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Abstract

Searching for biomarkers or signatures of microbial transformations of minerals is a critical aspect for determining how life evolved on Earth, and whether or not life may have existed in other planets, including Mars. In order to solve such questions, several missions to Mars have sought to determine the geochemistry and mineralogy on the Martian surface. This research includes the two miniaturized Mössbauer spectrometers (MIMOS II) on board the Mars Exploration Rovers Spirit and Opportunity, which have detected a variety of iron minerals on Mars, including magnetite ($Fe^{2+}Fe^{3+}_2O_4$) and goethite (α-FeO(OH)). On Earth, both minerals can derive from microbiological activity (e.g. through dissimilatory iron reduction of ferrihydrite by Fe(III)-reducing bacteria). Here we used a lab based MIMOS II to characterize the mineral products of biogenic transformations of ferrihydrite to magnetite by the Fe(III)-reducing bacteria Geobacter sulfurreducens. In combination with Raman spectroscopy and X-ray diffraction (XRD), we observed the formation of magnetite, goethite and siderite. We compared the material produced by biogenic transformations to abiotic samples in order to distinguish abiotic and biotic iron minerals by techniques that are or will be available onboard Martian based laboratories. The results showed the possibility to distinguish the abiotic and biotic origin of the minerals. Mossbauer was able to distinguish the biotic/abiotic magnetite with the interpretation of the geological context (Fe content mineral assemblages and accompanying minerals) and the estimation of the particle size in a non-destructive way. The Raman was able to confirm the biotic/abiotic principal peaks of the magnetite, as well as the organic principal vibration bands attributed to the bacteria. Finally, the XRD confirmed the particle size and mineralogy

Keywords	Bio-magnetite; Mössbauer spectroscopy; Raman spectroscopy; iron oxides; XRD

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Dear Ms Scalzo,

We thank you for your efforts in helping during the review process of this manuscript. We have carefully considered all the points which have been raised by the reviewer and feel that they have contributed to improving the quality of the manuscript. The details of our changes are included herewith:

Reviewer (Minor corrections)
→ *We appreciate the time and effort the reviewer has taken to evaluate the manuscript and are pleased with their opinion.*

We have considered the modifications that you recommended along the paper such as experimental campaigning to experiments, minor corrections on the references, small vocabulary corrections, etc.

Line 38: With the interpretation of the geological context and
We have added the geological context: (Fe content mineral assemblages and accompanying minerals).

Line 158: in situ measurements were carried out which correspond to samples where Mössbauer data was collected throughout the course of the experiment.
We rephrased the sentence is simple way: *in situ* Mössbauer measurements were carried out throughout the course of the experiment on each samples and it preparation.

Line 209: You could say here in which region of the spectrum
Based on the nature of Mössbauer spectroscopy, it is not common to refer to specific regions within a spectrum (e.g. as is the case for Raman), but instead discuss the presence of doublets or sextets. Therefore, we do not think defining the specific region of the spectrum will improve the interpretation of the data.

Line 212: You could say here in which region of the spectrum
See comment above

Line 296: Can you competently rule out the presence of remainders from the growth medium?
We removed this part by mentioning the existence of the organic substances due to the biochemical process.

Line 311: MIMOS II instrument, with the in situ experiments only looking at the very bottom layer of the iron layer which was undergoing microbial transformation. Is it possible that some conversion happens during the filtering stage?
The filtering removed the liquid fraction of the media which contains dissolved salts and the electron donor within a time frame of under 5 minutes. Consequently, there are no microbial transformation processes which could take place during or after filtration of the samples. As samples were measured directly after filtration under anoxic conditions, we do not consider that further crystallization took place in the samples.

Line 359: FH-AQDS-13 is calculated to be higher than those previously reported. Is there an implication for this?
We clarified by adding on the sentence: "..and showing a higher concentration of ferrous magnetite."

Figure 3. It has been corrected and improved.

Table 1. What is the difference between these two rows of "magnetite nanophases"? You may explain it in the table caption.
We have updated the figure caption to include:
"[a]Superparamagnetic magnetite - magnetic ordering prevented by small particle size.
[b]Magnetically ordered magnetite that has not reached full hyperfine splitting because of small particle size and/or impurities
[c]Values from Morris et al. (2008), averaging all magnetite and goethite measurements obtained in Gusev Crater with Mars Exploration Rover Spirit. Uncertainties quoted are standard deviations (2σ)."
"

HIGHLIGHTS:

Analysis of the mineral products of biogenic transformations of ferrihydrite to magnetite by the Fe(III)-reducing bacteria *Geobacter sulfurreducens* using the lab based MIMOS II from NASA-MER Mission.

A complete XRD and Raman mineralogical characterization has been carried on the different bio-mineralization processes for complementing the research.

The use of Mössbauer spectroscopy in combination with XRD and Raman spectroscopy presents an interesting combined system for the search of life, especially those focused on Martian mission.

Abiotic versus biotic iron mineral transformation studied by a miniaturized backscattering Mössbauer spectrometer (MIMOS II), X-ray diffraction and Raman spectroscopy

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Abstract

Searching for biomarkers or signatures of microbial transformations of minerals is a critical aspect for determining how life evolved on Earth, and whether or not life may have existed in other planets, including Mars. In order to solve such questions, several missions to Mars have sought to determine the geochemistry and mineralogy on the Martian surface. This research includes the two miniaturized Mössbauer spectrometers (MIMOS II) on board the Mars Exploration Rovers Spirit and Opportunity, which have detected a variety of iron minerals on Mars, including magnetite ($Fe^{2+}Fe^{3+}{}_2O_4$) and goethite (α-FeO(OH)). On Earth, both minerals can derive from microbiological activity (e.g. through dissimilatory iron reduction of ferrihydrite by Fe(III)-reducing bacteria). Here we used a lab based MIMOS II to characterize the mineral products of biogenic transformations of ferrihydrite to magnetite by the Fe(III)-reducing bacteria *Geobacter sulfurreducens*. In combination with Raman spectroscopy and X-ray diffraction (XRD), we observed the formation of magnetite, goethite and siderite. We compared the material produced by biogenic transformations to abiotic samples in order to distinguish abiotic and biotic iron minerals by techniques that are or will be available onboard Martian based laboratories. The results showed the possibility to distinguish the abiotic and biotic origin of the minerals. Mossbauer was able to distinguish the biotic/abiotic magnetite with the interpretation of the geological context (Fe content mineral assemblages and accompanying minerals) and the estimation of the particle size in a non-destructive way. The Raman was able to confirm the biotic/abiotic principal peaks of the magnetite, as well as the organic principal vibration bands attributed to the bacteria. Finally, the XRD confirmed the particle size and mineralogy

Keywords: Bio-magnetite, Mössbauer spectroscopy, Raman spectroscopy, iron oxides.

