 8.22×10^{-11} . This way, we are able

to compare the variances among solely the minerals. Averages indicate a greater number of cells on covellite, followed by bornite, and chalcopyrite last; the low *p* value shows this difference is statistically significant.

Groups	Num. ROI's	Cells/ μm^2	Variance
covellite	73	0.75	0.58
bornite	73	0.19	0.16
chalcopyrite	73	0.19	0.16
glass	73	0.08	0.07

Source of variation	SS	df	MS	F	<i>P</i> -value	<i>F</i> critical
Between groups	15.4	2	7.68	25.8	8.76×10^{-11}	3.04
Within groups	64.2	216	0.297			
Total	79.5	218				

Overall, average cell count numbers on the three mineral phases under study are close. Covellite attracted the largest volume of biomass, followed closely by bornite, with chalcopyrite (the most Fe-rich phase) last. Glass samples had very few cell counts despite the comparable biosignature readings from Raman spectroscopy. When counting cells, we noticed that glass tended to have visible EPS residue but few individual cells, which tended to be the opposite of the mineral samples. Preliminary ANOVA tests indicate that the cell counts on each mineral phase do not significantly differ; averages show that cell counts on mineral types are virtually the same. For future research, increasing the total number of regions of interest will yield a more accurate portrayal and clarify the relationship between geochemistry and adhesion preference.

3.5 FTIR Spectroscopy

ANOVA tests disproved the null hypothesis that the intensities of peaks in the amide III and tyrosine, guanine ranges are the same across mineral phases.

Considering this, we incubated a 24 hour sample for Fourier-Transform Infrared (FTIR) spectroscopic analysis. FTIR spectroscopy is considered complementary to Raman (Maquelin et al., 2002). Where Raman uses one frequency to generate a spectra, FTIR uses a range of frequencies in the electromagnetic spectrum to identify

the chemical makeup of a sample. For this experiment, we generated heat maps of our polished chip before and after incubation at key peaks: amide I ($1675, 1685, 1695\text{ cm}^{-1}$), amide III ($1240\text{-}1310\text{ cm}^{-1}$), nucleic acids (3200), and tyrosine/ guanine ($1515, 3200\text{ cm}^{-1}$). The results were complementary to our Raman ANOVA analysis; minerals had varying biomarker signal intensity.

Fig. 7 (below) shows the heat maps for amide III (1310 cm^{-1}). We can see that bornite initially has little to no signal in this range. The weak but visible signal in the post-incubation analysis verifies the introduction of amide III and thus biological materials onto the mineral surface. Chalcopyrite, which begins with some signal, also sees an increase over time. Covellite, however, is interestingly different. We see some areas that show the same increase in signal strength over time as bornite and chalcopyrite, however, the bottom half of the specimen does exactly the opposite. Further exploration would be needed to determine precisely why, but for the purposes of our experiment we are able to confirm an overall increase in biomarker signature intensity post-incubation.

Fig. 7 Heat maps of intensity of 1310 cm^{-1} on each mineral phase, before and after a 24-hour incubation cycle. Red indicates a high signal, blue indicates a weak signal.

3.6 Raman Spectroscopy in Astrobiology

In 2020, NASA's Mars Exploration Program will launch a new rover explicitly designed to investigate "high-priority science goals for Mars exploration," including seeking signs of habitable conditions or past microbial life in Mars's ancient history. To do this research, the rover is equipped with a suite of scientific instruments, including cameras, weather sensors, ground-penetrating radar, and spectrometers.

The Scanning Habitable Environments with Raman & Luminescence for Organics & Chemicals (nicknamed SHERLOC) is one of Mars 2020's many systems. SHERLOC's mission is to search for biosignatures using a camera, laser, and Mars's first ever Raman spectrometer. Considered complimentary to infrared spectroscopy, Raman is predicated on analyzing a material's spectral fingerprints (Marshall and Marshall, 2014; Rull et al., 2017). These fingerprints are caused by inelastic scattering of light when it interacts with matter and are measured in units of cm^{-1} , or wavenumbers. When the light of a single frequency or wavenumber, ν_0 interacts with a material, a small amount of it is sent scattering in different directions. The highest intensity in the spectral distribution of scattered light has the same incident wavenumber as ν_0 ; however, there is a visible spectral component at other wavenumbers. This Raman shift is caused by the incident light interacting with atomic and molecular vibration modes- modes which are characteristic of and can be used to identify the material being observed. Raman scattering tends to be a weak effect depending on the material's polarizability. However, Raman is still considered extremely useful and practical, especially for planetary exploration: it is nondestructive, fast, requires no sample preparation, requires no

physical contact with the sample, and the spectral bands provide definitive compositional characterization of the sample (Edwards et al., 2005; Franquelo et al., 2009; Haskin et al., 1997; Jorge Villar et al., 2006; Rull et al., 2017). On Mars, SHERLOC will use Raman spectroscopy to detect minerals, organic molecules, and potentially biosignatures.

