2
3 **Bioconstructions in ochreous speleothems from lava tubes on**
4 **Terceira Island (Azores)**

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15 **ABSTRACT**

16 The ochreous speleothems examined here were obtained from a lava tube on Terceira
17 Island (Azores) and show compact mineralized areas intermixed with zones in which
18 bacterial structures are evident. Bacterial layers of filament-like structures are common
19 throughout the deposits but differences in microstructure among the different
20 speleothems were observed, reflecting a broad morphological range of deposits.

21 The structures and minerals detected in the speleothems betray their biogenic origin.
22 *Gallionella* and *Leptothrix* were the two most frequently observed morphotypes and
23 probably the main contributors to speleothem formation. However, DGGE analysis
24 indicated the presence of another bacterial population (with a predominance of
25 proteobacteria) that could also contribute to iron hydroxide-oxide precipitation. The
26 sheaths of *Leptothrix* cells and stalks of *Gallionella* cells were associated with large
27 amounts of extrapolymeric substances (EPS), which play a role in biomineralization
28 processes. Independently of the taxa present, mineral deposits were composed of poorly
29 ordered Si-rich ferrihydrite, a typical mineral phase of biogenic Fe precipitates.
30 In this high-resolution microscopy study of interrelationships between mineral
31 precipitates and associated microorganisms and their structures, we attribute bacteria an
32 important role in constructing these speleothems. Through their metabolic activity,
33 these bacteria cause the precipitation of ferrihydrite but their mineral structures could
34 also act as nucleation points for passive mineral precipitation. Finally, the build-up of
35 bacterial mineral structures and later cementation processes seems responsible for the
36 formation of mineral layers that confer consistency to the speleothem. Our findings
37 point to bacterial activity as the main factor determining speleothem structure and
38 formation.

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1. Introduction

Microorganisms inhabit all possible environments in the biosphere including the Earth's subsurface and significantly impact its geochemistry through mineral precipitation and dissolution processes (Boston et al. 2001; Northup and Lavoie, 2001; Fortin and Langley, 2005; Takashima et al., 2008; Jones, 2010). In caves, mineral formations are often the result of secondary mineral deposits called speleothems. The idea of a role of living organisms in the development of some speleothems is fairly old but it was not until the second half of the last century that evidence appeared indicating that microorganisms may somehow control speleothem formation (Forti, 2001). Caves are natural geomicrobiological laboratories for research on biomineralization processes and the microorganism responsible for these processes, since microbial fabrics in this type of environment are protected from extensive diagenetic modification or destruction (Sanchez Moral et al., 2006). Resident microorganisms are commonly found in speleothems (Northup et al., 2000; Kasama and Murakami, 2001; Baskar et al., 2008) and bioprecipitation, biomineralization, and alteration of the rock substrate are some of the mechanisms whereby these microorganisms and their metabolism can contribute to mineral deposition in caves (Northup et al., 2000; Frankel and Bazylinski, 2003; Forti, 2005; D'Elia et al., 2007; Spear et al., 2007; Aubrecht et al., 2008; Baskar et al., 2009; Jones, 2009).

Iron-oxidizing bacteria actively take part in the redox cycling of iron and/or the precipitation of iron oxides in oxic and anoxic conditions (Kasama and Murakami, 2001; Barker and Banfield, 2003; Rentzt et al., 2007; Takashima et al., 2008; Baskar et al., 2009; Blöthe and Roden, 2009). Iron oxide precipitation also occurs as a result of passive reactions, with microbial cell walls and extracellular material acting as nucleation surfaces (Fortin and Langley, 2005). Since the rate of aqueous Fe(II)

oxidation and precipitation is quite low at acidic pH (Nordstrom and Southam, 1997), it is widely recognized that microorganisms are important mediators of the oxidation of Fe(II) in natural environments of low pH (Barker and Banfield, 2003; Bruun et al., 2010). At neutral pH, iron-oxidizing bacteria precipitate iron minerals at rates up to four orders of magnitude higher than the inorganic precipitation rate (Kasama and Murakami, 2001; Takashima et al., 2008). The community structure responsible for these oxidative processes has not been completely established, but morphologically distinct species such as *Gallionella ferruginea* and *Leptothrix ochracea* are generally observed associated with such processes (Kennedy et al., 2003; James and Ferris, 2004; Katsoyiannis and Zouboulis, 2004; Emerson and Weiss, 2004; Blöthe and Roden, 2009). Studies have shown that 50-80% Fe precipitation is attributed to the metabolism of microorganisms in laboratory experiments in the presence of *Gallionella*, *Leptothrix* and unicellular bacteria at 3-6 ppm of Fe concentration at pH of 6.9-7.2 (Emerson and Revsbech, 1994). In effect, Hallbeck and Pedersen (1991) reported that *G. ferruginea* relies on Fe(II) oxidation as an electron donor for autotrophic growth. However, the oxidation of *Leptothrix* has not been yet related to energy production or metabolic utility (Kennedy et al., 2003). Other not so morphologically distinct bacteria such as strains of the Proteobacteria subclass are also purportedly involved in these oxidative processes (Emerson and Moyer, 1997; Brunell et al., 2006; Emerson et al., 2007; Blöthe and Roden, 2009).