Introduction

The iron redox cycle is a significant process that takes place in most environments on Earth, which can be conducted by both abiotic and microbial processes. In anoxic, pH-neutral environments, microbial Fe(II)-oxidation is driven by either nitrate reducing bacteria (e.g. *Acidovorax* sp. BoFeN1), photoferrotrophic bacteria (e.g. *Rhodopseudomonas palustris* strain TIE-1) or neutrophilic microaerophilic bacteria (e.g. *Gallionella leptothrix*) (Kappler et al., 2005; Kappler and Newman, 2004; Kappler and Straub, 2005). The other half of the Fe cycle is driven by microbial Fe(III) reduction, either intracellularly by magnetotactic bacteria (Blakemore, 1975) or outside of the cell wall by dissimilatory iron reducing bacteria (DIRB) such as *Shewanella oneidensis* and *Geobacter sulfurreducens* (Lovley and Phillips, 1988, 1986). DIRB combine the oxidation of an organic substrate (e.g. acetate or lactate) or hydrogen with the reduction of poorly crystalline, short range ordered, Fe(III) minerals (e.g. ferrihydrite (Fe^{3+})$_2O_3$•0.5H_2O) and can lead to the formation of many different iron mineral phases and compounds including goethite, magnetite, green rust and siderite ($FeCO_3$) (Byrne et al., 2011). The mineralogical composition of these products of reduction depends on geochemical parameters, including Fe(III) reduction rate, pH, temperature and the presence of electron shuttles (e.g. riboflavin, quinones and humic acids) (O'Loughlin et al., 2010; Piepenbrock et al., 2011). Furthermore, a recent study suggests that magnetite can act as both an electron donor and an electron acceptor to different types of Fe metabolizers depending on the redox conditions (Byrne et al., 2015). In this regard, understanding the mineralogical products of biomineralization processes can help to detect signatures of microbial interactions with fluid, rocks, mineral deposits and subsequent diagenesis.

Several terrestrial environments where microbially driven Fe(III) reduction processes occur (Léveillé, 2009; Rothschild and Mancinelli, 2001) have been identified, such as Rio Tinto (SW Spain), Western Australian salt lake sediments (SW, Australia) and Ten Graben fault system of Green River (Utah, USA), have been proposed as terrestrial analogues to the types of conditions on Mars where iron cycling could have taken place (Nixon et al., 2012; Ruecker et al., 2016). These terrestrial analog systems allude to the existence of Fe(III)-oxyhydroxide bio-signatures and fossilized microorganisms, with Fe(III)-oxyhydroxides susceptible to recrystallization to more stable forms (Léveillé, 2009; Nixon et al., 2012; Parenteau et al., 2014; Ruecker et al., 2016). Utilizing Laboratory studies and natural analogues can be used to help identify bio-signatures preserved from the past (NASA´s Mars Exploration Rover Mission *Spirit*), current (NASA´s Mars Exploration Rover Mission *Opportunity*; Mars Science Laboratory (MSL) *Curiosity*) or future Mars missions (ESA-ExoMars rover; NASA Mars 2020) (Bish et al., 2013; Bost et al., 2015).

One of the most important techniques for characterizing Fe minerals on Mars is ^{57}Fe Mössbauer spectroscopy. This technique is capable of determining the oxidation state of iron, as well the mineralogy and the magnetic structure of different Fe mineral phases (Helgason, 2004). Indeed, two miniaturized Mössbauer spectrometers (MIMOS II) were part of the suite of analytical instruments carried on board the two Mars rovers Spirit and Opportunity, which landed on January 4, 2004 and January 25, 2004, respectively (Klingelhöfer et al. 2003). The data collected by these vehicles have provided a significant insight into the mineralogy of iron compounds on the Martian surface (Klingelhöfer et al., 2004, 2003, Morris et al., 2010, 2006, 2004). Raman and X-ray diffraction (XRD) techniques are among the payload of other current and future space missions (Bish et al., 2013; Bost et al., 2015, 2013). This includes

89 the CheMin XRD system on the NASA MSL Curiosity mission (Bish et al., 2013) and Raman
90 instruments such as the SHERLOC (Scanning Habitable Environment with Luminiscence for
91 Organics and Chemical Instrument) system, the Raman SuperCam that were selected for the
92 NASA Mars 2020 rover, and the Raman Laser Spectrometer (RLS) of the ESA ExoMars
93 rover (Grossman, 2013; Hutchinson et al., 2014; Léveillé, 2009; Wang et al., 2015).

94 In this study, we investigated the formation of biogenic magnetite nanoparticles
95 through microbial Fe(III)-reduction by the DIRB *Geobacter sulfurreducens* in the presence
96 and absence of the electron shuttling compound Anthraquinone-2,6-disulfonate (AQDS).
97 The presence of the electron shuttle is expected to greatly increase the speed of microbial
98 Fe(III)-reduction compared to experiments in which it is not present. The presence/absence
99 of AQDS should therefore have an impact on the type of mineral phases which are produced
100 during the reduction and affect their stoichiometry (i.e. Fe(II)/Fe(III) ratio). The
101 spectroscopic and mineralogical characteristics of these minerals were compared to those of
102 minerals formed from abiotic syntheses in laboratory and to natural samples. The main goal
103 of this study is to distinguish between iron minerals formed through abiotic and biotic process
104 by techniques that are or will be available onboard Martian based laboratories. In most cases,
105 Mössbauer spectroscopy can only be applied to dry powdered materials or embedded in a
106 solid matrix such as in ice or on a filter. Here, however, we use the MIMOS II instrument to
107 analyze in situ, a solid material that has deposited from an aqueous suspension. Our study is
108 analogous to experiments conducted by Zegeye et al. (2010) who performed *in situ*
109 monitoring of lepidocrocite bioreduction and magnetite formation by backscattering
110 Mössbauer spectroscopy (Zegeye et al., 2011, 2010).

111

Experimental setup

Preparation of ^{57}Fe enriched Fe(III)- oxyhydroxide

A starting precipitate of ^{57}Fe enriched (~12%) Fe(III)-(oxyhydr)oxide was prepared to enhance the signal-to-noise ratio of the Mössbauer spectrum obtained for sediment in water. Powdered naturally abundant isotope ^{NA}Fe(0) and pure ^{57}Fe(0) were separately dissolved in 0.7 M anoxic HCl in an anoxic glovebox (MBRAUN, 100% N_2 atmosphere) and continuously stirred overnight at 400 rpm leading to the formation of $^{NA}Fe^{2+}$ and $^{57}Fe^{2+}$, respectively. The two solutions were filtered (0.22 µm syringe tip filter) to remove any solid precipitates, then mixed in a volume ratio of 10% ^{57}Fe and 90% ^{NA}Fe and removed from the glovebox. The ^{57}Fe (i.e. containing ~12% ^{57}Fe) enriched solution was oxidized using 400 µl H_2O_2 (37%) and stirred at 400 rpm for 2 hours to form an aqueous solution of acidified Fe^{3+}. KOH (5 M) was added dropwise to the Fe^{3+} solution, leading to the precipitation of the Fe(III)-oxyhydroxide, at a final pH=7. The precipitate was washed of additional ions by centrifugation (4000 rpm, 20 min) three times, with the supernatant discarded and the precipitate re-suspended in ultrapure H_2O. After the final centrifugation step, the material was kept in liquid suspension and put into a 20 ml headspace vial. The suspension was made anoxic (but not dried) by applying vacuum (5 min) followed by flushing with N_2 gas (30 seconds), with both steps repeated three times. The final concentration of iron in the Fe(III)-oxyhydroxide suspension was determined using the ferrozine assay (Stookey, 1970) to be 421 mmol l^{-1}.

Fe(III)-reduction experiments

Two separate experiments were carried out using the ferrihydrite at different times, with one in 2013 and the second in 2014. For both sets of experiments, *Geobacter sulfurreducens* bacteria were grown until the late log phase in basal medium (Ehrenreich and Widdel, 1994), containing 25 mM acetate (electron donor), 40 mM fumarate (electron acceptor) and buffered with 22 mM $NaHCO_3$. The bacteria were then centrifuged three times (4000 rpm; 20 min) to remove the growth medium and washed with 30 mM $NaHCO_3$ in between centrifugation steps. The final cell suspension was made up to a volume of 2 ml and the optical density at λ = 600 nm (OD_{600}) measured at 0.385 abs and 0.372 abs (FlashScan 550, Analytik Jena, Germany) for experiments in 2013 and 2014 respectively.