The Raman spectrometer used in this study was a confocal Raman spectrometer, whereas SHERLOC is a Deep UV resonance Raman and fluorescence spectrometer. SHERLOC uses a type of Raman frequency that is sensitive to aromatic organics, making it highly effective at biomarker detection (10⁻⁶ w/w at <100 μ m scales). Our spectrometer used a much higher frequency laser (785 versus SHERLOC's 270 nm) at a low power (2mW). Initial trials showed that bornite would be “singed” very easily by the laser, forcing us to use the lower power. This likely had an impact on the weaker intensities in regions of interest, as biomaterial was thinly spread across the mineral surface and often overwhelmed by the mineral (or glass) spectra. SHERLOC is better prepared to circumvent similar issues, since it is more sensitive to organics than mineral substrate, however it is worthwhile to consider how substrate impacts the overall spectra.

We also polished our samples to reduce the amount of laser light absorbed. The objective depth of field (DOF) for our spectrometer is 9 μ m for a completely transparent sample. Mars 2020 will be analyzing unpolished particles of sand, clay, and solid rock, which could reduce DOF and add to the difficulties of detecting scarce or weak biomarkers.

3.7 Carbon on Mars

Currently, there is no quantitative estimate for total carbon on Mars. However, instruments designed to measure total organic and inorganic carbon (TOC and TI, respectively) have been proposed (Toon et al., 2019; Zent et al., 2006). Much progress has been made concerning Mars's atmosphere and sites accessed by rovers. Owen et al. (1977) used Viking 1 & 2 data to estimate atmospheric CO₂ as taking up 95% of Mars's lower atmosphere, and James et al. (1992) report approximately 30% of the Martian atmosphere is involved in the seasonal sublimation of carbon dioxide (Owen et al., 1977). The ExoMars Trace Gas Orbiter (TGO or ExoMars Orbiter) is a collaborative project between the ESA and Roscosmos tasked with discerning the source of methane in the atmosphere (biological or geological origin) and its decomposition products. TGO can detect methane concentrations of up to 100 ppt in ideal conditions (low amounts of airborne dust). As of April 2019, the orbiter has had no detections of methane on Mars, conflicting with earlier reports from the Curiosity rover. The Mars 2020 rover will be landing in Jezero crater, a site which MRO data suggests contains clay-rich sedimentary deposits. These clays are ideal targets for organic matter preservation.

Despite conflicting reports of atmospheric carbon, the soil of Mars is considered carbon-rich enough to have supported heterotrophic microorganisms (Gooding, 1992; Kazmierczak and Kempe, April 2003; McKay, 2010; Navarro-González et al., November 7, 2003; Preston and Dartnell, 2014). Soil from analogue sites, such as the Atacama desert, have shown to contain viable heterotrophs, and globular clusters of heterotrophic bacteria have been suggested as possible organic origins for carbonate globules in Martian meteorites (Kazmierczak and Kempe, 2003; Navarro-González et al., 2003).

4. Conclusions

Results from this experiment suggest that the model organism *P. fluorescens* may exhibit chemotaxis with regard to mineralogical substrate in some capacity when generating biofilm. Based upon cell count data, there appears to be a statistically significant preference for cells to adhere to covellite phases over bornite or chalcopyrite. This could be a result of surficial defects, as most covellite phases tended to appear as veins. Surface properties or overall structural complexity (chalcopyrite is tetragonal, covellite is hexagonal, bornite is orthorhombic) may also play important roles in controlling cell adhesion.

This work advances scientific understanding of the interactions of *P. fluorescens*, a proxy for biofilm-forming microbes, with sulfide minerals and provides a foundation for further work categorizing biomarker signals on sulfide minerals for astrobiological research. Additional experiments involving the analysis of biofilm-coated surfaces exposed to simulated Mars surface conditions are an exciting area of future research. For next steps, we would suggest conducting similar experiments with sulfur and iron-oxidizing bacteria instead of *P. fluorescens*. Future research may also focus on sulfides with a higher weight percent of iron, including end member pyrite. Mars is dominated by iron and sulfur, and it is therefore imperative that biosignatures on iron-sulfides—not only sulfates, as current literature tends to emphasize—are observed and catalogued.

