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Accepted author version posted online: 31 Oct 2014.

To cite this article: Carolina N. Keim, Hermínio A. Nalini Jr. & Jorge Carvalho de Lena (2015) Manganese Oxide Biominerals from Freshwater Environments in Quadrilatero Ferrifero, Minas Gerais, Brazil, Geomicrobiology Journal, 32:6, 549-559, DOI: 10.1080/01490451.2014.978513

To link to this article: http://dx.doi.org/10.1080/01490451.2014.978513

Geomicrobiology Journal, (2015) **32**, 549–559

ISSN: 0149-0451 print / 1521-0529 online
DOI: 10.1080/01490451.2014.978513

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Received July 2014; Accepted October 2014

Biogeochemical Mn cycling in aquatic environments is driven mostly by microbes, and includes reductive dissolution in anaerobic sediments as well as oxidation in aerobic regions. Oxidation is followed by precipitation, which occurs mainly on the extracellular structures of microorganisms. In this work, we studied the minerals precipitated on extracellular structures of native microorganisms from freshwater environments in Quadrilatero Ferrifero (Iron Quadrangle), Minas Gerais, Brazil, known to contain high levels of manganese. Light microscopy of biofilms and floating material showed diverse biomineralized structures. The most conspicuous were identified as the holdfasts of algae from the genus *Ulothrix*. Diatom frustules associated to manganese oxide precipitates were relatively common. In addition, both filaments and holdfasts produced by bacteria of the genus *Leptothrix* were found, as well as structures similar to those described as *Siderocapsa* and *Metallogenium*. Some previously unknown structures were also observed. Transmission electron microscopy of most of these structures showed the 'crumpled tissue-paper' morphology common in biomineralised manganese oxides. Energy-dispersive X-ray analysis (EDXA) showed that manganese and oxygen were the main components, along with minor amounts of Al, P, S, K, Ca and/or Ba. Our results bring new perspectives to the study of biomineralized manganese oxide structures from the environment. Moreover, they add information about the background of present-day microbial structures needed to better interpret fossilized microbial biominerals.

Keywords: biomineralization, holdfast, iron bacteria, *Leptothrix*, manganese oxides

Introduction

Several microorganisms are able to oxidize Mn(II) to Mn (IV) through Mn(III). Because Mn(IV) is less soluble than Mn(II), manganese oxide minerals readily precipitate onto the microbial extracellular structures. Manganese oxides are very reactive minerals, which abound in both terrestrial and marine environments. Biological Mn(II) oxidation is several orders of magnitude faster than purely chemical oxidation, and thus sedimentary manganese oxides are thought to be mostly biogenic (Tebo et al. 2004). In anaerobic environments, organic matter oxidation coupled to Mn(IV) reduction produces Mn(II), which is more soluble and readily diffuses in water (Lovley 1993).

When Mn(II) reaches aerobic environments, it can be oxidized to Mn(IV) by microorganisms. Because both the oxidation of Mn(II) and the reduction of Mn(IV) are carried out by heterotrophic microorganisms, the Mn biological cycle is driven by organic matter (Ehrlich 2012). This Mn biological cycle potentially leads to co-dissolution (Crowe et al. 2007) and/or co-precipitation of several heavy metals, which can have a large impact on the concentration of these metals in the water (Tebo et al. 2004).

Mn oxides are precipitated on the extracellular structures of diverse microorganisms, including bacteria (Adams and Ghiorse 1986; Emerson and Ghiorse 1992; Feng et al. 2010; Ghiorse and Hirsch 1979; Robbins and Corley 2005; Rouf and Stokes 1964; Sawayama et al. 2011), fungi (Emerson et al. 1989; Robbins and Corley 2005; Santelli et al. 2011), algae (Knauer et al. 1999; Robbins and Corley 2005), and lichens (Pentecost et al. 2010). Several of these manganese-oxidizing microorganisms have been cultured (e.g., Adams and Ghiorse 1986; Emerson and Ghiorse 1992; Rouf and Stokes 1964; Sawayama et al. 2011; Tyler and Marshall 1967).

Biomineralized iron oxides, manganese oxides, metal sulfides and carbonates are major carriers of several chemical elements in sedimentary basins (Borch et al. 2010; du Laing et al. 2009). Mineral precipitation in the environment is the result of interactions of microbial structures and metabolism, chemistry of water and minerals, and physical conditions such as temperature, pressure and light wavelength and

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intensity (Douglas et al. 2008; Skinner and Fitzpatrick 1992). Manganese oxide coatings have been observed in soils and sediments worldwide, and the probable source of these coatings are biomineralized biofilms and microbial mats (Ghiorse and Ehrlich 1992). In some aquatic environments, there is strong evidence for manganese oxidation and manganese oxide precipitation mediated by indigenous microorganisms (Douglas et al. 2008; Lünsdorf et al. 1997; Robbins et al. 1992; Robbins and Corley 2005). In addition, mineralized structures produced by microorganisms can remain in the geologic record for geologic time scales and still be recognized (Ghiorse and Ehrlich, 1992; Li et al. 2013). Very well preserved remains of microorganisms have been found in manganese concretions from a cave environment (Lozano and Rossi 2012). Thus, microscopic images of living, contemporary biomineralized structures can be compared to images of geologic samples and help to identify microfossils in old rocks.

Moreover, we think that studies on native microorganisms and their biomineralized structures can bring important clues to the understanding of diverse themes such as trace element cycling and biomineralization, as well as the origin of minerals and ores, including the manganese oxide coatings and concretions.

In this work, we used light and transmission electron microscopy, as well as energy-dispersive X-ray analysis (EDXA), to analyze mineralized extracellular structures of native microorganisms from Água Suja Stream (ASS) and Gualaxo do Norte River (GNR), which contain high levels of Mn in water and sediments. ASS is a tributary of GNR, and both flow through an area of gold, manganese and iron mining in Quadrilatero Ferrifero (Iron Quadrangle), Minas Gerais State, Brazil. Present and past iron and gold mining in the region released Mn to the hydrological system (Costa et al. 2003).

Materials and Methods

Samples of water and sediment were collected at Água Suja Stream (ASS) (20°17′18″S, 43°28′50″W) and Gualaxo do Norte River (GNR) (20°16′36″S, 43°25′54″W), in the municipality of Mariana (MG, Brazil). Geochemistry of water and sediments from the sampling sites have been studied previously (Costa et al. 2003) and are summarized in Table 1. Our sampling sites ASS and GNR correspond to the sampling sites 16 and 19, respectively, in the work of Costa et al. (2003). In ASS, water moves swiftly, showing a succession of rapids. Accordingly, most of the stream bed was covered by rocks and gravel, but finer sediments could be collected near the margins.