The Buracos cave developed in a lava tube on Terceira Island (Azores) (Fig. 1). The island harbours four polygenetic volcanoes (Pico Alto, Santa Bárbara, Guilherme Moniz and Cinco Picos) and a basalt fissure zone (Nunes, 2000, 2004; Fig. 1), which have been active in the last 50,000 years (Self, 1982). The Buracos cave is sited in the Malha Grande lava field, on the north flank of Santa Barbara Volcano. The cave name

(*buracos* = holes) is due to the presence of several skylights along the lava tube. Basaltic pyroclasts and lava flows are common, ranging in composition from basalts to comendites and pantellerites (Mungall and Martin, 1995; Self, 1976). The most recent eruption in 1761 gave rise to extensive flow towards the northern coastal slope. A geotherm system close to the Pico Alto volcano shows superficial manifestations at the Furnas do Enxofre Fumarolic field, close to the Guilherme Moniz central caldera, and thermal waters in the northeast (springs) and southeast volcano flanks (wells). Buracos cave is an isolated segment of the Gruta dos Balcões lava tube, the longest cave on Terceira island some 5.021 km in length (Pereira et al., 2004), that developed in the Malha Grande Lava field, 100 m to the west of the 1761 flow and less than 1 km northwest of the Furnas do Enxofre fumarolic field. The Buracos cave lies close to the surface at a 2-3 m depth and is covered by the basaltic rock and a water-saturated volcanic soil almost throughout the year. Abundant iron-rich speleothems including stalagmites, stalactites, columns and flowstones can be found in Buracos cave.

This study was designed to characterize the mineralogy, chemistry and the geomicrobiology of iron-rich speleothems from Buracos cave. Its main objectives were to examine the microbial contribution to the fabric of iron-rich deposits and clarify the role played by microorganisms in speleothem construction. The bacterial composition of these speleothems was determined through a combined microscopy/molecular approach.

2. Sampling and methods

2.1. Study Site

The rock forming the Buracos cave is porphyritic halocrystalline basalt containing clinopyroxene and olivine phenocrystals. The groundmass is microcrystalline and

consists of acicular plagioclase, olivine, clinopyroxene and scarce opaques. The basalt is scarcely altered, with only some pyroxenes and olivines having fractures presenting ferric-oxides. The lava flow is covered by pumice emitted about 3000 years ago (Nunes et al., 2008) and contains several basaltic lapilli deposits associated with the historic eruption of 1761 AD.

The temperature inside the cave remains very stable, near 14°C, and the humidity is very high the year round. In the rainy season, water flows through the ceiling cracks, runs off the stalactites and hits the floor. In the dry season, the dripping water from the ceiling is lower, but the environmental humidity remains high. All the speleothems appear wet.

Inside the Buracos cave, iron speleothems are stalagmites (Fig. 2A), stalactites (Fig 2A-C), columns and flowstones. All these formations show orange and reddish laminae (Fig. 2B). Stalactites and stalagmites are very abundant, sometimes growing together into columns or walls. The stalactites appeared commonly aligned along cracks (Fig 2C), with variable height (several centimeters to 2 meters). The flowstones, which reach 20–30 cm in thickness, coat entirely the walls and the floor of the cave. The low consistency of the surface oxide allows the distinction between active and inactive formations. The active formations crumble easily when they are touched.

2.2. Sampling

Representative samples of ochreous speleothems were collected from an area close to the entrance to Buracos cave. Half of the samples were obtained from stalactites and the other from stalagmites. To examine the most modern deposits, we chose stalactites of small diameter (approx. 5 cm) and surface layers of stalagmites. All the speleothems analyzed were soft, friable and porous and of a yellowish brown, dark brown and reddish colour. Their laminated wavy structure was determined by alternations of

friable, porous, yellowish brown components with thinner, harder dark brown reddish bands (1-3 mm).

2.3. Mineralogical and chemical techniques

Whole-rock powders were prepared to determine the mineralogical and chemical characteristics of the samples. Mineralogy was assessed by X-ray diffraction (XRD) of pressed powder mounts using a Philips semi-automatic PW 1710 diffractometer with monochromatic Cu K radiation. Geochemical analyses were performed at Acme Analytical laboratories Ltd. in Vancouver, Canada. Total abundances of major oxides and minor and rare earth elements were determined by ICP-emission spectrometry and ICP mass spectrometry following lithium metaborate/tetraborate fusion and dilute nitric acid digestion. Loss on ignition (LOI) was by weight difference after ignition at 1000°C. Details on research procedures applied to the samples is available at the ACMELABS homepage: <http://www.acmelab.com/>.