All Fe(III) reduction experiments were prepared in Plexiglas holders with a 500 µm bottom thickness. For 2013 experiments, cultures were prepared up to a volume of 15 ml in the glovebox using sterile, anoxic solutions and consisted of 50 mmol L^{-1} Fe(III)-(oxyhydr)oxide, 20 mM acetate and 30 mM $NaHCO_3$. One of the cultures additionally contained AQDS (10 µM) as an electron shuttle. Both culture experiments were sealed with a rubber butyl stopper to maintain an anoxic environment. The culture experiments were inoculated with 0.312 ml of *G. sulfurreducens* cell suspension immediately prior to loading on to the MIMOS II device. The cultures are subsequently referred to as FH+AQDS-13 and FH-AQDS-13.

For the 2014 experiment, the culture were prepared in a similar way, however it contained double the concentration of iron material, i.e. 100 mmol L^{-1} Fe(III)-oxyhydroxide, 40 mM acetate and 30 mM $NaHCO_3$. There was no AQDS present. The 2014 experiment was inoculated with 0.644 ml of the *G. sulfurreducens* cell suspension, so approximately twice as many cells as for the 2013 experiment. This culture is referred hereafter as FH-AQDS-14.

156 ***Analytical methods***

157 During the 2014 experiments, *in situ* Mössbauer measurements were carried out throughout
158 the course of the experiment on each samples and it preparation. These *in situ* Mössbauer
159 spectra were collected by placing the culture incubation vessels onto a MIMOS II in
160 backscattering geometry at the Mössbauer Group of Mainz University (Germany) and left
161 for the duration of the experiment. The culture vessels during the 2013 experiments were
162 left on the MIMOS II, however the recorded spectra were not of sufficient quality due to their
163 lower iron concentrations. Following the reduction phase of each experiment, the contents of
164 each of the reaction vessels were filtered with Millipore swinnex filter holders and Millipore
165 membrane filters (0.45 μm - HAWP04700). The entire solution of these experiments were
166 passed through the filter and then dried under an N_2 atmosphere. Filtered samples were sealed
167 in between two layers of Kapton tape to inhibit oxidation of the samples during measurement,
168 and Mössbauer spectra were collected with the MIMOS II instrument. Spectra were
169 calibrated against alpha-iron at room temperature and fitted with an in house routine (Mbfit)
170 using Lorentzian and Voigt line profiles. Mbfit is based on the least-squares minimization
171 routine MINUIT (James, 2004). All Mössbauer fitting parameters are shown in Table 1 (i.e.
172 center shift (CS), quadrupole splitting (ΔE_Q), hyperfine field (B_{hf})).

173 Dried samples from the 2013 experiments (i.e. FH-AQDS-13 and FH+AQDS-13) were
174 analyzed using X-ray diffraction (XRD), with data collected using a Bruker D8 Discovery
175 Diffractometer equipped with a molybdenum $K_{\alpha 1}$ X-ray tube (λ= 0.71 Å) at the Institut für
176 Anorganische Chemie und Analytische Chemie of Mainz University (Germany). Total
177 scattering was measured between 5° and 40° 2θ. A comparison with a natural rock magnetite
178 standard has been done. The natural magnetite used belongs to the NASA Athena Reference

179 Samples (Aref 043) (Schröder, 2003). Magnetite sample was ground in an agate mortar and
180 fixed onto a polymer tape (Scotch). The data were refined using the Rietveld method
181 (Rietveld, 1969) and the FULLPROF software (Rodríguez-Carvajal, 1993). A pseudo-Voigt
182 function was used to describe the peak shape and a polynomial function with five refinement
183 coefficients for the background.
184 Spectroscopic Raman measurement were performed at the Unidad Asociada Uva-CSIC
185 (Valladolid, Spain) on the dried samples from the 2013 experiments (i.e. FH-AQDS-13 and
186 FH+AQDS-13). The Raman mineralogical characterization of the sample was performed by
187 micro-Raman spectroscopy, using a microscope Nikon Eclipse E600 coupled to a
188 spectrometer KOSI Holospec f/1.8i with a resolution of 5 cm^{-1} illuminated by a laser REO
189 LSRP-3501, He-Ne 632.8 nm. The detection was performed by a CCD Andor DV420A-OE-
190 130 with a resolution of 5 cm^{-1}. The laser power used is 14 mW with a spot diameter of 15
191 μm. The kapton cover of prepared samples produce strong Raman fluorescence. The material
192 was carefully extracted from the Plexiglas holders inside a chamber/bell with a nitrogen
193 atmosphere to avoid abiotic oxidation. Moreover, some natural iron-oxides, sulfate and
194 carbonate from the sample collection of the ESA-ExoMars Raman Laser Spectrometer (RLS)
195 database were measured and used as a reference materials. The reference samples stem (1)
196 from several fieldtrips to terrestrial analogues including Rio Tinto (Spain), Tenerife (Spain),
197 Svalbard (Norway), Faroe Island, etc. (Lalla et al., 2016, 2015; Sansano et al., 2015) or (2)
198 were synthetized in the laboratory (Sansano-Caramazana, 2015). The spectral Raman
199 collection of the mineral phases has been obtained using standard Raman systems and the
200 RLS simulator system, processed and analyzed using the protocols for the ExoMars mission
201 (Hermosilla et al., 2012).
202 **Results**

Mössbauer analysis

Data were obtained for the FH-AQDS-14 experiment which was incubated at room temperature on a MIMOS II throughout the microbial Fe(III) reduction over the course of 19 days (Figure 1a and b) and are consequently referred to as *in situ* measurements. Figure 1a shows the recorded spectrum obtained for FH-AQDS-14 after 6 h, with a clear doublet visible despite the fact that the starting precipitate (ferrihydrite) is suspended in liquid media. Figure 1b shows the recorded spectrum for FH-AQDS-14 after 19 days of incubation on the MIMOS II, with the data clearly indicating the presence of both a doublet and sextet. After 19 days the microbial Fe(III) reduction in the *in situ* experiments was stopped and the precipitates were filtered. The filtered samples were then placed onto the MIMOS II again for further analysis, with Figure 1c showing the spectrum from the FH-AQDS-14 filtered sample at room temperature. It is clear from the figure that after 6 h, the sample displays a spectrum corresponding to ferrihydrite, whilst after 19 days, the sample shows a clear sextet which likely corresponds to magnetite. After filtration (Figure 1c), the sample displays a more characteristic spectrum of magnetite, though additional peaks corresponding to ferrihydrite and goethite appear to be present.

Figure 2 shows the data collected for FH-AQDS-13 (Figure 2a) and FH+AQDS-13 (Figure 2b) experiments measured at room temperature after filtration and loaded directly onto the MIMOS II instrument. The Mössbauer spectra for a reference sample of magnetite (Athena Reference Sample 043) is shown in Figure 2c for comparison and clearly shows the tetrahedral and octahedral lattice sites expected for the mineral. Figure 2 indicates that ferrihydrite FH-AQDS-13 was transformed mainly into pure magnetite, whilst FH+AQDS-13 was transformed to magnetite, but with traces of siderite.

XRD

XRD data shown in Figure 3 confirms that the only mineral phase present in sample FH-AQDS-13 at the end of the experiment was magnetite whereas sample FH+AQDS-13 contained both magnetite and siderite, confirming the Mössbauer results. The structure of magnetite is centrosymmetric and shows cubic symmetry with space group *Fd-3m*. The lattice parameter for FH-AQDS-13 and the FH+AQDS-13 samples show values around 8.30 Å which is comparable to known standards. The average crystallite sizes for both samples (also known as the coherent scattering domain) has been determined through the use of the Scherrer equation (Scherrer, 1918), being around 27-30 nm.