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APPENDICES

1. Polishing protocol

Polishing mineral samples can be an essential first step in many analyses. Getting a high-grade polish can be a challenge for beginners. The following protocol was developed with advice from Dr. Joseph Boesenberg, Brown University, for polishing copper sulfide minerals, but can be used for other mineral and rock samples. It is important to know where your samples lie on the Mohs hardness scale and whether they react with water or any other materials you may be using.

1.1 Goals

I aim to have small (5mm- 10mm wide) chips of bornite polished so that the surface is relatively scratch-free and a high enough grade for electron microprobe analysis.

1.2 Materials

Materials marked with an asterisk need at least one item per mounting ring. One mounting ring can hold 1-3 mineral chips. It is important to determine how many samples you intend to polish beforehand.

- Mineral samples
- Rock hammer, or another tool to break apart samples
- Plastic mounting ring forms
- Tweezers
- Scrap cardboard (or similar material)
- Double-sided tape
- Orthodontic or epoxy resin (must be soluble in acetone)

- DI water (though tap or filtered could also suffice)
- Monocrystalline Diamond Suspension (3 μm , 1 μm , 0.25 μm)
- 8" silicon carbide grinding disks of various grits (180, 400, 600, 1200),
Buehler*
- 8" TexMet C polishing cloth, Buehler*
- Polishing wheel with low-speed setting
- Light microscope
- Acetone
- Glass microscope slides
- Glass etcher or cutter
- Superglue
- Heat lamp

1.3 Mounting samples for polish

- Using a rock hammer, or other available tools, break apart mineral samples into chips 10 mm or smaller in width. Make sure to add an extra 3-4 chips onto the total number of chips for each mineral you need—as you adjust to the softness or hardness of each mineral, you may polish away entire chips by mistake.
- Place the scrap of cardboard on a flat surface. Lay a strip of double-sided tape across the cardboard, then secure the plastic mounting rings along the tape. Use the tweezers to place anywhere from 1-3 mineral chips inside the rings. Try to have the most-flat edge facing down against the tape inside the ring.

Make sure there is some amount of space between each chip when placing multiples in a single ring.

- Next, fill each mounting ring with resin powder. You do not need to cover the mineral chips entirely; a 1-2 mm thick coating should be enough to prevent the resin from breaking during polishing.
- Use the self-curing liquid from the orthodontic resin kit to wet the powder in the mounting rings. Make sure the powder is completely covered (it shouldn't take more than a couple of drops). It's okay if some leaks out of the bottom.
- Allow the resin time to harden. It will take around a half hour to air dry. The cardboard can also be carefully moved to a hot plate set on low to shorten the drying time. To test if the resin is hardened, you can tap it with a scoopula or pen. It should be completely solid.
- Carefully remove the mounting rings from the cardboard.

1.4 Polishing Samples

- Wet the 180 grit polishing circle with DI water (you do not need to use a polishing wheel). Take one of the mounting rings and use figure eight movements to drag the sample around. You can polish aggressively to achieve a flat surface. Try to remove any craters or small blemishes that appear on the surface. (Note: depending on the hardness of your mineral, you may need to start this step at a higher or lower grit. This step works well for bornite (hardness 3.5), but is too aggressive for covellite (hardness 1), which should use a 400 grit polishing circle at this stage.)

- Throw the 180 grit polishing circle away when you are satisfied with the surface area exposed. Polishing circles should not be reused. Gently rinse off your sample with water. Take out the 400 grit polishing circle and wet it. Using figure eight movements, polish the sample until all polishing wheel scratches from the 180 grit circle are removed. (Note: depending on the hardness of your mineral, you may need to start this step at a higher or lower grit. This step works well for bornite (hardness 3.5), but is too aggressive for covellite (hardness 1), which should use a 600 grit polishing circle.)
- Repeat the previous step using the 600 and 1200 polishing circles, occasionally checking samples under a light microscope to make sure polishing scratches from higher grits are being removed with each finer polish.
- Set your sample aside. Place the TexMet C cloth onto the polishing wheel and wet using 3 μm monocrystalline diamond suspension fluid. Turn the polishing wheel on to its lowest speed setting. Firmly grasp the sample and begin polishing it on the wheel using gentle figure eight movements. Be sure to occasionally rotate the sample in your grip to avoid wearing down only one side of the sample—you want the sample as flat as possible.
- Rinse your sample with water. Throw away the polishing cloth and replace it with another TexMet C cloth. Repeat the last step using 1 μm and 0.25 μm monocrystalline diamond suspension fluid. Regularly check samples under a light microscope to make sure polishing scratches from higher grits are being removed with each finer polish.