The chemistry of the water related to the speleothems was determined in a sample of stalactite drops by ICP-MS and ICP-OES (overrange values) at Activation Laboratories Ltd., Canada.

2.4. Electron microscopy

For the study of fresh fractures, inorganic and organic materials were observed and characterized in a FEI QUANTA 200 scanning electron microscope (SEM) equipped with an Analytical-Inca (Oxford Instruments) analysis system incorporating an energy dispersive [EDS] X-ray detector. This apparatus can be used in high vacuum, low vacuum and ambient modes and is equipped with secondary electron and backscattered

electron detectors. The fresh fractures of the samples were metalized with gold and observed at different magnifications.

EDS analysis was performed at 30 KV with a beam current of 60 μ A. The Oxford INCA software was used to acquire and process energy dispersive X-ray (EDX) spectra. Atomic ratio Fe/Si of mineral structures and precipitates were also calculated with this software for comparative purpose (Kasama and Muraki, 2001; Halbberg and Ferris, 2004).

To examine cross sections, speleothem samples were prepared according to the method of Wierzechos and Ascaso (1994). For the backscattered electron mode of scanning electron microscopy (SEM-BSE), the rock pieces were fixed in glutaraldehyde and osmium tetroxide solutions, dehydrated in a graded ethanol series and embedded in LR-White resin. Blocks of resin-embedded rock samples were then polished, carbon coated and viewed with a DMS 960 SEM microscope.

For low temperature (LT) SEM, small fragments of sample were mechanically fixed onto the specimen holder of a cryotransfer system (Oxford CT1500), plunged into subcooled liquid nitrogen, and then transferred to the microscope's preparation unit via an air-lock transfer device. The frozen samples were cryofractured and etched for 2 min at -90°C. After ice sublimation, etched surfaces were gold sputter coated and the specimens then placed on the cold stage of the SEM chamber. Fractured surfaces were observed under a DSM960 Zeiss SEM microscope at -135°C.

2.5. Molecular biology techniques

PCR. Total genomic DNA was extracted from representative speleothem samples using the UltraClean Microbial DNA Isolation kit (Mobio) for subsequent PCR amplification of 16S rRNA genes. Fragments of bacterial 16S rRNA genes suitable for subsequent

denaturing gradient gel electrophoresis (DGGE) analysis were amplified using the primer pair 341fGC and 907r (Muyzer et al., 1996) specific for eubacteria. 25 µl of the PCR mix [75 mM Tris pH 9.0/50 mM KCl/20 mM (NH₄)₂SO₄] contained 1 unit of Taq polymerase, 0.2 mM of each of the four dNTPs, 0.4 µM of each primer, 100 µg of bovine serum albumin, 1.5 mM of MgCl₂, 5 µl de 5xTaq Master PCR enhancer (Prime) and ca 10-50 ng genomic DNA. Annealing conditions were 60°C. Products were cleaned on a QIAGEN quick spin column (Qiagen). Both complementary strands were sequenced separately at the SECUGEN sequencing company (S. L. Madrid, Spain).

DGGE. We selected a fingerprinting technique (PCR-DGGE) and sequence analysis of the resulting 16S rRNA gene bands as the best way to survey the genetic diversity of the speleothems.

Acrylamide gels (6%) with 50 to 70% urea-formamide denaturing gradients were prepared following the manufacturer's instructions. Lanes were loaded with 22 µl of PCR product mixed with 15 µl of loading buffer, run at a constant 200 V for 7 h at 60°C and stained for visualization and photography with EtBr. Predominant bands were excised and incubated 1 h at 60°C in water prior to their use as targets for PCR amplification.

3. Results

3.1. Mineralogical and bulk chemical composition

X-ray powder diffraction analysis of the samples revealed a broad scattering band from 15 to 45 2θ°, peaking at around 35 -36 2θ° (Fig. 3), corresponding to Fe (hydr)oxides and the broad peak around 35 -36 2θ° defines the main reflection (about 2.50 Å peak).

Another broad small peak appeared at around 62-63 $2\theta^\circ$ (about 1.49 Å peak) defining the precipitates as incipient “2-line” ferrihydrite (Fig 3) (Jambor and Dutrizac, 1998). The widely reported nominal formula of ferrihydrite is $5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$. However, there is no accepted general formula since variable amounts of water can be replaced by adsorbed species in quantities that cannot be accommodated within the crystalline structure (Jambor and Dutrizac, 1998).

Bulk chemical analyses were conducted by ICP (Table I). These tests indicated that the precipitates contain high Fe_2O_3 (56-59%) concentrations along with SiO_2 (12-16%), concentrations defining the precipitates as poorly ordered Si-rich Fe-oxides/hydroxides. $\text{Fe}_2\text{O}_3/\text{SiO}_2$ ratios ranged from 3.44 to 4.72. The most significant impurities were Al, Mn, Ca and Mg, and most abundant rare elements (up 10 ppm) were Ba, Co, Sr, V, Zr, Y, La, Ce, Pr, Nd, Zn and Ni.