Raman analysis

Raman spectroscopy was carried out on selected areas of samples FH+AQDS-13 and FH-AQDS-13 (Figure 4) and compared to reference bands for several different iron minerals (Table 2) (Hanesch, 2009). The measured data has been processed by a Gaussian curve fitting analysis on each band to obtain the correct band position of the different minerals phases on the spectra. The fitting has been obtained by using the commercial program Bruker OPUS and following the standard procedure: spectra smoothing (if it is needed), background subtraction, normalization and interactive curve fitting (by Levenberg-Marquardt and/or Local Least Squares methods).

The results show the existence of several mineral oxides such as hematite, magnetite, siderite, and possibly poorly crystalline goethite in the band fitting (Figure 4 and Table 3). The bands in the region at 120 to 220 cm^{-1} belong unambiguously to the siderite being present on the different spectra. Secondly, the bands of the region 220-420 cm^{-1} have been assigned to the

248 vibration of hematite (~225, ~245, ~290 cm^{-1}), siderite (~287 cm^{-1}), goethite (~244, ~300
249 and ~385 cm^{-1}) and magnetite (310 cm^{-1}). However, the bands corresponding to
250 FH-AQDS-13 (Figure 4a and 4b) are more intense compared to FH+AQDS-13 (Figure 4c
251 and 4d) due to the influence of goethite presenting the strongest vibration in this band region
252 (according to the band fitting). On the other hand, the region 420-550 cm^{-1} presents the
253 vibration of goethite (~480 cm^{-1}), magnetite (~530 cm^{-1}), hematite (~500 cm^{-1}) and siderite
254 (~500 cm^{-1}). The bands on FH-AQDS-13 obtained by the curve fitting show a strong
255 influence caused by amorphous goethite (Figure 4a and 4b).

256 The region from 560 to 800 cm^{-1} can be assigned mainly to the magnetite (~650 cm^{-1}),
257 possibly goethite (681 cm^{-1}), hematite (~610 and ~660 cm^{-1}) and siderite (~720 cm^{-1}). Finally,
258 the region within the 1200-1650 cm^{-1} presents a mixture of bands that belong to
259 hematite/goethite magnon (~1350 cm^{-1}) and organics (~1314, ~1422, ~1545 and ~1575 cm^{-1}).

261 The magnetite bands are clearly visible at 300, 532 and 661 cm^{-1} which are in close agreement
262 with other studies (Hanesch, 2009; Shebanova and Lazor, 2003). The intense bands
263 corresponding to the A_{1g} mode at 667 cm^{-1} presents a variation of magnetite measured in other
264 studies. The A_{1g} oxide peak of magnetite is shifted toward higher wave numbers in the bio-
265 magnetite (700 cm^{-1}) compared to that of the inorganically produced magnetite (670 cm^{-1})
266 (Jimenez-Lopez et al., 2010). In previous studies, it has been found that a transition to an
267 orthorhombic phase on a starting oxidation process produces a displacement of the A_{1g} to
268 higher values (Shebanova and Lazor, 2003). The second magnetite band between 532 and
269 550 cm^{-1} is reported as the mode F_{2g} and is observed to be sensitive to possible defects in the
270 crystal structure as well as the presence of vacancies and interstitial cations. Furthermore,

271 other studies suggest that the F_{2g} band below to 500 cm^{-1} can be attributed to the occurrence
272 of hematite (de Faria et al., 1997; Hanesch, 2009; Shebanova and Lazor, 2003). The siderite
273 presents the strongest band at 182, 287, 731 and 1090 cm^{-1} (Hanesch, 2009; Rull et al., 2004).

274 The band fitting also reveals the existence of hematite and it presents as a combination of
275 several vibrations at 225, 245, 291, 410, 500 and 611 cm^{-1} with the magnon at 1321 cm^{-1}
276 (Hanesch, 2009). In the case of the possible goethite presence, the principal vibrations are at
277 244, 299, 385, 480 and 681 cm^{-1} being the most commonly used for the identification in
278 complex vibrational analysis (de Faria et al., 1997; Hanesch, 2009; Rull et al., 2004). The
279 bands at the aforementioned positions are very weak and broadened, due to poorly
280 crystallized goethite, which is combined with other oxide mineral phases (magnetite and
281 hematite).

282 When considering the Raman bands of biological component, the most intense vibrations
283 were detected between 1300 and 1600 cm^{-1}, which can be assigned to the active modes D
284 and G of the C-C bonds vibrations from carbonaceous materials. The band fitting
285 corresponding to this vibrational region presents several medium intensity vibrations at 1314,
286 1422 and 1565 cm^{-1} according to the best fitting analysis. The bands at 1314 and 1422 cm^{-1}
287 can be assigned to the D double modes D1 and D2, which is in agreement with other studies
288 (Ferrari, 2007). However, it has been found during band fitting that an influence of other
289 magnon vibrations of hematite/goethite phases on 1350 cm^{-1} (Hanesch, 2009; Rull et al.,
290 2007) is possible. The band G has also been observed (see Table 3), showing a distorted
291 structure clearly detectable by the broad bands. The vibrations at 2903 and 2961 cm^{-1}
292 correspond to the C-H vibrational regions, which could be assigned to symmetric and
293 asymmetric stretching of the $–CH_3$ from the organic matter (Böttger et al., 2012; Pereira et

al., 2007). Furthermore, the *G. sulfurreducens* Raman vibration bands can be assigned to the peaks detected along the different spectra measurements. In this regard, several vibrations have been identified considering the following regions: (1) the C-H stretching region bands on 2900-3000 cm^{-1}; (2) the CH deformation vibrational region between 1300 and 1450 cm^{-1}; and (3) the C=O stretching vibration zone in 1650 to 1800 cm^{-1} approximately (el-Kabbani et al., 1991; Kudelski, 2005).

Discussion

The *in situ* Mössbauer spectrum for FH-AQDS-14 after 6 h is dominated by the presence of a doublet corresponding to ferrihydrite. After 19 days the sample is still dominated by a central ferric doublet, although a sextet is clearly evident. In the filtered sample however (Figure 1c), it is clear that the Mössbauer spectrum displays two sextets corresponding to (1) tetrahedrally coordinated iron denoted Fe^{3+}_{tet}, and (2) octahedrally coordinated Fe which includes both Fe^{2+} and Fe^{3+}, often denoted $Fe^{2.5+}_{oct}$ with an additional goethite phase. This apparent difference in the sample before and after filtration is likely due to the nature of the MIMOS II instrument, with the *in situ* experiments only looking at the very bottom layer of the iron layer which was undergoing microbial transformation. A gradient in the reaction likely existed with most reduction taking place in the top portion of the sample where the mineral is more exposed to the solution (Dippon et al., 2015).When the sample was filtered, it was mixed and so the top fraction and bottom fraction were mixed and thus yielded the sextet as seen in Figure 1c. The goethite phase present in the filtered sample of FH-AQDS-14 was fitted with a sextet in which the hyperfine magnetic field (B_{hf}) value (Table 1) is smaller than that of well-crystalline goethite, indicating that the goethite has very low crystallinity or consists of very small particles.

