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Accumulation of a ferric mineral in the biofilm of *Montacuta ferruginosa* (Mollusca, Bivalvia). Biomineralization, bioaccumulation, and inference of paleoenvironments

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Abstract

Infrared absorption spectra of the amorphous and phosphorus-rich ferric mineral associated with the biofilm of *Montacuta ferruginosa*, a marine bivalve, were obtained by applying the KBr disc method. Phosphate absorption bands due to P–O stretching vibrations were observed at 1100 and 1020 cm^{-1}. This result, as well as the similarity of spectra with other well known (bio)minerals, suggests that the mineral is an amorphous iron oxyhydroxide gel with phosphate sorbed on its surface rather than a pure ferric phosphate. It is suggested that phosphate ions are of microbial origin because phosphate-producing enzymes were detected in vivo. Apart from iron, the mineral phase is devoid of other heavy metals. Despite its similarity with other abiotically precipitated phases, it is argued that the mineral is the result of in situ microbial biomineralization processes in the biofilm and not the result of a simple bioaccumulation process. This is supported by microscopic observations. A geological implication is that the simple presence of such iron minerals in ancient sedimentary environments, where microbes have not been fossilized, might be considered as an indicator of microorganisms performing biomineralization. By comparison with the present-day environment of *M. ferruginosa*, precise paleoenvironmental conditions may be inferred.

Keywords: Bacteria; Biofilm; Biomineralization; Iron; Paleoenvironment; Infra-red spectroscopy

1. Introduction

For about the first 2000 million years of Earth history, i.e. during the Archaean Eon (4600 to 2500 million years ago), the world was inhabited only by aquatic prokaryotes (Schopf, 1993). These early forms of life, which were probably anaerobes (Walker, 1987), were dominantly concentrated in benthic microbial mats, biofilms and stromatolites. Many of these ancient microbial communities interacted with metal ions and provoked the deposition of large quantities of sedimentary iron ores. On the early Earth, dissolved iron—Fe(II)—was considerably more abundant in the hydrosphere than today (Walker, 1987). Under these conditions, it was proposed that the deposition of banded iron formations (BIFs), enormous tracts of sedimentary iron ore deposited in the seas of Archaean and Early Proterozoic times (3500–1800 million years ago), were in fact microbiological in origin. Primary candidates

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responsible for BIF formation are anoxygenic photosynthetic bacteria, newly discovered in present-day marine muds, which use Fe(II) as the electron donor (Widdel et al., 1993). This example illustrates the importance of studying ancient and present-day microbial communities in parallel. Understanding present-day microbial communities, and studying microbially associated minerals, can influence ideas about the evolution of early life on Earth as well as the interpretation of mineral paragenesis and the sedimentary rock record (Préat et al., 1999a,b, 2000). It can even influence our ideas about early life on other planets like Mars, which possesses iron-rich soils and had liquid water flowing on its surface (Iberall-Robbins and Iberall, 1991; Kerr, 1996; McKay et al., 1996).

In this paper, we will focus on a present day marine microbial community associated with an iron-rich mineral deposit. The community studied forms a biofilm on the shell of *Montacuta ferruginosa*, a marine bivalve that lives in the burrow of the echinoid *Echinocardium cordatum* (Gage, 1966). This biofilm includes different morphotypes of bacteria and protozoa incrusted within a mineral rich in ferric iron (Gillan and De Ridder, 1995, 1997; Gillan et al., 1998, 2000). X-ray diffraction and energy-dispersive X-ray (EDAX) analyses indicated that the mineral is amorphous and rich in iron, phosphorus and calcium with traces of silicon (Gillan and De Ridder, 1995, 1997). The mineral forms granules whose diameter ranges from 0.05 to 1 μm (in the colloidal range).

Iron minerals are not easy to study in marine environments because they are present in low concentrations and as coatings on other mineral particles. Even less well known is the role of marine microorganisms in the generation of iron mineral phases. Bacterial cells can precipitate a variety of iron minerals, and usually the biogenic minerals have chemical compositions similar to those produced by abiotic precipitation from inorganic solutions (Konhauser, 1998). It is thus theoretically possible to predict the type of mineral that would form if the composition of the waters in which the microorganisms grow are known. However, microorganisms within biofilms are capable of maintaining microenvironments, at the biofilm/water interface, that are radically different from the bulk water in terms of pH, dissolved oxygen, and other elements. In addition, microorganisms are usually embedded in anionic exopolymeric substances (EPS) (sheaths, capsules) that greatly influence mineralization processes (Beveridge and Doyle, 1989). As a consequence, microorganisms in biofilms produce minerals that are not predicted by thermodynamic arguments based on the chemistry of the medium (Little et al., 1997). When microbes are observed in association with iron minerals in a biofilm, it is thus necessary to elucidate the mineral chemistry in detail before drawing any conclusions about the origin of the mineral (biogenic vs. abiotic).