3.2. Chemistry of water related to the speleothems

The stalactite drops analysis shows a very poorly-mineralized water of pH around 5.5 with a high (total) Fe concentration of 19.5 mg/L. Further elements detected in significant amounts were: SiO_2 (33.16 mg/L), Na (15.1 mg/L), Mg (5.89 mg/L), Ca (3.1 mg/L), K (2.83 mg/L), Mn (303 µg/L), Br (131 µg/L), Al (65 µg/L), Sr (30.3 µg/L), Ba (20.4 µg/L), Rb (10.7 µg/L), Zn (4.9 µg/L), Ce (3.85 µg/L), La (2.4 µg/L), Co (1.89 mg/L), Y (1.76 µg/L), Ni (1.5 µg/L) and V (1 µg/L). This water chemistry reflects strong leaching of basaltic pyroclasts and rocks by rainwater. This leaching could be promoted by the acid conditions of the soil (deep $\text{CO}_2(\text{g})$ or organic source) overlying the surficial basalts.

3.3. Structural features of the Fe-bioconstructions

1 The ochreous speleothems examined showed compact mineralized areas intermixed
2 with areas where bacterial structures associated with ferrihydrite deposits were
3 distinguishable. Bacterial horizons of filament-like materials were common throughout
4 the entire deposits but differences in microstructure among the different speleothems
5 were observed. Effectively, a wide morphological range of deposits could be
6 distinguished in the cross sections.

7 Microscopy revealed the laminated structure of most of the stalagmites. In some
8 zones, microstromatolites comprising alternating fine successive layers appeared at the
9 top of a stalagmite (Fig. 4). Between the different layers, micro-unconformities or
10 erosive surfaces were frequently found.

11 Through detailed observation of these microstromatolites, we were able to define the
12 following layers, which were also identified in other speleothems lacking a
13 microstromatolite structure:

14 - Type A or filamentous layer. This layer was composed of distinguishable filaments,
15 some of them prostrated or prone and lying parallel to subparallel to the deposition
16 surface; in other cases filaments appeared intermixed or disorganized. These layers were
17 200-350 μm thick.

18 - Type B or shrub layer. This layer was composed of shrub-shaped deposits formed
19 by several overlapping continuous sheets of erect branched filaments (arrows in Fig 4).
20 The thickness of each sheet varied but was in the range of 150 μm . This type of layer
21 was some 500-600 μm thick.

22 - Type C or columnar layer. This layer was made up of columns formed by
23 overlapping convex sheets. Columns were generally wider than 100 μm and their height
24 was 200 μm . Columns started to grow at isolated points and then packed together to
25 touch each other. Sometimes they tapered upward or ended in a rounded shape,

1 although they often appeared truncated and superimposed on each other. The bottom of
2 the columns was more mineralized and laminations could be observed (asterisks Fig. 4).
3 This layer type was around 300 μm thick.

4 - Type D or clotted layer. This layer was comprised of unorganized accumulations of
5 clots. Sometimes the clots led to the formation of mineral deposits of mound like
6 morphology (arrowheads in Fig. 4). Layer thickness was around 300 μm .

7 Other stalagmites did not show such clear laminated structures as the
8 microstromatolites described, but exhibited other bioconstructions such as small,
9 spherical, translucent mineral deposits (arrow in Fig. 5A). These mineral structures were
10 observed intermixed with bacterial remains and their size was in the range 20 μm to 200
11 μm (Fig. 5B).

12 Stalactites showed a concentric organization of alternating layers (Fig. 5C),
13 mineralized to a lesser or greater extent, which could be broken, truncated or could
14 generate convoluted forms. Some layers were composed of prone or erected filaments
15 as the type A layer, while others intensively mineralized layers showed no clear
16 structure. In some areas, different bioconstructions were locally found: 1) various types
17 of shrub layers (arrow in Fig 5D); 2) columns and mounds similar to these observed in
18 the stromatolites; 3) shrub-shaped mineral deposits forming small columns over 100 μm
19 in height (asterisks in Fig. 5E) and cements (arrow in Fig. 5E); and 4) multilobate or
20 spheroidal ooid accumulations of clotted texture and a diameter up to 500 μm (Fig. 5F).
21 Variations in morphologies and layering were observed in the stalactites and probably
22 reflect the influence of physico-chemical changes on stalactite growth. On the surface,
23 mineralization was less extensive and the bacterial mineral structures were easily
24 distinguishable (Fig. 6A).