Table 1. Main physical-chemical parameters of water at the sampling sites

	T (°C)	pH	EC (μS/cm)	Fe (mg/L)	Mn (mg/L)
MS	24.5*	6.7–7.2**	85.0–87.6**	14.3–20.0**	1.1–2.79**
GNR	25*	7.3–7.4**	75.7–146.0**	0.15–1.09**	0.24–0.09**

*Measured in the field during sample collection.
**From Costa et al. (2003).

Grass roots touched water from place to place near the margins. Vegetation around ASS was rather poor and no tree canopy covered the stream bed. On the other hand, GNR has slower water flux and some rapids from place to place. A canopy of trees covered most of the river bed, and their roots sometimes reached the water. No macrophytes or fish were observed in either place. The samples were maintained in plastic flasks in the lab, at room temperature and light for up to three months and will be called microcosms from now on.

About 10 days after collection, we observed dark, brown materials floating at the surface of some of the flasks containing GNR samples. These materials seemed rough at the naked eye and resembled a net. Samples were retrieved using a glass slide, mounted and observed by light microscopy, or broken into small pieces and transferred to Formvar-coated copper grids for whole mount transmission electron microscopy (TEM) and energy-dispersive X-ray analysis (EDXA). About ten days after collection, we observed a dark material covering the flask walls. This material was darker near the water-sediment interface, but could not be observed below the sediment-water interface. After 3 months, precipitation of this material in the microcosms was very slow. Ten to 60 days after collection, glass microscopy slides were put vertically into the flasks and retrieved after 5 to 75 days. For light microscopy, the slides were gently rinsed in filtered river water and mounted in river water using large coverslips. The samples were observed using a Zeiss Axioplan 2 light microscope in the Nomarski interference mode.

For transmission electron microscopy of thin sections, the slides were retrieved from the microcosms, gently rinsed in filtered river water, fixed in 2.5% glutaraldehyde in PIPES 0.1M pH 7.0, rinsed with the buffer, post-fixed in OsO_4 1% for 20 minutes, rinsed again, dehydrated in acetone 30%, 50%, 70%, 90% and 100%, and embedded in Polybed 812 resin (Polysciences). Liquid nitrogen was used to break off the glass slides and separate the glass pieces from the resin-embedded samples. Ultrathin sections were cut in a RMC PT-XL PowerTome ultramicrotome, collected with nickel slot grids and put onto Formvar films.

For whole mount transmission electron microscopy of biofilms that grew onto solid surfaces, Formvar-coated nickel grids were maintained in the microcosms for a few weeks and then retrieved, rinsed in distilled water and air-dried. Other samples were rinsed in distilled water, scraped from the slide surface using another glass slide, put onto Formvar-coated copper or nickel grids, and air-dried.

Both ultrathin sections and whole mounts were observed by a FEI Morgagni or a Jeol 1200 EX transmission electron microscopes at 80 kV. EDXA were done on both whole mounts and ultrathin sections, using a Noran EDS detector coupled to a Jeol 1200 EX transmission electron microscope operating at 80 kV for 100 or 200 seconds (live time). Measurements of size of microscopic structures were done in light microscopy images using the Image-Pro Plus 4.5 software (Media Cybernetics, Inc.).

Results and Discussion

Light microscopy observation of dark material floating in the microcosms or deposited onto glass slides revealed several types of morphologically distinct mineral structures, most of them indicative of biological origin. The GNR samples showed a larger morphological diversity of biomineralized structures than ASS samples.

We present the results according to the type of sample. In "benthic biofilm," we refer to the structures that coated solid surfaces maintained inside microcosms, and "floating biofilm" corresponds to floating material collected on the water surface.

Benthic Biofilm

Large discoid structures

In both environments, the most conspicuous mineral morphology had a hole in the center and grainy to filamentous textures at the periphery, forming an approximately discoid structure (Figure 1 a–b). Frequently, they were linked together (Figure 1c). The central hole had a smooth outline but was not perfectly circular (Figure 1 and 2a), whereas the periphery had irregular outlines (Figure 1a–b). In Gualaxo do Norte River (GNR), the whole structure was 22.4 ± 9.3 μm (N = 31) in diameter, whereas in Água Suja Stream (ASS) it was 27.9 ± 10.6 μm (N = 27). The central hole was 4.6 ± 1.7 μm (N = 37) in GNR, and 4.1 ± 1.2 μm (N = 69) in ASS. Observation of sand grains collected from the sediment surface showed several similar structures, confirming that they occur in rock and sediment surfaces in these environments (not shown).

Transmission electron microscopy of ultrathin sections of a large discoid structure showed prokaryotic microorganisms, diverse in morphology, embedded sparsely inside the mineral matrix (Figure 2b). The mineral matrix showed thin sheets with the "crumpled tissue-paper" morphology (Figure 2c) characteristic of biogenic manganese oxides (Feng et al. 2010; Klaveness 1977; Santelli et al. 2011; Spilde et al. 2005; Tan et al. 2010; Tipping et al. 1984). Occasionally, spiky, electron-dense precipitates were observed within this matrix (Figures 2b, 2d). The "crumpled tissue-paper" morphology was also observed in the mineral matrix of whole mount samples (Figure 2e).

Energy-dispersive X-ray analysis (EDXA) showed that manganese and oxygen were main constituents of these structures (Figure 2f). The elemental composition and the crumpled tissue-paper morphology found in these structures are common in biogenic manganese oxides from freshwater environments (Tan et al. 2010; Tipping et al. 1984), caves (Spilde et al. 2005), and also from the cultured bacteria *Pseudomonas putida* (Feng et al. 2010) and the cultured fungi *Plectosphaerella cucumerina* and *Stagonospora* sp. (Santelli et al. 2011).

Microorganisms embedded in the mineral matrix included cocci, rods, lobate and prosthecate prokaryotes (Figure 3). Most of them were found inside gaps within the mineral matrix. Many cells were coated by complex cell envelopes (Figure 3b). Frequently, rod-shaped cells arranged in filaments were found (e.g., Figure 3c). Lobate cells and/or cells containing membrane foldings were also frequent (Figure 3d, 3e). A few prosthecate cells could be observed (Figure 3f), maybe because of the difficulty to obtain longitudinal slices of prosthecae. Mineralized (Figure 3c) extracellular material was relatively common and resembled the fabric of the diffuse capsular layer in the sheaths of *Leptothrix cholodnii* SP-6 (Emerson and Ghiorse 1993). Not mineralized extracellular material was also observed (Figure 3b and 3e). Several cells contained intracellular inclusions (Figure 3a, 3d).