In the experiments carried out in 2013, sample FH-AQDS-13 (Figure 2a) appears to have more well-defined sextets than sample FH+AQDS-13 (Figure 2b). The broadened lines and additional broad sextet in these spectra can be explained by a lower degree of crystallinity possibly because of impurities in the magnetite structure, e.g. the incorporation of organic matter, and/or small particle sizes. In magnetically ordered phases, which present as sextets in Mössbauer spectra, these influences reduce the strength of the internal B_{hf}. A range of degrees of crystallinity then results in broad lines skewed towards smaller B_{hf} values. Small particle sizes, generally 30 nm or less, can additionally lead to an effect called superparamagnetism (Daniels and Rosencwaig, 1969). The particle sizes are then in the range of individual magnetic domains and random thermal movement of the particles leads to a net zero magnetic field and magnetic ordering appears to collapse, which manifests itself as a paramagnetic doublet in the Mössbauer spectrum instead of a magnetically ordered sextet (Daniels and Rosencwaig, 1969; Dezsi et al., 2008). Superparamagnetism appears to be visible in sample FH+AQDS-13 with the very broad lines in the Mössbauer spectrum (Figure 2b) in agreement with previous studies which have shown the samples prepared in the presence of electron shuttles (e.g. AQDS) tend to be smaller in diameter than those without (Byrne et al., 2011). Furthermore, the FH+AQDS-13 sample clearly contains siderite which is likely due to the enhanced rate of microbially driven Fe(III) reduction, due to the presence of AQDS, resulting in an excess of Fe^{2+} in solution which can further react with the CO_3^{2-} present in solution from the buffer system and lead to the precipitation of siderite (Cornell and Schwertmann, 2003; Hansel et al., 2003; Liu et al., 2009).

The parameters obtained from fitting the magnetite spectra closely correspond to the values expected for magnetite (Table 1). All of the hyperfine parameters determined are in general

smaller than expected, however this is likely due to the sample exhibiting poorer magnetic ordering at room temperature than well-crystalline, stoichiometric magnetite (Figure 2c). Neglecting potential differences in *f*-factor for the different Fe lattice positions, the stoichiometric ratio (x=Fe(II)/Fe(III)) of magnetite can be determined by comparing the relative areas of the two sextets according to the following equation (Da Costa et al., 1995; Daniels and Rosencwaig, 1969):

$$x = \frac{0.5\, Fe_{oct}^{2.5+}}{0.5\, Fe_{oct}^{2.5+} + Fe_{tet}^{3+}} \quad (1)$$

Using equation 1 it is seen that magnetite formed in FH+AQDS-13 (x = 0.25) is more oxidized than perfectly stoichiometric magnetite (x = 0.5). The iron in magnetite formed in FH-AQDS-13 (x = 0.65) is more reduced than stoichiometric magnetite. The difference is thought to be due to the faster reduction rate in the presence of AQDS, leading to the formation of superparamagnetic magnetite nanoparticles. However, this result also suggests that the presence of electron shuttles might in fact lead to the formation of rather oxidized magnetite. Since electron shuttles such as AQDS are organic compound, it might indicate that magnetite found in regions with low stoichiometry can be linked to the presence of organic substance. The relative ratio of Fe(II)/Fe(III) in the magnetite in FH-AQDS-13 is calculated to be higher than those previously reported and showing a higher concentration of ferrous magnetite (Veeramani et al., 2011). Moreover, the mineral phases measured are in agreement with Veeramani *et al.*, (Veeramani et al., 2011), though here we have also identified the presence of siderite.

The XRD results for our samples show smaller values for the lattice constant(8.30 Å) than the 8.397 Å associated with stoichiometric magnetite (ICDD, n.d.; “Magnetite -

362 www.mindat.org," 2001). Maghemite has a smaller lattice constant of 8.33 Å, which is more
363 similar to our results, suggesting the samples to be partially oxidized which agrees with the
364 Mössbauer results for sample FH+AQDS (Wohlfarth and Arrott, 1982). It has also been seen
365 that the lattice parameters of pure and metal-substituted bio-magnetites tend to be smaller
366 compared to those of chem-magnetites where the bio-magnetite likely has a relatively more
367 compact crystal structure with fewer uncoordinated iron ions on the surface (Moon et al.,
368 2010). The average crystal size is around 27-30 nm for the FH+AQDS-13 and FH-AQDS-13,
369 being consistent with the Mössbauer observations of smaller B_{hf} values, broadened and
370 skewed lines, and superparamagnetism. Also, this result confirms the interpretation of other
371 studies (Byrne et al., 2011; Veeramani et al., 2011).

372 In the Raman results, the variation of A_{1g} mode corresponding to the magnetite could
373 becaused by the curve fitting or may be due to structural changes generated during the
374 microbial Fe(III) reduction where the oxidation process produces a displacement of the A_{1g}
375 to higher values. An estimation can be done by curve fitting relative band intensity of I_{685-90}/I_{650} produced by the magnetite and the bio-magnetite, the results show the existence of two
377 Raman bands where the I_{685-90} is more intense (See Table 3b – Figure 3). Also, the
378 FH-AQDS-13 presents the possible existence of goethite due to the higher intensity of the
379 bands in the region between 220-420. Furthermore, it can be affirmed by the $I_{220-520}/I_{560-800}$,
380 which for FH-AQDS-13 is at approximately 0.5 and FH+AQDS-13 is 0.35 (Figure 3).

381 In the region from 2800 to 3100 cm^{-1}, the FH-AQDS-13 bands are more intense compared to
382 those of FH+AQDS-13 spectra and confirm that the *G. sulfurreducens* transformed the
383 starting material.

In general, the combination of Mössbauer, Raman and XRD technique allowed the detection of several iron mineral phases and mineral structures. The magnetite was the main mineral that formed as a result of microbial Fe(III) reduction by *G. sulfurreducens*. The Raman based vibrational technique was able to detect hematite with the possibility of minor phases of goethite, clearly distinguishable by the main vibrations and the curve-fitting. Mössbauer spectra indicate oxidized magnetite in the FH+AQDS-13 samples. Oxidation of magnetite leads to maghemite and eventually hematite formation, but these phases are difficult to differentiate (Vandenberghe et al., 2000).

The combination of siderite, magnetite, hematite and non-crystalline goethite suggests a relatively complex mineralization process that is strongly dependent on biological mechanism. Similar formations of mixed minerals can be achieved by chemical processes, however they often present with other phase combinations including ferrihydrite-goethite-lepidocrocite-magnetite (Hansel et al., 2005) or goethite-hematite-maghemite-magnetite-akaganeite (Ahn et al., 2012). Furthermore, the starting conditions of microbial Fe(III) reduction, such as the use of different starting substrates including hydrous ferric oxide, goethite, hematite, magnetite, carbonates, and Fe(III)-clays, or the variation of geochemical parameters including Eh, pH, and temperature can yield stark differences in the types of minerals produced via primary or secondary mineralization pathways (Jimenez-Lopez et al., 2010; Perez-Gonzalez et al., 2010).

Here we suggest that the use of Mössbauer-Raman-XRD analyses in combination with other structural studies could potentially be used to obtain a suitable range of criteria to help develop fingerprints to distinguish biological and abiotic magnetite. The biogenic magnetite and goethite from our experiments differs significantly from the magnetite and goethite

observed with the Mars Exploration Rovers in rocks and soils on Mars (Table 1), where the magnetite is suggested to be of primary, igneous origin (Morris et al., 2008).

Our biogenic magnetite is non-stoichiometric and, in the case of FH+AQDS-13 appears to be oxidized. The broad lines reflect in Mössbauer spectra reflect low crystallinity, potentially resulting from impurities such as organic matter, and/or small particle sizes. The presence of organic matter is confirmed by Raman spectroscopy and small particle sizes are indicated by XRD. Taken together, the evidence extracted from the different techniques could be used to establish some criteria for bio-signatures. Table 4 presents a resume of the main features of each technique that could be used for the detection of bio-signatures in planetary exploration.