According to our previous analyses, two chemistries are possible for the iron mineral of *M. ferruginosa*: (i) amorphous ferric phosphate or (ii) amorphous iron oxyhydroxide with phosphate sorbed on the mineral surface. In the first case, it is possible that bacteria are directly responsible for the nucleation of the mineral since ferric phosphates only form at very low pH (Arlidge et al., 1963; Mann, 1989) and are not normally present in North Sea water and sediments (Slomp et al., 1996). Such a process is called microbial biomineralization (Lowenstam and Weiner, 1983). If this is the case, iron colloids would probably be observed in close contact with cells, in sheaths and capsules, because mineral nucleation should be restricted to favourable low-pH microenvironments. In addition, the colloids would be more or less homogeneous in size and/or form, as the microenvironment is well defined. In case (ii), microbial biomineralization would be rejected because amorphous iron oxyhydroxides are minerals that are thought to form mainly abiotically in seawater (Byrne and Kester, 1976). In this case, the iron-encrusted biofilm would probably be the result of a passive accumulation of abiotically precipitated iron colloids, a process that may be called bioaccumulation. In this case various morphotypes and various sizes of colloids would be expected in sheaths and capsules, essentially at the surface of the biofilm.

For geochemists and geomicrobiologists, it is important to distinguish between bioaccumulation and biomineralization processes, especially if paleoenvironmental conditions are inferred from the type of mineral observed. If an ancient bioaccumulation process is recognized, precise paleoenvironmental con-

ditions cannot be inferred because minerals were not in situ precipitated by the microbes; on the contrary, if an ancient biomineralization process is recognized, precise paleoenvironmental conditions (in terms of pH and Eh) may be inferred because we can compare with present-day microbial communities where biomineralization processes have been demonstrated.

The aim of the present work is to determine whether microbial biomineralization or bioaccumulation is responsible for the iron accumulation in the biofilm of *M. ferruginosa*. The iron mineral was thus analysed with diffuse reflectance infrared (DRIR) spectroscopy in order to elucidate its chemistry, and observations under the electron microscope were performed in order to study the spatial relationships of iron colloids and microbial cells. The phosphatase activity of the biofilm was also determined in vivo because phosphatases are phosphate-releasing enzymes, and a high phosphatase activity in the biofilm may be linked to the precipitation of iron phosphates. Some microorganisms are known to accumulate several times their own weight of precipitated metals by this mechanism (Macaskie et al., 1992). The geological implications of our findings are then discussed.

2. Materials and methods

Specimens of *M. ferruginosa* (Montagu, 1803) were collected intertidally from the burrows of *E. cordatum* (Pennant, 1777) (Echinoidea, Spatangoida) at Wimereux (Pas-de-Calais, France) during July 1996 and January 1998. The bivalves were briefly rinsed in filtered seawater and their iron-encrusted biofilms were sampled with a scalpel blade.

For DRIR spectroscopy, standard protocols were used (Russell, 1979; Nanzyo, 1986). Biofilms from 10 individual bivalves were used in the analysis. Biofilms were centrifuged, rinsed two times with