3.4. Microbial components of the bioconstructions

Our fresh-fracture SEM study of the speleothems confirmed observations of less dense layers alternating with fully mineralized layers. In the less dense layers, filamentous mineral structures and mineral precipitates could be distinguished (Fig. 6A and 6B). Fe-mineral precipitates were comprised of small particles of non-defined and variable shape. Some of them were rounded precipitates smaller than 1 μm , frequently 0.3-0.5 μm , grouped together to form lumps (asterisks in Fig. 6B). EDS analysis of fresh fractures of the filamentous structures served to detect the presence of carbon (atomic ratio $\text{Fe/C}=0.84 \pm 0.1$), which we interpret as existence of organic matter.

Filamentous mineral structures were of variable length (up to 30 μm) and thicknesses of 5 μm when thicker inorganic coatings were observed in the speleothems. The filaments were straight or twisted and of variable thickness. Straight filaments in many cases had an inner channel of diameter 0.18 μm . Twisted mineralized structures resembled the stalks of *Gallionella* (Fig. 6B) and straight ones the sheaths of the iron-oxidizing bacterium *Leptothrix* (Fig. 6A). The atomic ratio Fe/Si of these filamentous mineralized structures revealed differences among the different layers of the speleothems. In the layers of *Leptothrix*, the ratios obtained varied between 1.75 and 4.76, and in the *Gallionella* layers between 2.06 and 3.46. The highest atomic ratio Fe/Si of the totally mineralized areas was around 19.27.

In some surface areas of the speleothems, the abundant presence of extrapolymeric substances associated with bacterial structures was observed by LTSEM (Fig. 6C-D). Areas showing different EPS densities related to the presence of different morphotypes were also observed. Thus, the *Leptothrix* morphotype displayed an EPS network associated with the tubular sheaths (arrows in Fig. 6C) while *Gallionella* stalks were completely covered by a denser EPS network (arrows in Fig. 6D). Mineralization of the

EPS related to the cells was also observed in some areas. Mineralized cells were frequently observed within a mineralized EPS matrix (arrow in Fig. 6E). The mineral deposits corresponding to EPS mineralization became a dense mineralized mesh (arrows in Fig. 6F).

Gallionella helical-like stalks showed the accumulation of granulose ferrihydrite deposits in their fibres (Fig. 6B and 7A). The stalk became completely covered by these deposits (Fig. 7B) in some areas. Sometimes it was possible to distinguish a gradient of mineralization when moving away from a mineralized compact layer, with decreases in compaction and increases in independent mineralized structures (Fig. 7C). Different phases of mineralization could be discerned in this spatial gradient. Stalks with some mineral deposits were observed in phase I (Fig. 7A). Individualized mineralized bacterial structures that were however completely covered by ferrihydrite deposits were the predominant components of phase II (Figs. 7B, 7D). In phase III, groups of mineralized bacterial structures formed compact masses but individual mineralized remains were also visible (Fig. 7C). Phase IV corresponded to the compact mineralized layer (Fig. 7C). Dense spheres deposits seemed also to be formed by the accumulation of *Gallionella* mineral encrusted stalks showing an outer radially organized band (Figs 5F and 7E). In all stages, mineral precipitates associated with the stalks showed Fe and Si as their main components (Fig. 7F).

Most intense mineralization associated with the *Leptothrix* morphotype consisted of the formation of a thick mineral layer rich in Fe and also containing Si coating entirely the filamentous structures (Fig. 7G and 8A). EPS were also visualized in the proximity of the mineralized structures (arrows in Fig. 8B). Higher atomic Fe/Si ratios (2.44 ± 0.11) due to higher amounts of Fe were detected in the sheaths covered by a wide external mineralized layer compared to naked sheaths (1.86 ± 0.105). The accumulation

of mineral-encrusted sheaths produced smooth mineral deposits (arrow in Fig. 8C). In other areas, only the build-up of broken naked sheaths was observed (arrow in Fig. 8D). Spherical translucent mineral deposits, observed in some speleothems, seemed to be associated with the presence of *Leptothrix* and mineralized EPS (Fig. 5B).

Alternative layers composed of the two morphotypes could be observed (Fig. 8E), but it was also frequent to find both morphological types intermixed to generate mixed mineral deposits (Fig. 8F).

Our DGGE results indicated that these bioconstructions contained different bacterial phylotypes (Fig. 9). Different DGGE profiles were obtained for each speleothem, although some bands were common to both. The main DGGE bands (in terms of intensity) were excised, reamplified and sequenced, though we could not always obtain good sequences. Four different sequences were obtained from the excised stalagmite DGGE bands and 3 from the stalactite DGGE bands, corresponding with the most predominant bands in both DGGE profiles. BLAST analysis of the sequences indicated that most band sequences corresponded to Proteobacteria (Table II). *Leptothrix* and *Gallionella* sequences were not obtained.