The question, however, is whether the features observed here would be preserved over geological timescales. Over time, magnetite might mature and increase in crystallinity while organic matter can undergo decay, thus potentially eliminating any evidence over extended timescales.

Conclusions

The MIMOS II instrument, of which two have previously been sent to Mars onboard Martian rovers, has been used to characterize different mineral phases produced by the microbial reduction of Fe(III)-oxyhydroxide and determined the dominant phase produced to be magnetite. Other techniques on current or future Mars rover missions such as XRD and Raman have been used to complement these results. Mössbauer, XRD, and Raman confirm the existence of magnetite for the samples incubated with only ferrihydrite (FH+AQDS-13 and FH-AQDS-13) and magnetite combined with siderite for the sample incubated with an added electron shuttle (FH+AQDS-13). The values of the crystal lattice show values under

8.397 Å and crystalline sizes under 30 nm being in the range of superparamagnetism as confirmed by the Mössbauer analysis.

Raman analysis shows the formation of different mineralization products, including goethite (possibly), hematite, magnetite and siderite which is only somewhat in agreement with the Mössbauer analyses that did not observe hematite in samples. Some differences between the bio-magnetite and natural magnetite have been detected by Raman spectroscopy on the bands at approximately 650 and 540 cm^{-1}. In all spectra recorded, organic compounds were detected which correspond to the DIRB *G. sulfurreducens*.

The combination of these three different analytical techniques could be used as a critical approach for observing the products of Fe-biomineralization processes on future space missions, especially those focused on Mars. In this regard, these techniques, used in combination with morphological studies, will help to establish a criterion of the biological origin of biogenic magnetite and other Fe minerals.

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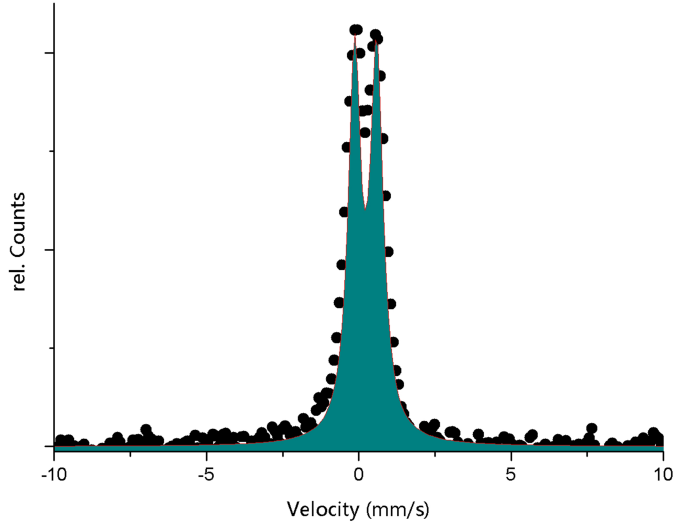
671

List of Figure

a)



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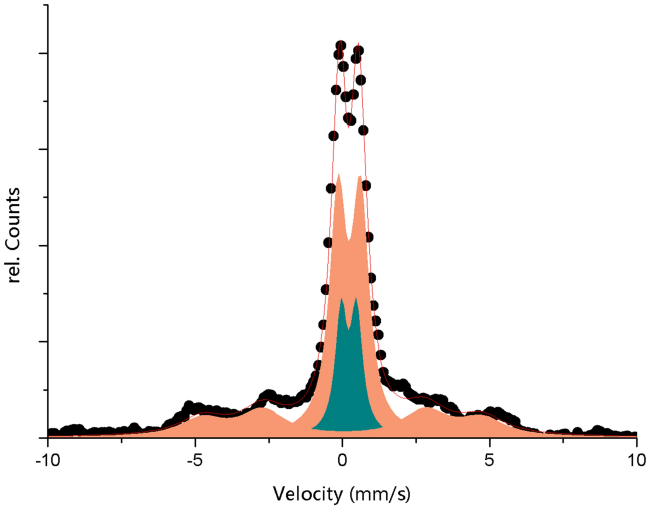

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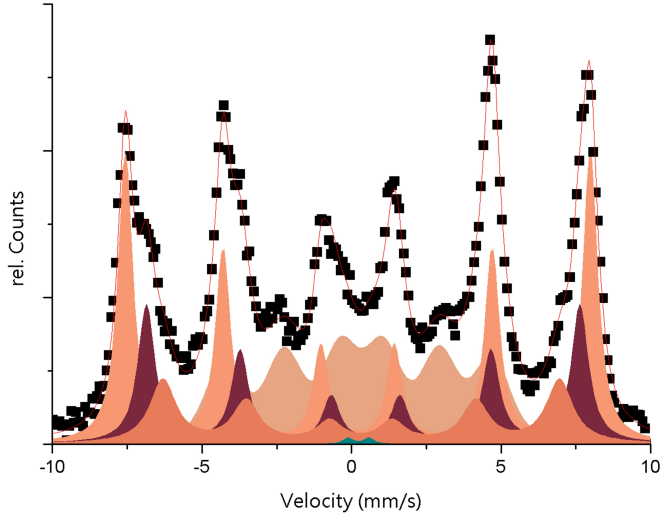
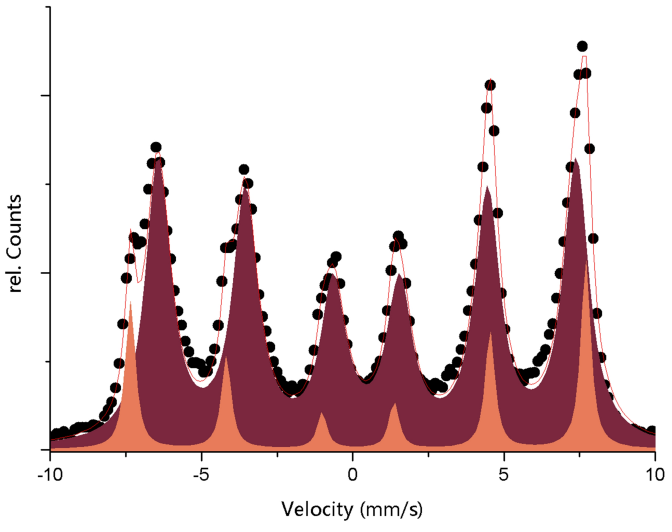


Data
Magnetite (tet^{3+})
Magnetite ($oct^{2.5+}$)
Goethite nanophase
Magnetite nanophase
Ferrihydrite
Siderite
Fit

a)



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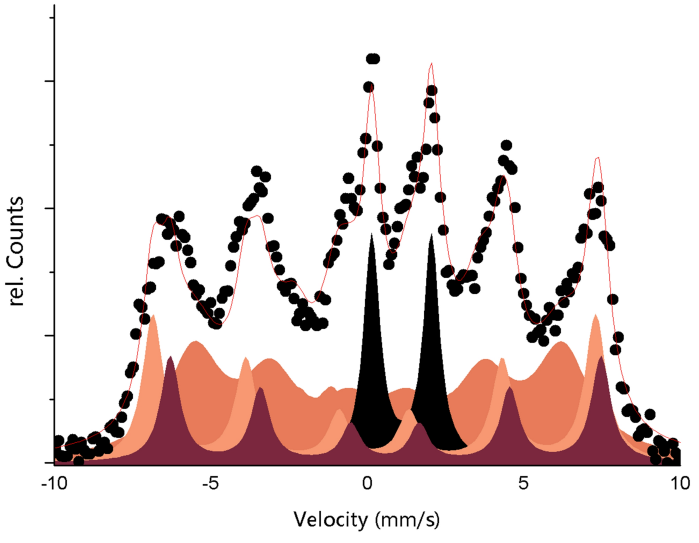