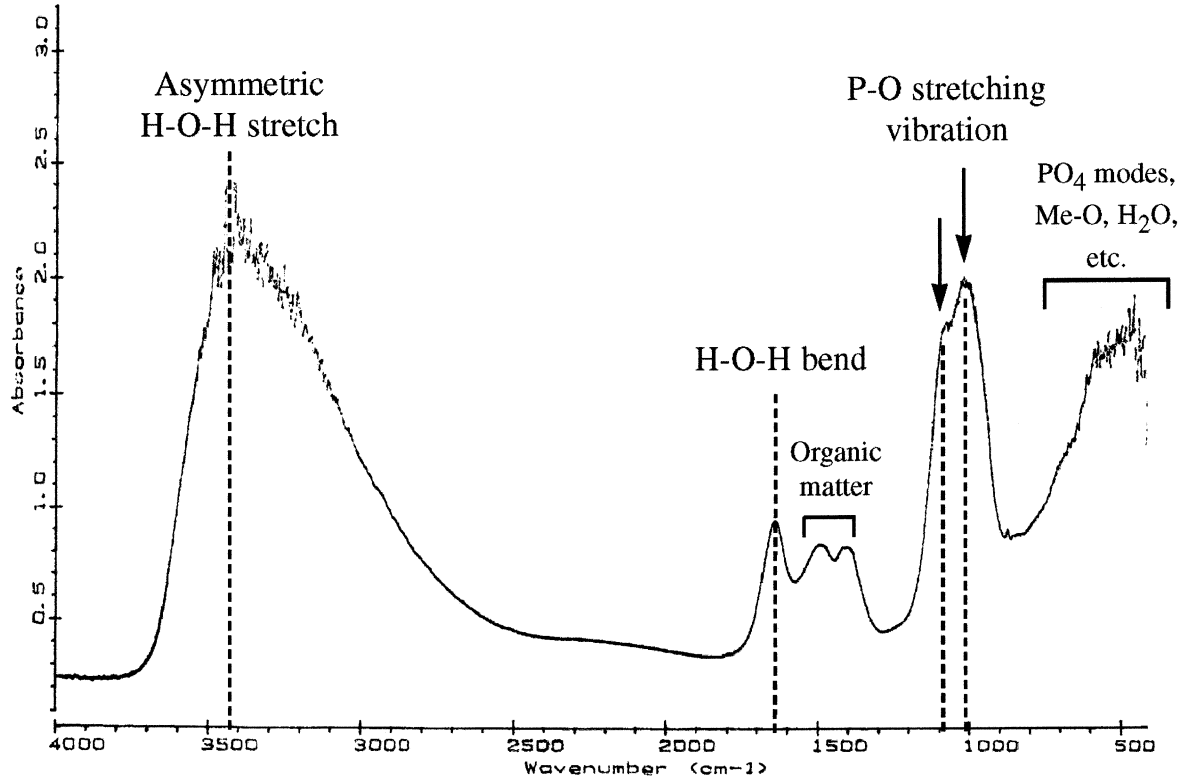


Fig. 1. DRIR spectrum of the iron mineral of *M. ferruginosa*. The arrows indicate the splitting of the P—O absorption band. Me—O, metal–oxygen stretches.

MilliQ water and air-dried. The KBr disc method was used: 3 mg of the iron-encrusted air-dried biofilms were ground with 300 mg of KBr in a mortar and pestle. The mixture was then transferred to an evacuable die and pressed at about 10,000 psi. The pellet obtained was placed in a Nicolet 680 Spectral Workstation and spectra were recorded at room temperature from 500 to 4000 cm^{-1}.

For light microscopy (Nomarski and epifluorescence microscopy) fragments of living biofilms were stained with a solution of 0.01% acridine orange and 0.01% glutaraldehyde as a fixative. Acridine orange specifically stains microbial cells (Austin, 1988). Specimens were then observed under a Leitz Diaplan Nomarsky microscope equipped with an I2/3 filter block for epifluorescence. Under the Nomarsky microscope large microorganisms and minerals are both visible. In the epifluorescence mode, and with acridine orange staining, all microorganisms are visible but not the iron minerals. It is thus possible to detect bacteria embedded in a mass of iron minerals.

For transmission electron microscopy (TEM), biofilms were fixed for 2 h in 1% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4). The biofilms were then rinsed for 30 min (3×10 min) in buffer, dehydrated in graded ethanol (70%, 90%, 100%), placed 5 min in propylene oxide, embedded in Spurr's resin and thin sectioned. Thin sections were observed under a Philips EM 300 TEM microscope. Some sections were contrasted with uranyl acetate and Reynold's lead citrate (Gillan and De Ridder, 1997).

To detect the presence of phosphatase activity, 17 individual biofilms were sampled as above and pooled in a sterile NaCl solution (30 g/l). Biofilms were then divided into two parts. The first part (control) was heated 10 min at 100°C to kill the microorganisms; the second part was not heated. The two parts were then tested for phosphatases with the API ZYM multitest system (API System, La Balmes-Grottes, France) according to the manufacturer's instructions. The API ZYM assay is a widely used semi-quantitative microbiological assay based on colorimetric reactions (Humble et al., 1977). Strips of microtubes inoculated with bacteria are incubated 24 h at 20°C. The reaction tubes are then graded 0 (enzyme not detected) to 5 (high concentrations of enzyme present), depending on the intensity of colour compared with representations on a colour chart. The phosphatases tested were alkaline phosphatase, acid phosphatase and phosphoamidase.