Ehrlich (1999) suggested that microorganisms that grow slower than iron and manganese oxide minerals may become encased inside these minerals, and this is possibly the case of at least some of these microorganisms. Since the ultrastructure of most cells was compatible with a living state (Figure 3) and two or three cells were sometimes observed inside the same void (e.g., Figure 3c), encasement did not seem to be deleterious to most cells. Similarly, St-Cyr et al. (1993) found bacterial microcolonies inside manganese-rich minerals from an iron plaque of a submerged plant, and Miyata et al. (2007) found both living prokaryotes and cell remains inside manganese oxide precipitates formed in long-term mixed cultures. Douglas et al. (2008) observed a biofilm of manganese oxide-depositing bacteria in a wet state, and showed that the appearance of the outer surface was the

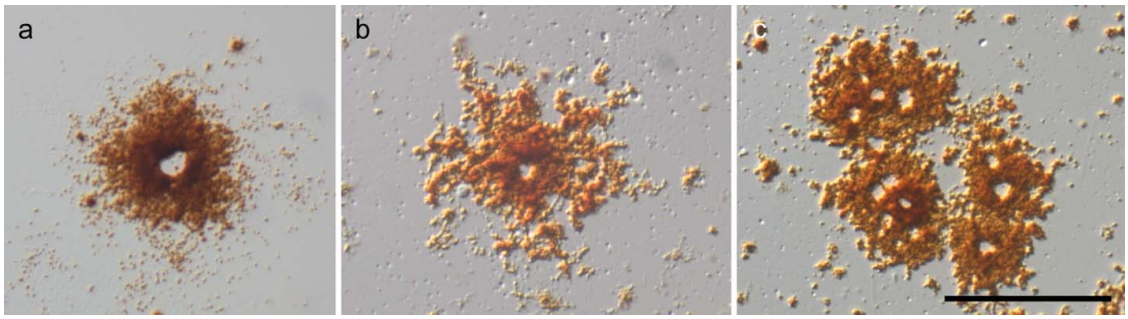

Fig. 1. Nomarski interference light micrographs of large discoid structures with a hole in the center. Some of them show a grainy texture (a), whereas others had filamentous extensions (b), or presented textures intermediate between grainy and filamentous (c). Sometimes they were linked together (c). Bar = 50 μm. Images obtained from GNR (a) and ASS samples (b and c).

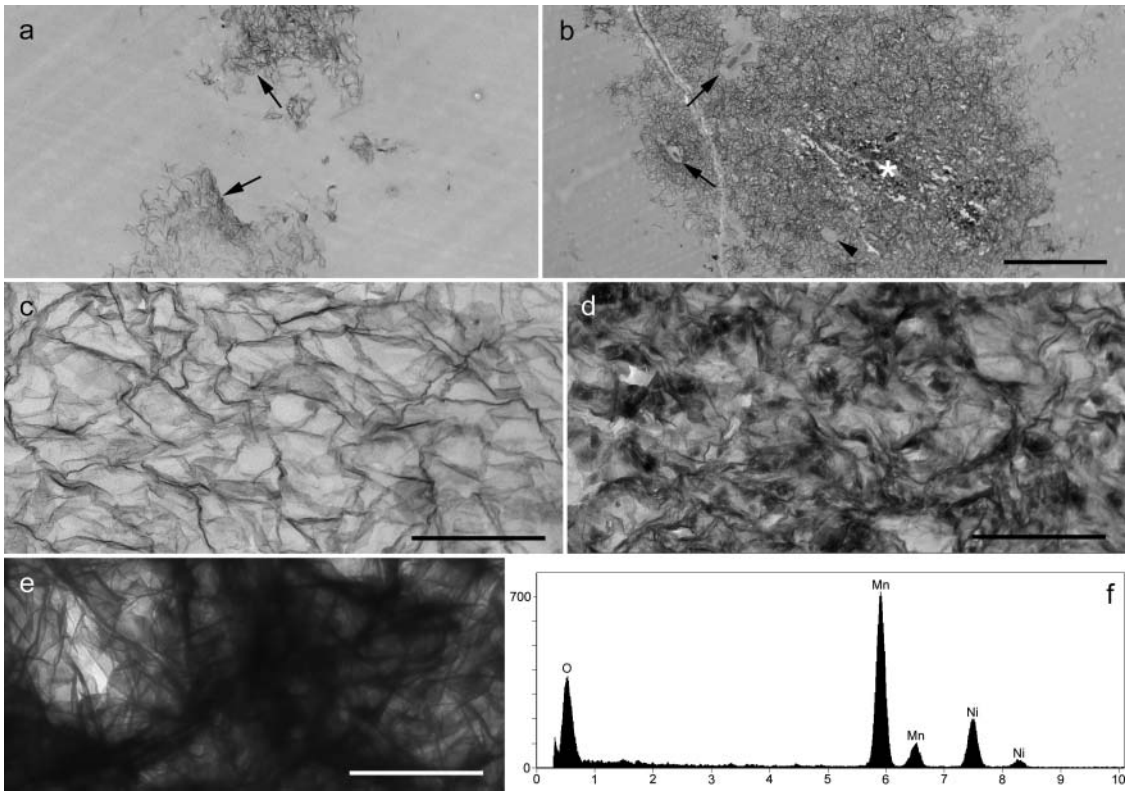

Fig. 2. Transmission electron micrographs and energy-dispersive X-ray analysis (EDXA) of large discoid structures from ASS samples. (a–d) Ultrathin sections, either stained with uranyl acetate and lead citrate (a–b), or unstained (c–d). (a) Low magnification image showing the sharp border of the inner hole (arrows). Bar = 2 μm. (b) Border region showing some prokaryotic cells (arrows) and cell remnants (arrowhead) within the mineral matrix, which is more electron-dense at some inner regions (asterisk). Bar = 5 μm. (c–e) Higher magnification images. Bar = 0.5 μm. (c) Mineral matrix showing a 'crumpled tissue-paper' morphology. (d) Electron-dense region of the mineral matrix, containing a denser packing of minerals and also electron-dense patches formed by fine-grained minerals. (e) Sample scraped and deposited onto a Formvar film (whole mounts), showing another view of the 'crumpled tissue-paper' morphology. (f) EDXA of a large discoid structure showing mainly manganese and oxygen peaks. Nickel peaks are from supporting grid.

same as bacterial exopolysaccharide (EPS) in a wet state. The fact that the lining of voids where we have found cells was frequently more electron-dense than the surrounding matrix (Figure 3a) may suggest that the EPS containing manganese oxide was soft enough to be pushed as the microbes grew.

Although we have found a large morphological diversity of prokaryotic cells, none of them showed specific interactions with the crumpled tissue-paper type mineral matrix, suggesting that they were not responsible for biomineralization of the large discoid structures. On the other hand, size and shape suggest that the large discoid structures are, in fact, holdfasts of a eukaryotic microorganism. Tazaki (1997) found structures similar in size, morphology and ultrastructure in a freshwater pond, but interpreted them as the holdfasts of the bacterium *Leptothrix discophora*, which are smaller and have a different morphology (Carlile and Dudeney 2001).