- The sample should be extremely reflective with very few scratches or blemishes left on the surface (some polishing scratches are unavoidable, but there should be large areas of unscratched surface). If there are polishing scratches still on the surface, continue polishing until they are gone. If scratches persist, move to a coarser grit grade until they are removed and work back down to a finer polish.
- Soak samples in acetone until resin becomes slimy and loose enough to free mineral chips. If acetone does not dissolve, try cutting the resin to expose a greater surface area.
- Repeat the last two sections (mounting and polishing) so that you are polishing the opposite side of the mineral chip. If you only need one side highly polished, you can simply polish using the 180 grit circle until you reach the desired thickness for the mineral chip. Re-soak samples in acetone to remove resin, rinse with water, and let dry.

1.5 Mounting Polished Samples

- While allowing samples to dry, use the glass etcher to cut the microscope slides into approximately 1 cm x 1 cm squares.
- Check that samples are dry before continuing. Place a small amount of superglue onto the center of the glass slide. Using tweezers, place your sample with the side you wish to analyze facing up. Gently press down on the surface using a Kim wipe. Allow to sit under a heat lamp for 5 to 10 minutes. Test the bond of the glue to the glass and sample using tweezers—it should not move.

- Store samples where appropriate and clean work station.

1.6 Conclusion

The procedure, as written, works very well for the mineral bornite and chalcopyrite. Softer minerals such as covellite should be polished starting with finer grit polishing circles. Most minerals can and should be polished by hand until you switch to using diamond suspension fluid or diamond paste. (If using diamond paste, spread four small dollops on each quarter of the TexMet C cloth and wet with DI water.) If done correctly, the polished sample should have few scratches visible with a light microscope. You should also be able to see your reflection on the sample surface.

2. Incubation Protocol

2.1 Materials

- BD Difco™ Dehydrated Culture Media: LB Broth, Miller
- Scoopula
- Scale
- Weighing paper
- 1 L purified water
- 2 L glass media bottle with cap
- Autoclave-safe plastic bin
- Autoclave
- Fume hood
- Liquid culture (*P. fluorescens*)

- Sterile 6-well plate
- Sterile 3 ml disposable transfer pipette
- Ethanol
- Latex gloves
- Kim wipes
- Autoclave bag

2.2 Mixing LB Broth

- Using the scale, weighing paper, and scoopula, measure out ~25 g of Difco Dehydrated Culture Media.
- Fill glass media bottle with 1 L purified water and add 25 g of culture media. Screw on the bottle cap and shake and swirl to thoroughly mix the water and media.
- Place glass bottle in an autoclave-safe plastic bin. Unscrew cap and rest it on the edge of the bottle lid so that half the opening is exposed.
- Autoclave at 121°C for at least 15 minutes.
- Screw cap back onto bottle while still inside the autoclave to keep sterile. Allow the bottle and liquid media time to completely cool.
- Once cool, label container and put away. Liquid media can be stored at room temperature.

2.3 Incubating Cultures

- Place bottle of LB culture media, sterile 3 ml disposable pipettes, Kim wipes, ethanol, and sterile 6-well plate into the fume hood. Put on latex gloves and wash hands with ethanol. Wipe down the section of the fume hood you plan to

work on using Kim wipes and ethanol. Clean the outside of the 3 ml pipettes and 6-well plate with Kim wipes and ethanol. Anytime you accidentally touch a non-sterile surface with your gloves or with a tool, you need to clean it again using ethanol—it is imperative to keep out any unwanted contamination.

- Unwrap your 6-well plate, placing it so that the underside is facing up. Unscrew the culture media bottle, placing the bottlecap underside face up. Unwrap one of your 3 ml pipettes. Carefully use the pipette to transfer ~3 ml of liquid culture media into each of the well plates. Screw the bottle cap back on, throw away the pipette and wrappings. Rewash gloved hands with ethanol.
- Unscrew the liquid culture bottle, placing the bottlecap underside face up. Unwrap one of your 3 ml pipettes. Completely fill the pipette with liquid culture. Carefully transfer ~3 drops of liquid culture into each of the well plates. Screw the bottle cap back on, throw away the wrappings. Place the pipette in the autoclave bag to be disposed of later. Rewash gloved hands with ethanol.
- Cover the 6-well plate with its lid and appropriately label. Taping the edges of the well plate together is recommended if you plan to leave the well plate undisturbed for more than a few hours.
- Carefully move the well plate to a safe place where it can be stored at room temperature. Fume hoods can be too cold and dry, evaporating the liquid before the culture has time to grow.