3. Results

3.1. DRIR analysis

It can be seen in an example DRIR spectrum obtained from the iron mineral of *M. ferruginosa* that a broad absorption maximum is present at 3420 cm^{-1} (Fig. 1). This is due to the asymmetric H—O—H stretch of molecular water. The band at 1640 cm^{-1} is the H—O—H bend; it is neither sharp nor displaced in frequency as is often the case with

Fig. 2. Light micrograph (Nomarsky mode) of three morphotypes of filamentous bacteria occurring in the biofilm. m1 to m3, morphotype 1 to 3. Scale bar = 10 μm.

Fig. 3. Epifluorescence view of Fig. 2. m1 to m3, morphotype 1 to 3. Scale bar = 10 μm.

Fig. 4. Light micrograph (Nomarsky mode) of the iron mineral of *M. ferruginosa.* Scale bar = 10 μm.

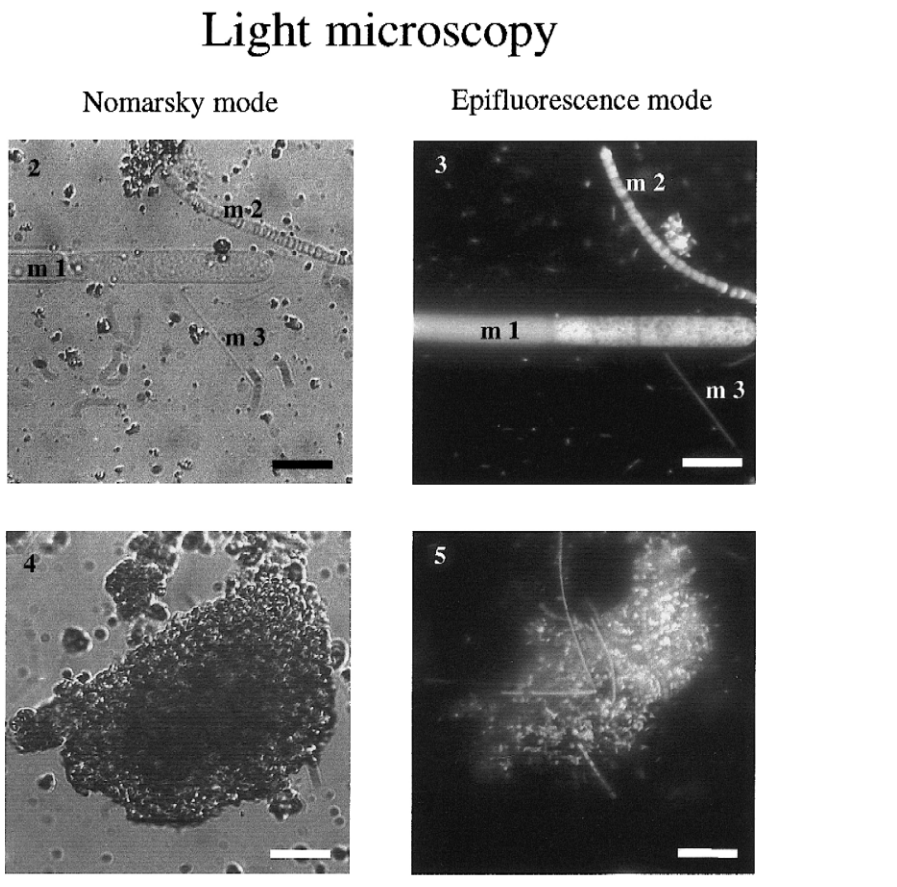
Fig. 5. Epifluorescence view of Fig. 4. Scale bar = 10 μm.

Fig. 6. Electron micrograph of a bacterial cell encrusted with various sized iron granules. c, cytoplasm; Ir, iron granules. Thin section contrasted with U and Pb. Scale bar = 500 nm.

Fig. 7. Electron micrograph of a bacterial cell encrusted with iron granules of a homogeneous size. These granules are in the sheath region. c, cytoplasm; Ir, iron granules; w, bacterial wall. Thin section contrasted with U and Pb. Scale bar = 500 nm.