4. Discussion

The structures and minerals observed in the speleothems revealed their biogenic origin. The mineral deposits observed were the result of agglutination of specific bacterial structures and close associated ferrihydrite deposits. The microorganisms detected form biofabrics organized in arrays of cells and associated mineral microstructures that confer structural strength to the mineralized component (Spear et al., 2007). The

morphotypes *Gallionella* and *Leptothrix* were most frequently observed in these speleothems.

In some samples, the morphotype *Gallionella* dominated and could be identified by its distinctive stalk in the form of bundled fibres arranged as a double helix (Fig. 6B). Some stalks were almost free of iron precipitates (Fig. 7A) and presumably represent younger segments of stalk closer to the apical cells (Hallberg and Ferris, 2004); others were completely encrusted with these precipitates (Fig. 7B). Iron precipitate formation thus appears to start inside the fibre, where the organic structure may act as a template for crystallization (Warren and Ferris, 1998). Later crystals begin to develop on the outside and the whole stalk becomes encrusted (Hallberg and Ferris, 2004). *Leptothrix* formed very fine tubular structures composed of Fe-oxihydroxides (Fig. 6A) that could transform into heavily encrusted forms (Fig. 8A). Impregnation and encrusting of the sheaths and stalks with iron probably takes place after the cells have left the envelopes or stalks, when a passive process cannot be ruled out (Emerson and Weiss, 2004). The presence of the sheath and build-up of ferrihydrite in the stalk offers several advantages for persisting in this environment, since it prevents the living cell from becoming encrusted (Fortin and Langley, 2005; Takshima et al., 2008). In addition, other mechanisms such as solubilization by complexation, creation of specific cellular pH microenvironments, modification of the cell surface charge, and production of cellular exopolymers that act as precipitation templates could also be involved in cell protection (Schädler et al., 2009).

We did not detect an evident population of living cells of the genera *Gallionella* or *Leptothrix* in the speleothems. This is probably why these species did not appear as predominant bands in the DGGE profiles obtained. Other authors have also reported difficulties in detecting the presence of these species using molecular methods

(Takashima et al., 2008; Bruun et al., 2010). The latter authors proposed the presence of sheaths as the reason for not being able to recover DNA from *Leptothrix*-like cells (Bruun et al., 2010). In addition, most of the cells of these genera present in the speleothem are probably not alive because only the associated mineral structures were visualized. Living cells seem to be present only in outer layers. Our DGGE analysis yielded a profile dominated by proteobacteria (Table II). Several proteobacteria have also been incriminated in Fe oxidation processes (Bruneel et al., 2006; Emerson et al., 2007). Thus, a significant number of proteobacterial 16S rRNA gene sequences may be found in the clone library of a circumneutral-pH groundwater Fe seep (Blöthe and Roden, 2009). Our DGGE analysis also revealed the presence of other uncultured bacterial groups related to the Fe cycle, such as those of the *Actinobacteria* group. The production of mineral structures showing *Gallionella* and *Leptothrix* morphotypes and their capacity to precipitate ferrihydrite makes them likely candidates as main contributors to the formation of these speleothems. Effectively, *Gallionella* sp. and *Leptothrix* sp. are the most commonly observed bacteria associated with biogenic iron oxides in neutral pH environments (Emerson and Weiss, 2004). Hence, iron oxide particles in the close vicinity of *Leptothrix* sheaths and *Gallionella* stalks and their exopolymers may be referred to as extracellular biogenic iron oxides (Fortin and Langley, 2005). However, we cannot rule out a role of a unicellular unsheathed species detected by DGGE, which although not responsible for the iron precipitates encrusting *Leptothrix* sheaths and associated with *Gallionella* stalks could contribute to iron precipitates showing a patchy distribution (Fortin and Langley, 2005).

Leptothrix and *Gallionella* are large producers of exopolymeric substances (EPS) associated with the sheath and stalk, respectively (Fig. 6C and 6D). EPS are important surface reactants that can bind or nucleate Fe minerals (Fein et al., 1997; Yee and Fein,

2001; Daughney et al., 2001; Chatellier and Fortin, 2004; Chan, 2004; Fortin and Langley, 2005; Miot et al., 2009). The close association between EPS and mineralized cells as well as the detection of mineralized EPS (Fig. 6E and 6F) support the hypothesis that they play a key role in ferrihydrite accumulation. In effect, the presence of EPS and features of these microorganism surfaces have been described as the main factors determining organic Fe precipitation rates in Fe-stalactites (Kasama and Murakami, 2001). The type of mineralization observed in both morphotypes is clearly determined by their different cell structures but could also be influenced by the observed differences in the EPS network (Fig. 6C and 6D).