Little et al. (1997) reported structures similar in both morphology and elemental composition growing on stainless steel maintained in freshwater. Robbins and Corley (2005) also observed very similar structures in a manganese-rich stream and showed that they corresponded to the holdfasts of filamentous green algae of the genus *Ulothrix*. Indeed, we have found green algae probably belonging to the genus *Ulothrix* in ASS samples (not shown), however no direct evidence for their direct participation in the construction of such structures was found. Because of the high numbers and large size of these holdfasts, we suggest that they are the major sites of biogenic MnO_x precipitation in both GNR and ASS. Robbins and Corley (2005) found similar results in a freshwater creek.

Filamentous Sheaths

Straight, thin filaments with one rounded end linked to very thin filaments were observed in GNR (Figure 4a). The rounded end probably correspond to a holdfast, although much smaller than the small disks described next. The brownish color and smooth edges suggest that they are evenly mineralized. These sheaths are similar to those of *Leptothrix ochracea*, which is an uncultured, sheathed iron bacterium (Fleming et al. 2011), although *L. ochracea* have not been described to produce holdfasts. Thus, this may correspond to an undescribed sheathed bacterial species.

In ASS samples, filamentous bacterial sheaths were abundant near the water-sediment interface. Two types of

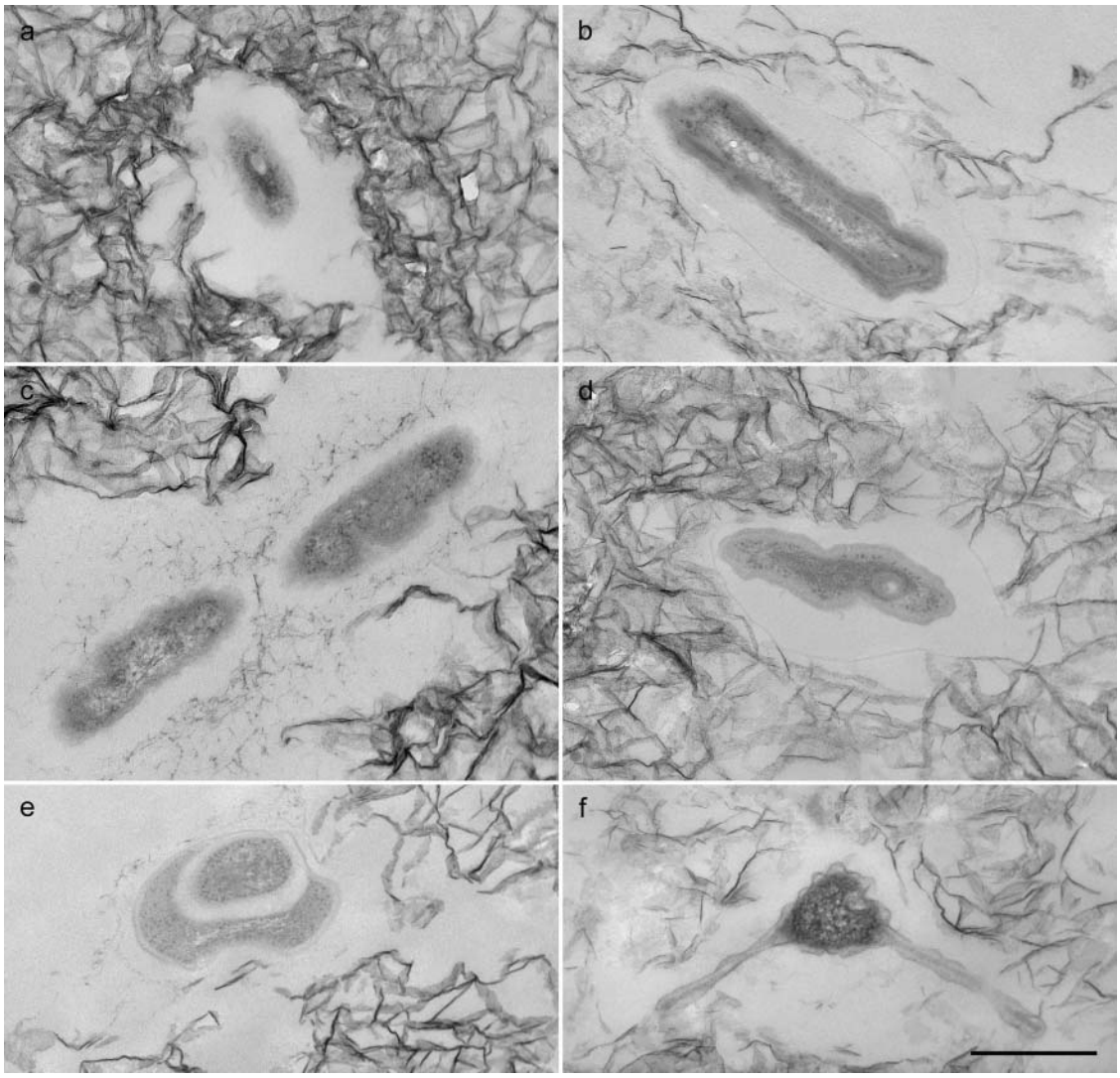

Fig. 3. Transmission electron micrographs of gram-negative prokaryotes within voids of large discoid structures from an ASS sample. (a–c) Rod-shaped prokaryotic cells. (a) The mineral is denser close to the cell. (b) Cell showing a complex, layered cell envelope with peculiar, acute angles at the poles. (c) Cells arranged in line inside a sheath formed by thin, mineralized filaments. (d–e) Lobate cells. (d) Cell containing many dark spots in the cytoplasm and coated by a thick envelope. (e) Cell coated by a thin exopolysaccharide matrix. (f) Prosthecated cell showing two thin cell extensions. Bar = 0.5 μm.

filaments with different widths and degrees of mineralization were observed (Figure 4b). The wide filaments were 1.5 $\pm$ 0.1 μm in internal diameter (N = 12) and 5.4 $\pm$ 1.8 μm in external diameter (N = 28); in contrast, the internal diameter of the narrow filaments was about 0.25 μm, whereas the external diameter was 1 to 3 μm. Both were rough and tortuous, branched, and grew attached to the substrate (in this case, the microscope slide). Transmission electron microscopy showed empty sheaths impregnated with minerals (Figure 4c). The size of these sheaths suggests that they correspond to the narrow type observed by light microscopy. Minerals showed irregular growth, which probably gives the rough appearance to the sheaths at the light microscope. The minerals presented the 'crumpled tissue paper' appearance characteristic of some biogenic manganese oxides, including the large discoid structures shown in Figures 1–3.