Fig. 8. Enlarged view of the iron granules of Fig. 7. Thin section contrasted with U and Pb. Scale bar = 50 nm.

coordinated water (Lowenstam and Rossman, 1975). The absence of a broad structure in the 2500–3400 region is another argument for the absence of coordinated water. There was no evidence for isolated hydroxide in the 3500 to 3700 cm^{-1} region, although the KBr pressed disk technique is not the



most appropriate method for the detection of OH groups (Russell, 1979). Absorption bands due to P—O stretching vibration of phosphate were observed at 1100 and 1020 cm^{-1}. The broad region of absorption near 600 cm^{-1} contains the remaining phosphate modes, metal–oxygen stretches and librational modes of water. The small absorption bands at 1400 and 1500 cm^{-1} probably correspond to organic materials.

3.2. Microscopic and phosphatase analyses

Under the light microscope, various morphotypes of filamentous bacteria (Figs. 2 and 3) are associated with masses of mineral granules (Figs. 4 and 5). When the biofilm is stained with acridine orange, the abundance and morphological variety of microbes in the mineral masses becomes evident (Figs. 4 and 5). Under the electron microscope, the mineral appears as granules 50 to 500 nm wide (Figs. 6–8). At a quick glance, it seems that individual cells are encrusted with granules heterogeneous in size and shape (Fig. 6). However, in the sheath region of many cells (against the bacterial cell-wall), sub-spherical colloids are homogeneous in size (50 to 60 nm) and distribution (Figs. 7 and 8). Such cells were observed throughout the thickness of the biofilm, from inner to outer biofilm regions. Colloids are not an artifact of contrasting because they are also observed without the U and Pb treatment (pictures not shown).

All three types of phosphatases were detected at high concentrations in the biofilm (maximum score in biofilm of 5; controls, 0).

4. Discussion

The absorption bands due to the P—O stretching vibration of phosphate, observed at 1100 and 1020 cm^{-1} (Fig. 1), are similar to the absorption bands of phosphate sorbed on iron oxyhydroxide gels (Nanzyo, 1986). The splitting observed indicates the presence of phosphate with a low symmetry, i.e. phosphate adsorbed at the surface as a bridging binuclear type complex. On the contrary, if phosphate would have been surrounded by iron, like in amorphous iron phosphate, a single absorption band for P—O stretching vibration would have been observed at 1060 cm^{-1} (Nanzyo, 1986). As a consequence, the DRIR spectrum obtained here indicates that the iron mineral of *M. ferruginosa* is not a ferric phosphate. The DRIR spectrum is very similar to the spectrum of synthetic iron oxyhydroxide colloids with phosphate sorbed on their surface (Nanzyo, 1986). Surprisingly, the spectrum resembles the one obtained for the dermal granules of the holothurian *Molpadia intermedia* (Lowenstam and Rossman, 1975). Like the mineral in the present study, these dermal granules are made of spherical subunits (about 130–240 nm in diameter in the case of *M. intermedia*) that are X-ray amorphous, rich in water, ferric ion and phosphate with smaller amounts of calcium and silicon. The structural model that was proposed for these granules is that they consist of cores (about 11 nm wide) of poorly crystalline hydrous ferric oxides surrounded by a phosphate surface layer, and cemented together by divalent cations and additional phosphate (Lowenstam and Rossman, 1975). The iron mineral of *M. ferruginosa* also resembles the iron core of bacterioferritins (Mann, 1989). Other amorphous iron (bio)minerals with high phosphate levels have been described in the marine environment, for example in some polychaetes, polyplacophorans and gastropods (Lowenstam, 1972). In summary, in the case of *M. ferruginosa*, most of the phosphate is probably adsorbed at the surface of the mineral that consists of small cores of iron oxyhydroxides. The amorphous nature of the mineral may be related to high levels of phosphate (Mann, 1989) or to continuous redox cycling of Fe(III) to Fe(II), a process that is thought to occur in microenvironments like biofilms (Wells et al., 1995). The surface-adsorbed phosphate groups are possibly of microbial origin because of the high phosphatase activity of the microbes in the biofilm (API ZYM testing). The phosphate might also come from the large phosphate inclusions (volutin) that are present in the filamentous bacteria (Gillan and De Ridder, 1997). As previously suggested, it is possible that the production of phosphate is a mechanism of defense against iron toxicity (Macaskie et al., 1992).