Independently of the taxa present, mineral accumulations were composed of poorly ordered Si-rich ferrihydrite. Ferrihydrite is one of the most common minerals found in association with active microbial iron oxidation in circumneutral freshwater systems (Konhauser, 1998; Langley et al. 2009). According to our knowledge of ferrihydrite, it seems that Si is incorporated in the iron oxihydroxides by microbial activity (Emerson and Moyer, 1997). Ferrihydrite is the normal precipitate if the oxidation/precipitation reaction is fast and there are small amounts of silicate present (Cornell and Schwertmann, 1996). According to Kennedy et al. (2003), the adsorption of Si to iron oxide-encrusted bacterial surfaces give stability of two-line ferrihydrite, in that the natural conversion to more crystalline iron oxides such as hematites and goethite is avoided. This stability of ferrihydrite explains the well-preserved morphology of bacterial sheaths and stalks observed in this study. We also detected distinct Fe/Si atomic ratios for different layers, suggesting that during speleothems formation this ratio could change. Silicon was homogeneously dispersed throughout the initial precipitates, presumably via Fe-O-Si bonding, but in compact red layers, the ratio was

1 higher due to Fe-rich late-stage cementation. This later cementation could be key for the
2 consistency of the speleothems.

3 The structure of the speleothems analyzed seems to be conditioned by the type of
4 bacteria present and level of mineralization. The biomineralization patterns shown by
5 the two morphotypes revealed the role of bacterial metabolism in the mineralization
6 process although with differences between morphotypes, which could relate to different
7 metabolic and morphological features (Miot et al., 2009). The EPS matrix associated to
8 both morphotypes could also play a major role in developing specific textures (Handley
9 et al., 2008). Hence, it was possible to correlate some of the bioconstructions to a given
10 morphotype. For example, translucent spherical structures were ascribed to *Leptothrix*
11 (Fig. 5B), and dense spheres (Fig. 5F) and some shrubs and columnar formations to
12 *Gallionella*. Other formations could be the result of a mixture of both morphotypes (Fig.
13 4). Thus, the greater or lesser presence of a given morphotype during speleothem
14 formation could condition the presence of different textures and the formation of
15 specific layers. Bacterial mineralized structures and their associated mineral deposits
16 such as shrubs, mounds and columns were frequently organized according to the
17 boundaries of the layers. In addition, the upper limits of some of the growth layers were
18 truncated or severely corroded, possibly because of subaerial exposure stages (Fig. 4).
19 Fully mineralized layers formed through the accumulation of these mineral bacterial
20 structures and later cementation processes. The observation of bacterial remains in all
21 the layers enables us to rule out the possibility of periodic pure inorganic precipitation
22 as the origin of the compact mineral layers, but we cannot discard the contribution of
23 some inorganic passive precipitation. Acid leaching of basaltic soils and rocks causes
24 intense Fe and Si enrichment of subsurface water. Variations in redox conditions during
25 water flow into a cave, causes oversaturation of the water related to Fe-(hydr)oxides and

1 could lead to mineral precipitation at neutral pH (Kirby and Elder Brady, 1998). The
2 amount of Fe in the cave water (19.5 mg/l) was sufficient for the inorganic precipitation
3 of ferrihydrite but not for this to become the dominant process (Phoenix et al., 2003).
4 Since microbially mediated Fe⁺ oxidation can inhibit the inorganic chemical oxidation
5 of Fe (Emerson and Revsbech, 1994), the inorganic contribution to the mineralization
6 observed could be low. A lack of substances essential for bacteria, such as Fe(II),
7 dissolved oxygen or nutrients, could regulate their life cycle and activity and,
8 consequently, the deposition of mineral bacterial structures and speleothem formation
9 (Takashima et al., 2008). However, since the biogenic minerals detected are not only the
10 result of direct bacterial metabolic activity but also contribute to passive sorption and
11 nucleation reactions (Kasama and Murakami, 2001; Baskar et al., 2008), the
12 contribution of Si and Fe in interstitial water could also be a determining factor in the
13 mineralization phase and consequently in speleothem microstructure. Variations in the
14 water deficit or acid contributions (related to seasonal changes or CO₂ flux) cause water
15 pH modifications promoting changes in organic/inorganic precipitation reactions.

16 Many authors argue it is difficult to clearly differentiate between true biogenic
17 Fe-oxides and those formed as a result of abiotic reactions in natural samples containing
18 neutrophilic Fe-oxidizers (Fortin and Langley, 2005). However, by exploring
19 relationships between mineral precipitates and microorganisms and their structures
20 using high-resolution microscopy techniques, we infer an important role of bacteria in
21 the construction of these speleothems.