These filaments may correspond to sheaths of two different bacteria of the genus *Leptothrix*. Filamentous bacteria such as these are common in iron- and manganese-rich freshwater environments (e.g., Robbins et al. 2000; Robbins and Corley 2005) and have been found colonizing stainless steel in brackish water (Kielemoes et al. 2002). Some are straight and plain (Robbins and Corley 2005), as found in GNR samples, whereas others are tortuous and had rough surfaces (Adams and Ghiorse 1986; Kielemoes et al. 2002), as found in ASS samples. These filaments also resemble those from cultivated *Leptothrix* sp. (e.g., Sawayama et al. 2011). Literature is rather confusing, because the same nomenclature used for environmental microorganisms was used for cultured strains. Here, we will use the term

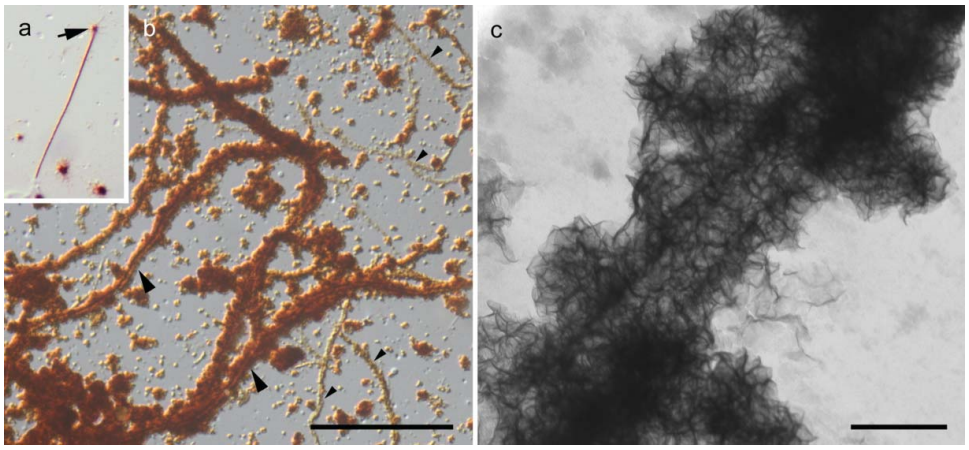

Fig. 4. Microscopy of filamentous structures. (a–b) Nomarski interference light microscopy showing (a) a straight filament which grew from a tiny holdfast (arrow) and (b) tortuous, rough filamentous sheaths attached to the glass slide. There were thick (large arrows) and thin (small arrows) filamentous sheaths, both covered by brown minerals and branched. Bar = 50 μm for (a) and (b). (c) Transmission electron microscopy of whole mounts showing an empty bacterial sheath covered by minerals with the 'crumpled-tissue paper' morphology. Bar = 0.5 μm. Images obtained from GNR (a) and ASS samples (b–c).

"*Leptothrix* sp." to design the bacteria that produce sheaths and/or holdfasts encrusted by manganese oxides.

Small disk-shaped structures

Small, dark, disc-shaped structures were found in both sites (Figure 5). These were clearly different from the discoid structures shown in figure 1 in size, shape and texture: they were smaller, had circular shapes and smooth texture. Small disks from ASS were 6.7 ± 0.8 μm in diameter (N = 27), and the central hole was 1.5 ± 0.3 μm in diameter (N = 27). Those from GNR samples were usually smooth, whereas those from ASS samples were frequently linked to thin filaments that extended from the disks, mainly at the plane of the glass slide (Figure 5). Through-focus light microscopy allowed a rough estimative of thickness around 2 μm. In addition, it showed that these disks had small rims at both the inner and outer borders. The inner hole had a sharp outline, whereas the periphery showed some roughness at the plane of the slide (Figure 5).

Accordingly, transmission electron microscopy of whole mounts showed a sharp outline in the inner hole and a rough outline at the periphery (Figure 6a). The periphery presented fibrillary extensions and, occasionally, small electron-dense crystalline precipitates (Figure 6b). In less dense discs, the mineral matrix seems fibrous (not shown). Selected area electron diffraction produced diffuse rings, showing that this structure contains amorphous and/or microcrystalline minerals (not shown). Energy-dispersive X-ray analyses showed that manganese and oxygen were the main components of the disks, along with minor amounts of calcium, aluminum and barium (Figure 6c). These results suggest that the mineral in the small disks consists of amorphous or microcrystalline manganese oxide.

The morphology, size and elemental composition of these small disk-shaped structures strongly suggest that they correspond to the holdfasts of *Leptothrix discophora*, or a related microorganism. The holdfasts of *L. discophora* have been described in environmental samples (Carlile and Dudeney 2001; Robbins et al. 1992; Robbins et al. 2000; Robbins and Corley 2005; Schmidt and Robbins 1992; Schwers 1912), but were never found in samples of cultivated strains (Carlile and Dudeney 2001; Spring 2006). Other bacteria from the same genus, called *L. lopholea*, are able to produce holdfasts in culture (Spring 2006; Spring and Kämpfer 2005).

The bacterium *Leptothrix discophora* was described morphologically in 1912, under the name *Megalothrix discophora*, as rod-shaped cells enclosed in a thick sheath impregnated with iron oxides, which attach to solids through a conspicuous, disk-shaped, manganese oxide-containing holdfast. The disks were 10–12 μm in diameter with a central hole 1–1.5 μm in diameter and 0.3–0.5μm in thickness

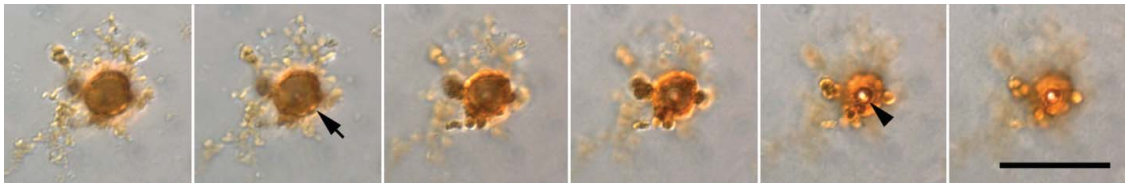

Fig. 5. Nomarski interference light micrographs of a small disk-shaped structure with a hole in the center, obtained from an ASS sample. The images are shown in through-focus series. Note the inner hole (arrowhead) and an outer rim (arrow). Bar = 20 μm.