The iron mineral of *M. ferruginosa* resembles the mineral phases that spontaneously precipitate in seawater (amorphous oxyhydroxide but not ferric phosphate; Byrne and Kester, 1976). It might thus be concluded that the mineral is simply abiotically ac-

cumulated and is not the result of in situ microbial biomineralization processes. In addition, colloids of various sizes were found in association with bacterial cells, and this could be interpreted as the result of a simple microbial (bio)accumulation process, as opposed to active biomineralization. However, this is not supported by the following three observations. (1) Our microscopic investigations have shown that iron colloids are closely associated to cells throughout the thickness of the biofilm, from inner to outer regions (see also Gillan and De Ridder, 1995), and many bacterial cells have iron colloids throughout the thickness of their sheath. If iron minerals were simply abiotically accumulated, they would mostly be present at the surface of the biofilm and at the surface of sheaths. (2) Some cells present sheaths encrusted with sub-spherical colloids showing an homogeneous size; this also suggests an in situ microbial formation of the colloids. (3) It is known that pure forms of colloidal iron oxyhydroxides are rare in seawater; other heavy metals are generally associated (Wells and Goldberg, 1994). However, only iron is abundant in the mineral of *M. ferruginosa* (Gillan and De Ridder, 1997). As a counter example, the shell of the marine gastropod *Hydrobia ulvae* is covered with an iron-rich microbial biofilm featuring a mineral rich in manganese, magnesium and aluminium in addition to ferric iron (Gillan and Cadée, 2000). In accordance with the life-style of *H. ulvae*, it was hypothesized that abiotically precipitated iron colloids are simply accumulated in its biofilm. These observations, taken together, support the argument that the biofilm microbes of *M. ferruginosa* are not abiotically accumulating precipitated iron colloids, but that other processes are responsible for a direct nucleation of the colloids in situ (microbial biomineralization), in bacterial sheaths and capsules. This biotic nucleation in the biofilm likely accounts for the presence of a relatively pure mineral phase in the biofilm. Thus, although the mineral of *M. ferruginosa* resembles abiotic phases that are known to precipitate spontaneously from solution, its composition (its "purity"), its morphology, and its distribution in the biofilm are all in accordance with an in situ growth mode.

A wealth of data on other biofilm and planktonic systems suggests that the formation of amorphous iron oxyhydroxide gels on bacterial surfaces is common (Beveridge and Doyle, 1989; Tebo, 1995; Fortin et al., 1997; Konhauser, 1998; Tazaki, 1998). In fact, if we take into account the antiquity of bacteria and their numbers in natural waters (10^3 to 10^6/ml and up to 10^9/g of sediments, Austin, 1988) it is possible that most of amorphous iron oxyhydroxide colloids found in sediments are in fact of microbial origin. Even if this is not entirely true, microbes are known to act as "stabilizing agents" for metastable phases such as colloidal iron oxyhydroxides, because they impede their transformation into crystalline minerals, such as goethite, ferrihydrite and akageneite (Mann, 1989; Crichton, 1991; Kuma and Matsunaga, 1995). It is known that the vast majority of microorganisms that have populated the Earth through time have left no direct record (Knoll and Awramik, 1983). We are thus forced to look at indirect traces of microorganisms, like the microbial biominerals. As such, the geological implications of our work are two-fold: (i) the simple presence of pure amorphous iron oxyhydroxide in an ancient sedimentary environment might be considered as an indicator of microorganisms performing an in situ biomineralization process, even if the microorganisms have become invisible because they have not been fossilized; (ii) many iron ores that are thought to be the result of ancient, high-temperature, hydrothermal processes may, in fact, be the result of ancient low-temperature microbial activity. With regard to the first implication, precise paleoenvironmental conditions may be inferred because we can compare with the present-day environment of *M. ferruginosa*, i.e. an aphotic environment with weak water currents that simultaneously carry oxygen and waste products (organic matter from *E. cordatum* faeces) as well as two independent sources of ferrous iron (Gillan and De Ridder, 1997).

Many new microbial ecosystems and biominerals undoubtedly await description. Exploring the diversity of microbial biofilms and populations represents one of the major challenges of the future for understanding the genesis of sedimentary rocks, and the biogeochemical environments in which they were deposited. Interactions between the geology of the Earth and microbial ecosystems are likely much more intricate than had been imagined a few decades ago, and our understanding of the ways in which microbes interact with each other, and with their

mineral-rich environment, is only now beginning to emerge.

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