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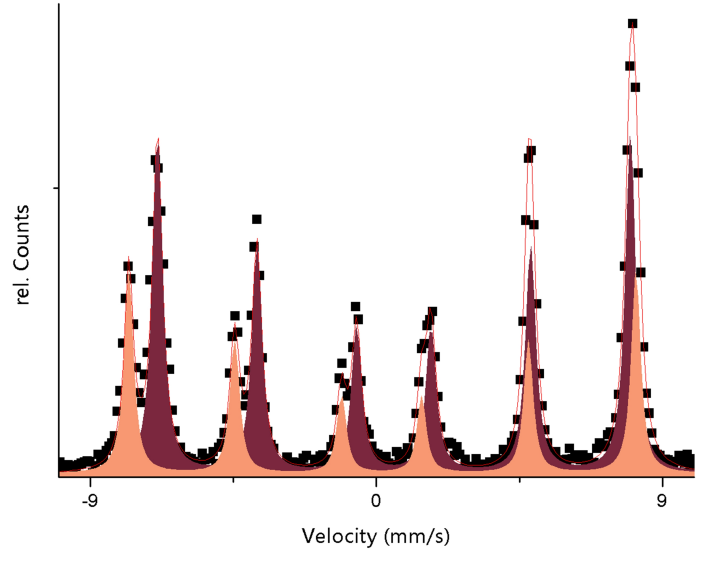
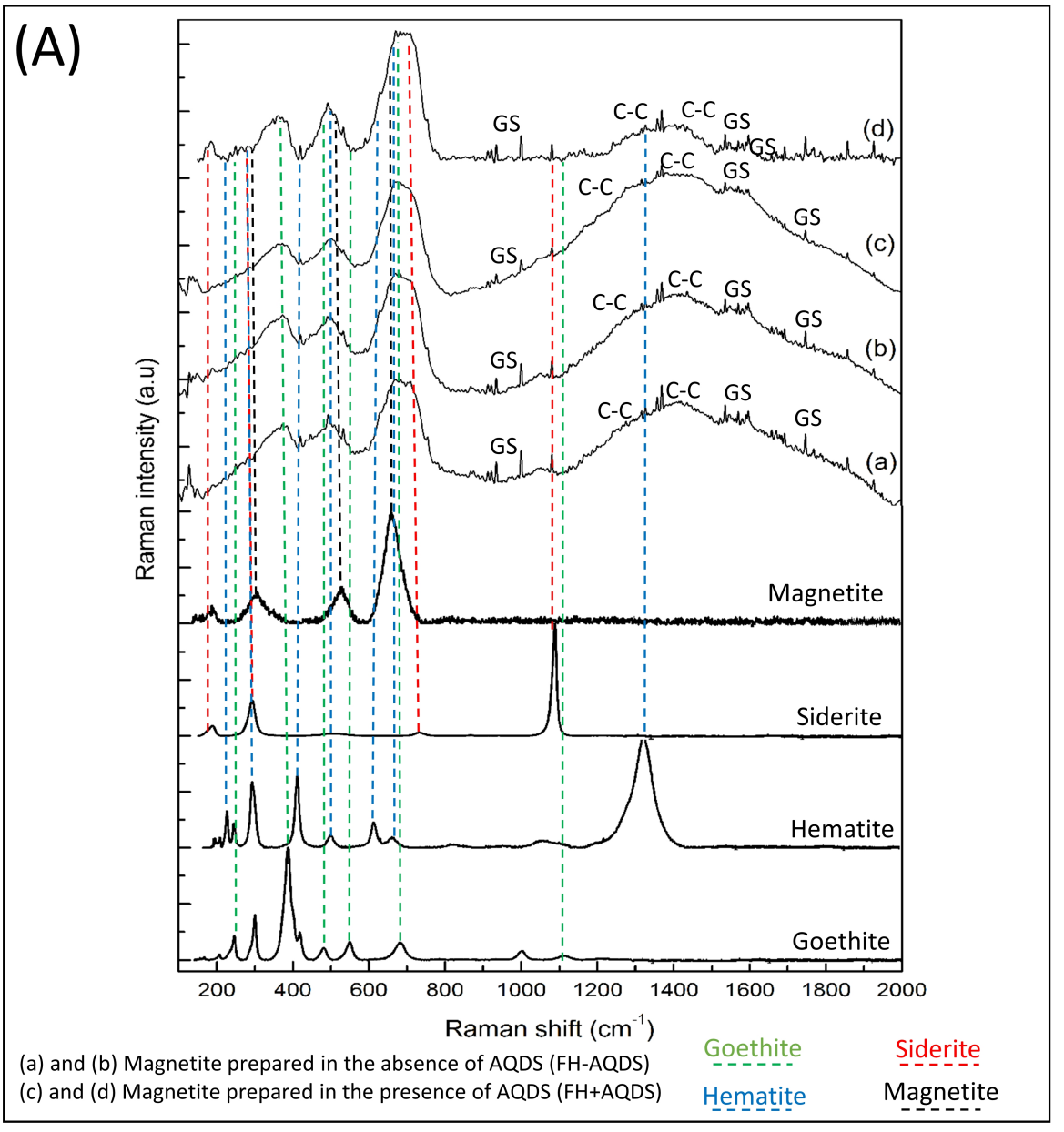
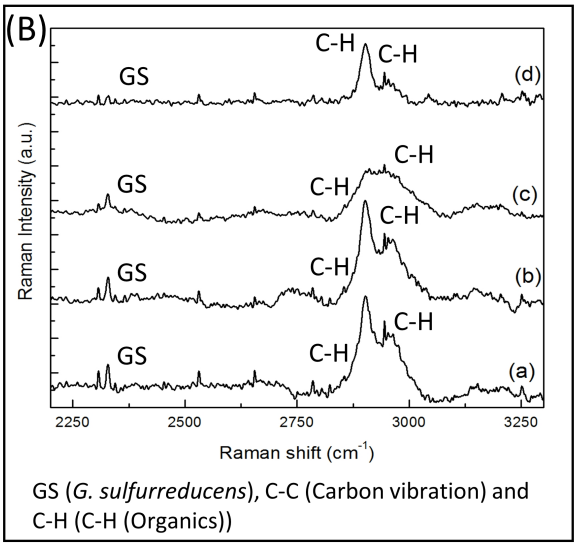
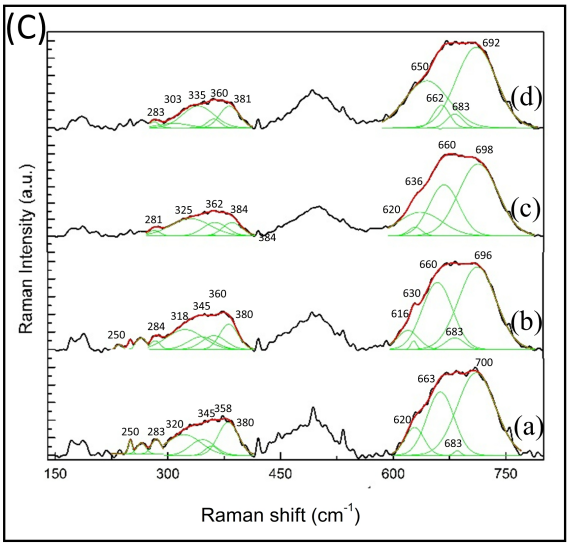