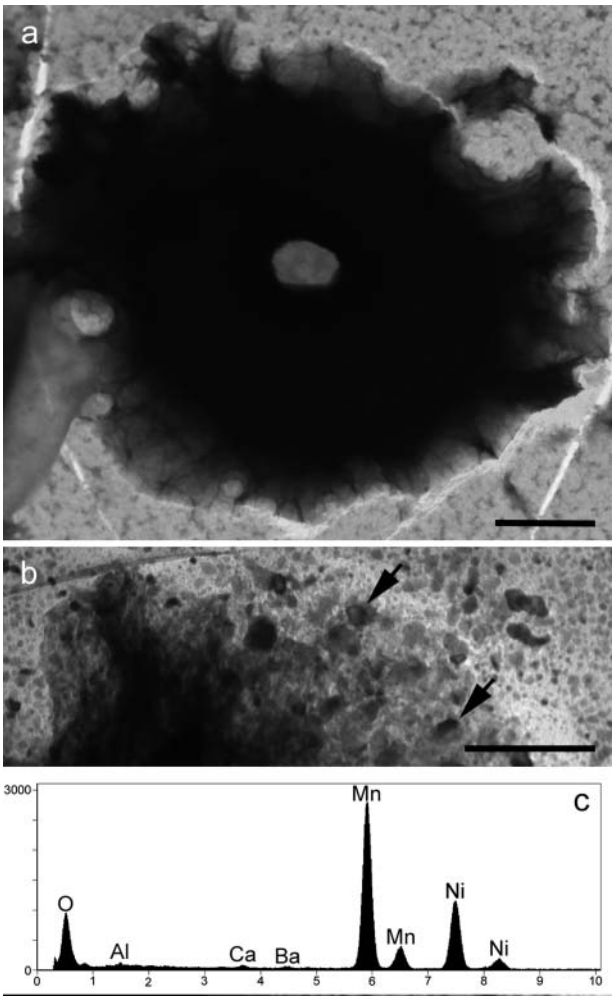

Fig. 6. Transmission electron microscopy (TEM) and EDXA of a small disk-shaped structure from ASS sample, in whole mounts. (a) TEM of a whole disk showing the well-defined outline and fibrillary projections at the external outline. Bar = 2 μm. (b) Higher magnification of the border of the same structure showing tiny, dark precipitates (arrows). Bar = 0.2 μm. (c) Energy-dispersive X-ray spectrum of a similar structure, showing mainly manganese and oxygen peaks, as well as minor aluminum, calcium, and barium peaks. Ni peaks are from supporting grid.

(Schwers 1912), which are close to the disk size observed in this work.

The related, cultivated bacteria *L. discophora*, *L. cholodnii* and *L. mobilis* are able to oxidize manganese, and in the sheath-producing *L. discophora* and *L. cholodnii* strains, the sheaths become impregnated with manganese oxides. The loss of the ability to oxidize manganese or to form sheaths has been documented in several *Leptothrix* strains. The same probably happened to the disk-shaped holdfasts (Spring 2006). The loss of important phenotypic characteristics in cultured strains illustrates the importance of microscopic observations of environmental samples if one wants to understand biogeochemical processes driven by microbes in the environment.

Other structures

The benthic biofilm from GNR samples showed also structures composed by thin filaments radiating from a single point, forming a bunch (Figure 7a). The whole structure was 29.6 ± 11.5 μm in diameter (N = 17), and the individual filaments were 0.9 ± 0.2 μm in width (N = 86). These resemble *Metallogenium* (Gregory et al. 1980; Jaquet et al. 1982; Klaveness 1977; Miyajima1992; Neretin et al. 2003; Robbins and Corley 2005; Zavarzin 1981), structures described as microbial cells (Walsh and Mitchell 1973; Hanert 2006; Jaquet et al. 1982), but after considered to be not living since no evidence for living cells were observed inside the hollow center (Gregory et al. 1980; Klaveness 1977). In addition, similar structures have been found in cultures of manganese-oxidizing fungi (Emerson et al. 1989) and bacteria (Miyajima1992), as well as in mixed cultures (Margulis et al. 1983). This is still a controversial issue, since some studies did find cells (Jaquet et al. 1982), whereas others did not find evidence for cells in these structures (Emerson et al. 1989; Gregory et al. 1980; Klaveness 1977; Miyajima 1992). Thus, they can be regarded as biominerals because they depend on microorganisms to occur.

Club-shaped filaments radiating from a single point (Figure 7b) were relatively abundant in GNR Samples. The whole structure was 24.2 ± 8.4 μm in diameter (N = 11.5), and the large tips of the filaments were 4.9 ± 2.5 μ in width (N = 103). Although they also bear similarities with *Caulobacter*, and *Caulobacter*-like cells have been found in manganese-rich environments before (Ferris et al. 1999) and also in fossil ferromanganese concretions (Lozano and Rossi 2012), the fact that we have found a large variation in the tip width suggest that these structures does not contain *Caulobacter* cells, but may comprise a second type of *Metallogenium*.

GNR samples also contained star-shaped structures attached to the glass slide (Figure 7c). These structures contained 1 to 12 tips, were 4.9 to 18.4 μm in width, and had 16.2 to 44.2 μm in distance between the farthest tips (N = 6). These could correspond to large star-shaped cells. Star-shaped bacterial cells of the genera *Prosthecomicrobium* and *Ancalomicrobium* are morphologically similar to these structures, although they are much smaller and grow unattached (see Staley 1968). Another possibility is that these structures consist of several sheathed, encapsulated bacteria that grow from a single point toward the periphery, attached to the glass slide. The morphology of star-shaped structures containing a single tip and a round end are very similar to some reported images of *Leptothrix discophora* (Carlile and Dudeney 2001), which support this interpretation. In addition, descriptions of *L. lopholea* agree with observed structures (Spring and Kämpfer 2005). If this is the case, these structures could be considered one more *Leptothrix* sp. in GNR samples.

Very small, mineralized, hemispherical or globular structures (Figure 7d–e) were very common in samples from both sites. Some were smooth (Figure 7d) and sometimes contained a small clear area, corresponding to a prokaryotic cell or a hole on the mineralized hemisphere. Those from GNR samples were 3.1 ± 0.7 μm in diameter (N = 52), whereas those