You should begin to see a cloudy biofilm taking form after approximately 24 hours, with best results being between 48 and 72 hours.

3. Supplemental data

3.1 *P. fluorescens* growth curves

Figure 1. Liquid media from incubating cultures was removed from the well-plate using a 3ml transfer pipette at the time of sample extraction. The media was placed into a UV-cuvette and its absorbance (arbitrary units) measured using a NanoDrop ND 1000 UV/VIS Spectrophotometer with excitation wavelengths of 405 and 415 nm. Readings were all completed within +/- 30 minutes of sample extraction. This figure shows the average absorbance of each timestep with 405 nm excitation.

3.2 Sulfur in Jezero Crater

Jezero crater is located along the Martian dichotomy and was once host to a deep lake, as evidenced by a sizable delta, inverted channels, and point bars. The Jezero watershed and floor units have spectral signatures indicative of olivine, pyroxene, carbonates, smectite clays, and sulfates (Goudge et al., 2015; Salvatore et al., 2018). Salvatore et al. (2018) averaged areal abundances on TES (Thermal Emission Spectrometer onboard MGS) data from Jezero crater, as seen in table 1 below.

Table 1. Average areal abundances of sulfate, in percentage, of 60,000 iterative linear unmixing procedures on TES data in Jezero crater, as reported by Salvatore et al., 2018.

Unit	Sulfate avg areal abundances (in %)	Median model error	Percent of runs above detection limit
Massive basement	12.9	2.0	73.5
Altered basement	12.7	1.8	84.3
Mottled terrain	11.0	3.5	70.8
Light toned floor material	17.1	2.7	98.6
Pitted capping unit	13.1	2.6	87.6
Crater floor	11.0	2.1	65.9
Syrtis basalts	13.0	2.3	73.8

BORNITE						PLANETARY			
Bornite	StdDev	Chalcopyrite	StdDev	Covellite	StdDev	Shergotty ^[3]	EH-Enstatite Chondrite ^[5]	EL-Enstatite Chondrite ^[5]	
S	255400	0.8846	339300	1.223	310300	1.075	1300	56000	31000
Fe	110800	0.3837	285400	1.029	8758	0.03034	151000	305000	181000
Cu	623900	2.161	358100	1.291	662800	2.296	16	215	120

NORMALIZED TO EH			
Bornite	Chalcopyrite	Covellite	
S	4.561	6.059	5.541
Fe	0.3633	0.9357	0.02871
Cu	2902	1666	3083

NORMALIZED TO EL			
Bornite	Chalcopyrite	Covellite	
S	8.239	10.95	10.01
Fe	0.6122	1.577	0.04839
Cu	5199	2984	5523

NORMALIZED TO SHERGOTTY			
Bornite	Chalcopyrite	Covellite	
S	196.5	261	238.7
Fe	0.7338	1.89	0.058
Cu	38990	22380	41430

Figure 2. Major elemental abundances of polished bornite and accessory minerals (ppm), and standard deviations (n=12), compared against elemental abundances for Shergotty meteorite (Mars meteorite, pigeonite, augite, maskelynite; Ti-magnetite, whitlockite), EH-Enstatite Chondrite (high iron enstatite chondrite; enstatite, iron-nickel, sulfides) and EL- Enstatite Chondrite (low iron enstatite chondrite; enstatite, iron-nickel, sulfides). Minor elemental abundances BD not included.