5. Conclusions

- The speleothems examined are comprised of accumulations of specific bacterial structures (mainly *Gallionella* and *Leptothrix* morphotypes) and ordered Si-rich ferrihydrite deposits (incipient 2-line ferrihydrite). Successive microbial growth and its associated mineral precipitation causes the growth of these speleothems.
- The structure of the speleothems analyzed seems to be conditioned by the type bacteria present (structural and metabolic features) and level of mineralization. Some of the bioconstructions can be assigned to one morphotype such as the translucent spherical structures to *Leptothrix* and dense spheres and some shrubs and columnar formations to *Gallionella*. Other formations could be the result of mixture of both morphotypes
- Bacterial activity seems to be the main determinant of speleothem structure. Through their metabolism, bacteria precipitate siliceous ferrihydrite but their mineral structures and associated EPS matrix also act as nucleation points for a subsequent passive mode of mineral precipitation. The observation of bacterial remains in most of the speleothem layers precludes the possibility of periodic climatic pure inorganic precipitation. Factors affecting the life cycle and activity of the bacteria could conditionate the deposition of mineral bacterial structures and consequently the speleothem structure.
- Accumulation of bacterial mineral structures and later inorganic cementation are finally responsible for the formation of consistent mineral layers, conferring consistency to the speleothems.
- Independent of the taxa present, the mineral precipitates were siliceous ferrihydrite but differences in Fe/Si atomic ratios have been found. The compact mineralized structures were richer in Fe than independent bacterial structures, indicating a Fe-rich late cementation.

- Acid leaching of basaltic soils and rocks caused intense Fe and Si enrichment of subsurface water, leading the observed biogenic siliceous ferrihydrite precipitation at slightly acid or circumneutral pH environment.

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Legends

Fig 1. Geological map of Terceira Island (adapted from Nunes 2004) showing the location of Buracos cave.

Fig 2. (A) Stalagmites and stalactites from Buracos cave. (B) Sample of the lower apical section of a small stalactite in the Buracos cave. (C) Aligned stalactites along a crack in Buracos cave.

Fig 3. Powder X-ray diffraction patterns for the ferrihydrite, showing a broad scattering band between 15 and 45 $2\theta^\circ$ with two broad main peaks.

Fig 4. SEM-BSE image of a microstromatolite structure from a stalagmite in which different layer types (A, B, C, D) may be distinguished. Arrows indicate clotted mineral accumulations, arrowheads shrub-shaped accumulations and asterisks columnar formations.

Fig 5. (A) SEM-SE image of a stalagmite showing spherical translucent mineral deposits (arrow). (B) Detailed SEM-SE image of the deposits observed in A. (C) SEM-BSE image of a stalactite showing the concentric organization of alternating layers. (D) SEM-SE image of shrub-shaped mineral deposits (arrows) in a stalactite. (E) SEM-BSE image of the columnar layer of a stalactite showing columns (asterisks) and cementation (arrows). (F) SEM-BSE image of spheroidal ooid deposits of clotted texture in a sample taken from a stalactite.

Fig 6. Electron microscopy images of bacterial structures. (A) SEM-SE image of a fresh fracture of a superficial layer of *Leptothrix* filaments. (B) SEM-SE image of a fresh fracture of an area harbouring the *Gallionella* morphotype. Asterisk indicates rounded Fe minerals. (C, D) LTSEM images of *Leptothrix* (C) and *Gallionella* (D) morphotypes and their associated EPS matrix (arrows). (E, F) Bacterial structures and EPS matrix showing the first phases of mineralization (arrows) visualized by SEM-BSE (E) and LTSEM (F).

Fig 7. (A-E) SEM-BSE images of layers rich in *Gallionella* morphotypes. *Gallionella* helical-like stalks showing the initial accumulation of granulose ferrihydrite deposits in their fibres (A) and their complete covering by these deposits (B). (C) Gradient of mineralization associated with the presence of a mineralized compact layer. (D) Detailed image of the mineral structures comprising phases II and III. (E) Detailed image of an ooid mineral deposit formed by compaction of *Gallionella* mineral encrusted stalks. Note the outer part shows a radial organization. (F-G) Fe and Si distribution map obtained by EDS analysis of structures of *Gallionella* (F) and *Leptothrix* (G).

Fig 8. (A-B) Images of mineralized *Leptothrix* filaments visualized by SEM-BSE (A) and LTSEM (B). Arrows indicate EPS. (C) LTSEM image of accumulated mineral encrusted *Leptothrix* filaments. (D) SEM-BSE image of accumulated broken naked *Leptothrix* sheaths (arrow). (E) SEM-BSE image of alternating mineralized *Leptothrix* (L) and *Gallionella* (G) layers. (F) SEM-BSE image of mixed mineral deposits containing both morphological types. Black arrows note *Gallionella* deposits and white *Leptothrix* deposits.

1

2 **Fig 9.** Denaturing gradient gel electrophoretic profile of PCR-amplified 16S rRNA gene
3 fragments obtained from representative stalagmite (STM) and stalactite (STC) samples
4 using the bacterial primers 341fGC and 907r.

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4 Table I: Total abundances of major oxides obtained by ICP-emission spectrometry

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7 Table II: Codes (DGGE bands), accession numbers and closest relatives for the bacterial

8 DGGE bands shown in Fig. 9.

9

AZORES ISLANDS

Terceira

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9