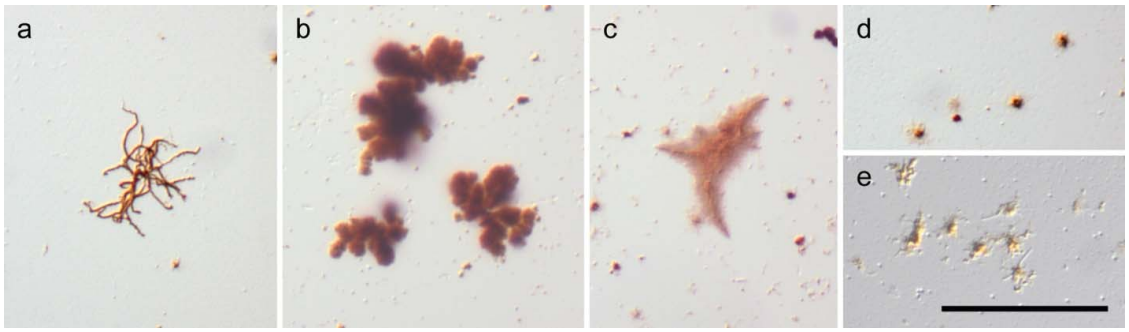

Fig. 7. Nomarski interference light micrographs of biomineralized structures. (a) Thin filaments arising from a single point. (b) Large club-shaped filaments arising from a single point. (c) Star-shaped structure. (d) Small mineralized structures, hemispherical in shape, with smooth surfaces. (e) Similar structures linked to thin, not mineralized filaments which can be straight, helical, or club-shaped. Bar = 50 μm. Images obtained from GNR (a–d) and ASS (e) samples.

from ASS were 3.7 ± 0.8 μm (N = 34). These probably correspond to single cocci or short rods coated by manganese oxide minerals, and bear similarity to *Siderocapsa* (Hanert 2006). Hemispherical structures of similar size but surrounded by very thin, probably not mineralized, straight or curled filaments were observed in both environments (Figure 7e). Those from GNR were 3.7 ± 1.0 μm in diameter (N = 37), whereas those from ASS were about 4.3 μm. The whole structure appearance, and specially the presence of filaments, suggest that they correspond to budding bacteria like *Hyphomicrobium* or *Pedomicrobium*, which have been reported to oxidize manganese and precipitate manganese oxide extracellularly (Ghiorse and Hirsch 1979; Tyler and Marshall 1967).

Floating Biofilm

Floating biofilms appeared only in some GNR samples. At the naked eye, the floating biofilm was dark brown and very opaque, with tiny, irregular holes. It has been reported the observation of the manganese-oxidizing bacteria *Leptothrix discophora* in floating, oil-like films that frequently occur in manganese-rich environments (Robbins et al. 1992; Robbins et al. 2000). However, the floating material that appeared in our samples was discontinous and very dark, not iridescent and continuous as the oil-like material known to contain manganese bacteria.

At the light microscope, a monolayer of long rod-shaped prokaryotes was observed near the border of the floating biofilm (Figure 8a). Dark spots were sparsely distributed (Figure 8a), and they shared pretty much the morphology of the hemispherical structures of the benthic biofilm shown in Figure 7d, and also the structures known as *Siderocapsa* (Hanert 2006). Larger structures, composed by a central disk and some protuberances at the periphery, were relatively common (Figure 8b). They were 7.6 ± 0.7 μm in diameter (N = 23). Similar structures were rarely observed in the benthic biofilm of GNR samples (not shown).

The most prominent mineralized structures in these samples were very large, densely mineralized and had no specific morphology (Figure 8c). They had a grainy texture and, in most of them, the periphery was composed by wide filaments. Although the voids between them were sometimes occupied by diatom frustules, they did not present obvious connections with microorganisms present in the sample. Another prominent feature of the floating biofilms was the presence of frustules of diatoms, most belonging to the genera *Cymbella*, *Navicula*, *Encyonema*, *Nitzschia*, *Gomphonema*, and *Achnanthidium*, associated to small manganese oxide precipitates (Figures 8d-f). Some contained chloroplasts, indicative of viability (Figure 8d). Robbins et al. (1992) and Robbins and Corley (2005) found diatom frustules and associated manganese oxide minerals in benthic biofilms from a freshwater stream. On the other hand, Douglas et al. (2008) found manganese oxides associated to bacteria, but not to diatoms, in a microbial mat from a small brine pond.

Rarely, structures composed by bunches of thin filaments radiating from a single point were observed in these samples (Figure 8e). They were very similar to that observed in the benthic biofilms of GNR (Figure 7a), and also resembled *Metallogenium*. Filamentous bacterial sheaths of different widths and degrees of mineralization were found in the floating biofilm (Figure 8f–i). Some were straight, smooth, apparently not mineralized, and rigid, as evidenced by the sharp bends seen in Figure 8f. It is possible that these sheaths were produced by the same bacteria that produced the sheaths in the benthic biofilm shown in Figure 4a, identified as *Leptothrix* sp. in this work. Other filamentous sheaths were tortuous, mineralized, and rough, and would also be identified as *Leptothrix* sp. (Figures 8g–i). Roughness increased with width, suggesting that these sheaths could be produced by the same species, which were observed at diverse degrees of mineralization. These may correspond to the wide sheathed bacteria found in the benthic biofilm (Figure 7b), although here they grew unattached.

EDXA of the large structures shown in Figure 8c showed mainly manganese and oxygen peaks, along with small amounts of calcium and barium (Figure 8j). This type of structure comprised most of the manganese oxide in the floating biofilm samples. Because of the unspecific morphology, lack of specific connections with microbial cells, and the fact that floating biofilms did not appear in all samples, it is probable that they arise as result of secretion of manganese-oxidizing factors like enzymes (e.g., Adams and Ghiorse 1987), quinones (Johnson and Tebo 2008), or superoxide (Learman et al. 2011), and/or macromolecules that serve as template for mineralization (Li