Data
Magnetite (tet^{3+})
Magnetite ($oct^{2.5+}$)
Magnetite nanophase
Siderite
Fit


(A)
Raman intensity (a.u)
GS
C-C
(d)
(c)
(b)
(a)
Magnetite
Siderite
Hematite
Goethite
200
400
600
800
1000
1200
1400
1600
1800
2000
Raman shift (cm-1)
(a) and (b) Magnetite prepared in the absence of AQDS (FH-AQDS)
(c) and (d) Magnetite prepared in the presence of AQDS (FH+AQDS)
Goethite
Siderite
Hematite
Magnetite


(B)
Raman Intensity (a.u.)
GS
C-H
(d)
(c)
(b)
(a)
2250
2500
2750
3000
3250
Raman shift (cm-1)
GS (G. sulfurreducens), C-C (Carbon vibration) and
C-H (C-H (Organics))


(C)
Raman Intensity (a.u.)
(d)
(c)
(b)
(a)
150
300
450
600
750
Raman shift (cm-1)

List of Tables:

Table 1

Sample	Mineral Phase	CS (δ) mm s^{-1}	Linewidth (S) mm s^{-1}	ΔE_Q mm s^{-1}	B_{hf} T	Relative area %	Fe(II)/Fe(III) in Fe_3O_4
FH-AQDS-14 (*in situ*, 6 h)	Ferrihydrite	0.34	0.25	0.74		100	
FH-AQDS-14 (*in situ*, 19 days)	Ferrihydrite	0.34	0.34	0.77		11.4	
	Magnetite nanophase[a]	0.33	0.24	0.53		21.7	
	Magnetite nanophase[b]	0.13	0.92	-0.03	29.5	66.9	
FH-AQDS-14 (filtered)	Ferrihydrite	0.34	0.20	0.74		0.5	
	Magnetite (tet- Fe^{3+})	0.30	0.30	0.00	48.1	40.1	
	Magnetite (oct- $Fe^{2,5+}$)	0.54	0.34	- 0.04	44.9	21.7	
	Magnetite nanophase[b]	0.43	0.54	0.00	41.2	15.8	
	Goethite nanophase	0.39	0.35	-0.06	27.6	21.9	
FH-AQDS-13 (filtered)	Magnetite (tet-Fe^{3+})	0.30	0.19	0.00	46.6	20.8	0.65
	Magnetite (oct- $Fe^{2,5+}$)	0.56	0.52	-0.00	42.8	79.2	
FH+AQDS-13	Magnetite (tet-Fe^{3+})	0.33	0.40	0.00	44.0	18.6	0.25
	Magnetite (oct- $Fe^{2,5+}$)	0.69	0.36	0.01	42.8	12.1	
	Magnetite nanophase[b]	0.45	1.07	0.01	36.6	35.7	
	Magnetite nanophase[b]	0.71	0.59	0.04	18.0	11.4	
	Siderite	1.21	0.31	1.90		22.2	
Athena Reference Sample 043	Magnetite (tet-Fe^{3+})	0.29	0.2	0.01	49.4	37.1	0.46
	Magnetite (oct- $Fe^{2,5+}$)	0.66	0.2	0.00	46.1	62.9	
Average Mars magnetite[c]	Magnetite (tet-Fe^{3+})	0.31±0.03		0.01±0.03	50.0±0.5		
	Magnetite (oct- $Fe^{2,5+}$)	0.66±0.06		-0.01±0.08	46.7±0.8		
Average Mars goethite[c]	Goethite	0.38±0.02		-0.19±0.10	37.3±2.9		

[a]Superparamagnetic magnetite - magnetic ordering prevented by small particle size.

[b]Magnetically ordered magnetite that has not reached full hyperfine splitting because of small particle size and/or impurities

[c]Values from Morris et al. (2008), averaging all magnetite and goethite measurements obtained in Gusev Crater with Mars Exploration Rover Spirit. Uncertainties quoted are standard deviations (2σ).

Table 2.

Mineral Phase	Principal Raman bands (cm^{-1})
Goethite	244, 299, **385**, 480, 681
Hematite	**225,** 245, 290-300, 412
Lepidocrocite	**250**, 348, 379, 528, 650
Siderite	184, 287, 722, **1082**
Ferrihydrite	370, 510, **710**
Magnetite	310, 540, **670**
Maghemite	350, 512, **665**, **730**

Table 3 a.

Raman band position (cm^{-1})	Raman assignment
172 (w)	*****
186 (w)	Siderite
283 (m)	Hematite/Goethite/Siderite
340 (m)	Magnetite
378 (m)	Hematite/Goethite
420 (vw)	Goethite
451 (sh)	****
474 (m)	Goethite
495 (s)	Siderite/Magnetite
533 (sh)	Magnetite
650 (sh)	****
667 (vs)	Magnetite
717 (vs)	Siderite
755 (sh)	*G. sulfurreducens*
1000 (w)	*G. sulfurreducens*
1082 (w)	Siderite
1314 (m)	Hematite/Goethite
1358 (w)	*G. sulfurreducens*
1370 (w)	*G. sulfurreducens*
1422 (m)	Carbon vibration
1565 (m)	Carbon vibration
1598 (w)	*G. sulfurreducens*
2328 (w)	*G. sulfurreducens*
2903 (s)	C-H(Organics)
2961 (m)	C-H (Organics)

**** Raman band not assigned

Table 3 b.

Mineral Phase	Principal Raman fitting bands (cm^{-1}) of the Mineral and organics detected			
	Raman Spectra (a)	Raman spectra (b)	Raman spectra (c)	Raman Spectra (d)
Goethite	283, **376**, 420, 475	284, **373**, 471	285, **383**, 420	283, **385**, 420, 480
Hematite	**250**, 283, 493, 609, 1348	**250**, 283, 401, 497, 609, 1337	**250**, 283, 403, 480, 1358	**250**, 283, 491, 609, 1331
Siderite	**187**, 505, 711, **1083**	**188**, 504, 703, **1083**	**187**, 700, **1083**	**184**, 712, **1083**
Magnetite	308, 533, **653** or **691** *(bio-mag)**	315, 534, **652** or **679** *(bio-mag)**	308, 534, **64**2 or **685** *(bio-mag)**	315, 533, **652** or **690** *(bio-mag)**
Organics	1327, **1370**, 1400, 1537,**1598**, 2328, 2344, 2884, **2902**, **2963**	1279, **1386**, 1431, 1549, **1598**, 2307, 2328, 2874, 2902, **2963**	**1287**, 1427, 1559, **1600**, 2328, 2878, **2905**, 2940, 2974	1270, **140**1,1438, 1469, 1542, **1597**, 2874, **2903**, 2947, **2969**, 3043

*****Refers to the magnetite produced by *G. sulfurreducens*. The curve fitting relative band intensity of $I_{685\text{-}90}/I_{650}$ shows for: (a) ~1.50, (b) 1.27, (c) 1.63, (d) 1.2.

Table 4.

Technique	Structure and crystallinity	Particle size	Detection of organics	Oxidation states	Stoichiometry	*Geological context
Raman spectroscopy	Yes	No	Yes	No	Potentially	Yes
XRD	Yes	Yes	No	No	No	Potentially
Mössbauer Spectroscopy[a]	Yes	Yes	No	Fe	Yes	Yes

[a]Mössbauer spectroscopy is limited to Fe-bearing materials.

***Geological Context: Mineral assemblages and accompanying minerals**