DataSet/Point	S	Mn	Fe	Co	Se	Cu	Zn	Ni	As	Sn	Total
1 / 1 .	25.48	0	10.95	0.1	0	62.9	0	0.19	0.37	0	99.99
2 / 1 .	30.95	0.01	0.72	0.07	0.02	66.37	0	0.05	0.09	0	98.28
3 / 1 .	34.14	0	28.73	0.05	0	36.27	0	0.01	0.02	0	99.22
4 / 1 .	25.47	0	11.15	0.14	0.06	63.14	0	0.15	0.36	0	100.47
5 / 1 .	25.64	0.01	11.09	0.13	0.01	61.82	0	0.17	0.35	0.03	99.25
6 / 1 .	25.59	0	10.99	0.16	0.02	62.54	0	0.15	0.4	0.01	99.86
7 / 1 .	25.65	0	11.07	0.14	0	61.94	0	0.17	0.38	0.01	99.36
8 / 1 .	25.25	0	11.15	0.14	0.02	63	0	0.22	0.4	0.01	100.19
9 / 1 .	25.55	0	11.12	0.19	0	62.6	0	0.22	0.31	0.02	100.01
10 / 1 .	25.48	0	10.94	0.18	0.01	61.93	0	0.26	0.54	0	99.34
11 / 1 .	25.29	0	11.17	0.1	0	63.23	0	0.11	0.23	0.01	100.14
12 / 1 .	25.52	0	11.08	0.08	0.04	62.09	0	0.12	0.23	0.02	99.18
13 / 1 .	34.6	0.01	30.02	0.04	0.01	34.53	0	0.01	0.01	0.05	99.28
14 / 1 .	34.52	0.01	29.8	0.06	0	34.48	0	0	0.05	0.02	98.94
15 / 1 .	34.45	0	29.69	0.05	0.03	33.96	0	0.02	0.05	0.03	98.28
16 / 1 .	31.39	0	23.72	0.45	0.02	40.3	0	0.71	1.5	0.03	98.12
17 / 1 .	34.35	0	29.77	0.09	0.06	34.77	0	0.03	0	0.02	99.09
18 / 1 .	33.22	0	25.97	0.26	0	38.49	0	0.19	0.34	0.04	98.51
19 / 1 .	34.13	0	29.66	0.14	0	35.16	0	0.1	0.27	0.01	99.47
20 / 1 .	32.69	0	25.66	0.24	0	39.56	0	0.24	0.57	0.05	99.01
21 / 1 .	26.01	0	10.98	0.27	0	61.02	0	0.38	0.77	0	99.43
22 / 1 .	25.51	0	11.24	0.08	0.06	62.42	0	0.1	0.2	0	99.61
1 / 1 .	34.23	0	29.22	0.06	0	34.53	0	0	0	0.04	98.08
2 / 1 .	34.21	0.01	29.73	0.04	0	33.95	0	0	0	0.03	97.97
3 / 1 .	34.48	0	29.31	0.06	0	34.77	0	0	0.01	0.02	98.65
4 / 1 .	34.63	0.01	29.79	0.04	0.02	34.79	0	0.01	0.07	0.09	99.45
5 / 1 .	34.54	0.01	29.26	0.06	0	34.27	0	0.01	0.01	0.03	98.19
6 / 1 .	31.05	0.01	1.01	0.1	0.02	66.63	0	0.05	0.08	0.03	98.98
7 / 1 .	31.15	0	1.21	0.23	0	64.86	0	0.27	0.55	0	98.27
8 / 1 .	30.82	0	0.85	0.52	0.04	63.67	0	0.79	1.63	0.01	98.33
9 / 1 .	31.53	0.01	0.5	0.05	0	66.44	0	0.02	0.09	0	98.64
10 / 1 .	30.9	0	0.8	0.12	0	66.47	0	0.11	0.27	0	98.67
11 / 1 .	31.25	0	0.81	0.06	0.04	67.1	0	0.04	0.04	0.01	99.35
12 / 1 .	30.89	0	0.68	0.12	0.06	66.6	0	0.13	0.3	0.01	98.79
13 / 1 .	34.69	0	29.09	0.07	0.07	34.25	0	0.01	0.01	0.05	98.24
14 / 1 .	31	0	0.83	0.04	0	67.12	0	0.03	0.02	0.02	99.06
15 / 1 .	30.81	0	1.33	0.11	0	66.32	0	0.06	0.15	0.02	98.8
16 / 1 .	30.5	0	1.07	0.08	0	67.34	0	0.05	0.14	0.01	99.19
17 / 1 .	31.49	0	0.7	0.05	0	66.41	0	0.05	0.14	0.05	98.89
18 / 1 .	31.38	0	0.53	0.07	0	66.33	0	0.05	0.09	0	98.45

Figure 3. EMPA raw data. Purple= bornite; Blue= covellite; Yellow= chalcopyrite; Green= Major element. Units are in wt%. Light gray font indicates abundance below detection (BD). The dark gray font is above detection.

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