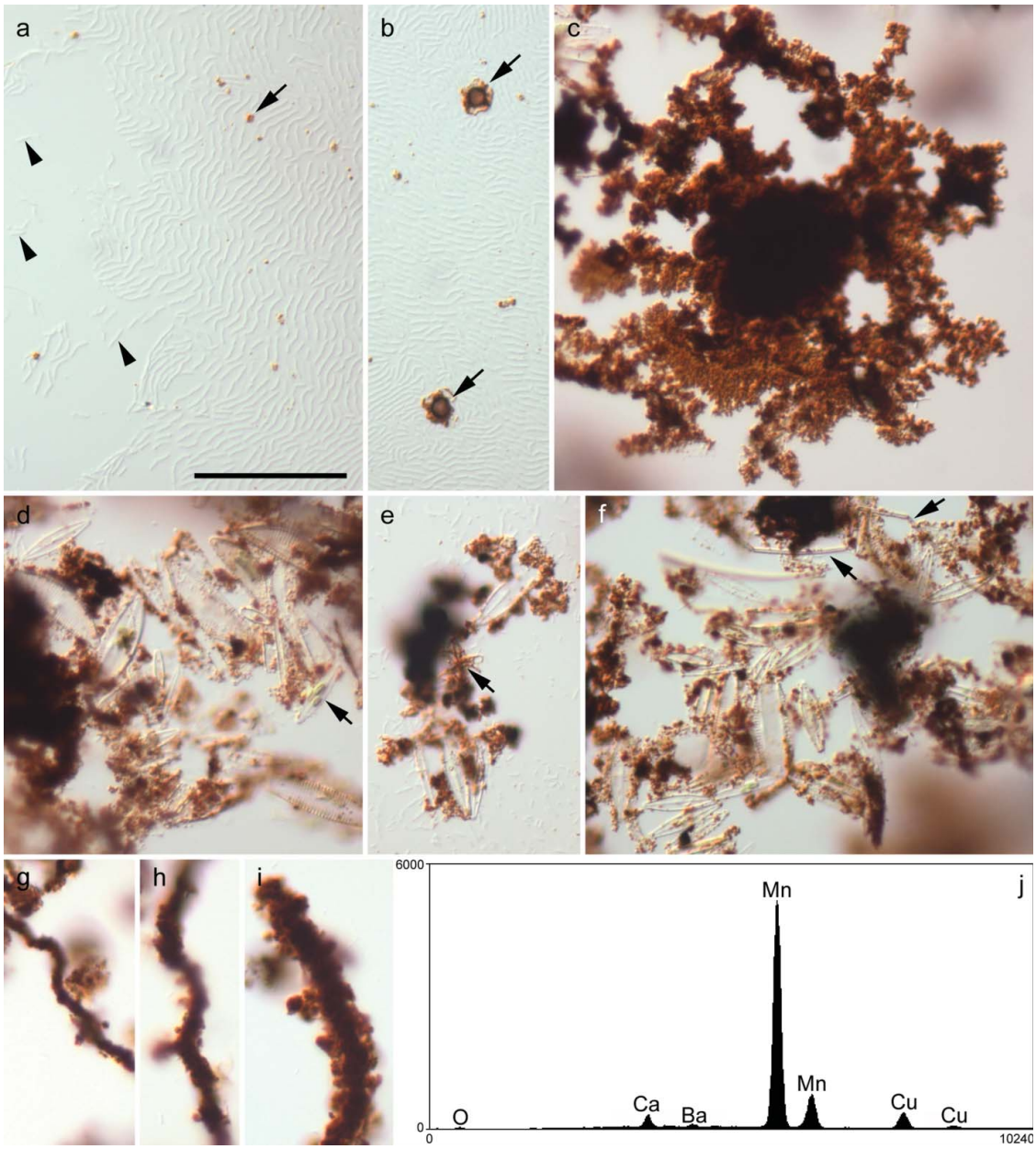

Fig. 8. Nomarski interference light micrographs and EDXA of biomineralized structures of floating biofilms from GNR. (a) Monolayer of long, thin, rod-shaped prokaryotes probably comprising a single species, shown here at the border of the sample (arrowheads). A few small biomineralized structures are also seen (arrow). (b) Circular structures devoid of central holes (arrows). (c) Large mineralized structure, denser at the center and filamentous at the borders, without a hole in the center. (d–f) Regions containing many diatom frustules associated to manganese oxide minerals, including (d) living diatoms containing green chloroplasts (arrow), (e) a filamentous structure resembling *Metallogenium* (arrow), and (f) thin, apparently not mineralized, straight filaments with some bends suggesting rigidity (arrows). (g-i) Rough, tortuous, mineralized filaments. Filament width and roughness increases from (g) to (h) and then (i). Bar = 50 μm. (j) EDXA of a large mineralized structure similar to that shown in (c) showing mainly manganese peaks, as well as minor calcium and barium peaks. Cu peaks are from supporting grid.

et al. 2013), by microorganisms from the water or from the floating biofilm.

Elemental Composition of Manganese Oxide Biominerals

EDXA showed that all structures analyzed contained relatively large amounts of Mn and O (Figures 2f, 6d, and 8j). In addition, the large disks contained small amounts of K and Ca, the small discs contained P, S, Al, K, Ca and/or Ba (e.g., Figure 6c), and the large manganese oxide structures in the floating biofilm contained Ca and/or Ba (Figure 8j). Similarly, Raymond et al. (1992) found Ba, Ca, Fe, Al and P as well as Mn in mineral phases of rock varnish. Tazaki (1997) found mainly Fe, Si and Al and minor amounts of K,

Ca and Mn in structures similar to the large holdfasts shown in Figure 1. Despite the fact that iron concentrations are larger than manganese concentrations in the water of both sites (Table 1), microbial structures concentrated manganese, such that iron was not detected in the EDXA spectra.

Conclusions

Similarly to macro-organisms, microorganisms that live in aquatic environments can be free-living (planktonic) or grow attached to surfaces (benthic). Some of them alternate between the two lifestyles. Because we rinsed our slides with filtered river water, our benthic biofilm samples contain exclusively those microorganisms able to grow attached to surfaces, as well as their remains. Robbins and Corley (2005) used a similar technique and found the holdfasts of *Leptothrix* and *Ulothrix*, as well as *Leptothrix* filaments and *Metallogenium*. The floating biofilm is a special case, because it could have arisen by action of planktonic microorganisns, and then benthic microorganisms can grow attached to pre-existing parts of the biofilm. Although there were marked differences between benthic and floating biofilms, some structures were very similar in the two microenvironments: the structures similar to *Siderocapsa* (Figures 7d and 8a), the new structures shown in Figure 8b, and the filamentous sheaths observed in Figures 4a–b and 8f–i.

In both environments (ASS and GNR), we observed that manganese oxide minerals were formed mainly by biomineralization. The same has been observed in other environments (e.g., Douglas et al. 2008; Robbins and Corley 2005). In our samples, manganese was precipitated on extracellular structures, such as holdfasts, sheaths and capsules. These structures have been known as the main mineral-precipitating structures in microorganisms (Ghiorse and Ehrlich 1992; Lowenstam and Weiner 1989). With continuous manganese oxide deposition and aging, these materials can form manganese oxide coatings, which are widespread in manganese-rich environments (Ghiorse and Ehrlich 1992). The dark brown color observed in the flask walls and glass slides with the naked eye suggest that our samples were at an initial phase in the production of manganese coatings.

Biomineralization processes usually produce minerals with specific morphology, size and composition, which can remain in the geologic record for geologic eras (Ghiorse and Ehrlich 1992; Li et al. 2013). However, the recognition of microbial fossils in the geologic record is not straightforward and a large volume of data on present-day microorganisms is needed. The results shown in this work adds to the knowledge of morphology and composition of micro-biominerals and may help in future studies on fossil microbes.

Acknowledgments

The authors thank Weliton J. da Silva for identification of diatom genera, and Felipe Pitzer for help with measurements of microscopic structures. We also thank Douglas A. Pabst and José Dias Corrêa Jr. for help in collecting samples.

Funding

This work was supported by CNPq and FAPERJ Brazilian agencies.

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