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Origin of abundant moonmilk deposits in a subsurface granitic environment

Ana Z. Miller^{1*}, Angela M. Garcia-Sanchez¹, Pedro M. Martin-Sanchez², Manuel F.C. Pereira³, Jorge E. Spangenberg⁴, Valme Jurado¹, Amelia Dionísio³, Maria J. Afonso⁵, Helder I. Chaminé⁵, Bernardo Hermosin¹, Cesareo Saiz-Jimenez¹

¹Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS-CSIC), Sevilla, Spain.

²Bundesanstalt für Materialforschung und -prüfung (BAM), Department 4 Materials & Environment, Berlin, Germany.

³CERENA, DECivil, Instituto Superior Técnico, Universidade de Lisboa, Lisboa, Portugal.

⁴Institute of Earth Surface Dynamics, University of Lausanne, 1015 Lausanne, Switzerland.

⁵Laboratório de Cartografia e Geologia Aplicada, Instituto Superior de Engenharia do Porto (ISEP), Politécnico do Porto, Porto; Centro GeoBioTec|UA, Aveiro, Portugal.

***Corresponding author:** A.Z. Miller

Postal address: Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS-CSIC), Av. Reina Mercedes 10, 41012 Sevilla, Spain.

E-mail address: anamiller@irnas.csic.es

Running title: Origin of moonmilk in a granitic water gallery

ABSTRACT

Subsurface granitic environments are scarce and poorly investigated. Most common speleothem minerals found in these cavities are opal-A and other secondary minerals such as evansite, struvite, hematite, goethite and gypsum. Moonmilk is a whitish secondary mineral deposit of varying composition, mainly calcite, commonly found in karstic caves and various soil types worldwide. However and to the best of our knowledge, this study is the first record of moonmilk in an urban subsurface granitic environment. A multidisciplinary approach was used to characterize the abundant moonmilk deposits and associated microbial communities coating the granite walls of the 16th century Paranhos spring water gallery in Porto urban area (NW Portugal). Moonmilk morphology and texture, mineralogical composition, stable isotopic composition and microbial diversity of moonmilk samples have been studied to infer on the processes involved in the abundant formation of moonmilk in this granitic environment. The moonmilk deposits are composed of very fine needle fibre calcite (NFC) crystals with different morphologies and density. Calcified filaments of fungi hyphae or bacteria were distinguished by FESEM. Stable isotopic analysis revealed a meteoric origin of the NFC, with an important contribution of atmospheric CO₂, soil respiration and HCO₃⁻ from dissolution of mortar carbonate phases of the repairing materials introduced in the granite joints. The DNA-based analyses revealed the abundance of Actinobacteria, mainly represented by *Pseudonocardia hispaniensis*, Thaumarchaeota and Ascomycota, dominated by *Cladosporium*. This microbial composition is consistent with the groundwater quality degradation and contamination sources of the Paranhos water gallery, including garages, petrol stations and wastewater pipeline leakage. Whether the identified microorganisms are involved in the formation

of the NFC or not is difficult to demonstrate, but we evidenced both abiotic and biogenic genesis for the calcite moonmilk of this subsurface granitic environment.

Keywords: moonmilk, needle fibre calcite, water gallery, biomineralization; carbonate precipitation

INTRODUCTION

Caves are natural entrances into the subsurface that can be formed in a wide variety of rock types by different geological processes. These underground open spaces harbor a significant amount of microbial communities including mineral-utilizing microorganisms, and represent unique environments for investigating the largely unexplored subsurface life. Karstic caves, generally formed in limestone or other soluble rocks such as dolostone and gypsum, are the most common cave types (Northup and Lavoie, 2001; De Waele *et al.*, 2017). In contrast, cavities in granite rock are scarcer and less investigated (Vidal Romaní and Vaqueiro Rodriguez, 2007; Osborne *et al.*, 2013; Sallstedt *et al.*, 2014; Vidal Romaní *et al.*, 2015; Zepeda Mendoza *et al.*, 2016). These caves result mainly from run-off infiltration through the granite massif discontinuities that causes chemical weathering of the rock complemented with mechanical or physical erosion. The weathering products include more resilient relic minerals, as quartz grains, and newly formed minerals, such as illite, biotite/muscovite, chlorite, and iron oxy-hydroxides. These weathering products are released by disintegration and easily removed by percolating waters opening the granite discontinuities gradually. Once the granite cavities are defined, dissolution and precipitation processes promoted by very slow water flow into the granite fractures, act leading to the formation of authigenic minerals, which are deposited as speleothems.

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3 Their formation is controlled by geochemical conditions such as redox, pH and host
4 rock composition, in addition to the hydrodynamic conditions such as fluid flow and
5 saturation. The degree and nature of microbial involvement in speleothem formation is
6 still unclear (Barton and Northup, 2007), i.e., whether the microorganisms are actively
7 involved in the formation or simply buried during mineral precipitation. Speleothem
8 microbiota can be highly varied, containing bacteria, fungi, algae, testate amoeba,
9 collembolan and mites among other organisms (Vidal Romaní *et al.*, 2013).

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12 The knowledge on microbial diversity and functionality from subsurface granitic
13 environments is limited. It is confined to artificial induced environments, such as
14 granitic aquifers, mines and boreholes drilled for research purposes and nuclear waste
15 disposal (Pedersen, 1993, 2000; Szewzyk *et al.*, 1994). Granite cavities are of great
16 interest as they provide a natural laboratory to study the microbial species adapted to
17 subsurface granitic environments and their interactions with minerals (Pedersen, 1993;
18 Szewzyk *et al.*, 1994; Vidal Romaní *et al.*, 2010; Miller *et al.*, 2012a,b). In granite
19 caves, opal-A (an amorphous hydrated polymorph of silica) is the most common
20 speleothem mineral. Other secondary minerals such as evansite, bolivarite, struvite,
21 pigotite, taranakite, allophane, hematite, goethite, halite, gypsum, anhydrite, plumbo-
22 aragonite and calcite have been reported (Vidal Romaní and Vaqueiro Rodriguez, 2007;
23 Vidal Romaní *et al.*, 2010; (Sanchez-Moral *et al.*, 2011). Among these minerals, special
24 attention will be given in this paper to the formation of calcite associated with a specific
25 speleothem type, i.e., moonmilk found in a subsurface granitic environment.

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27 Moonmilk is a whitish secondary mineral deposit of varying composition and high-
28 water content commonly found in subterranean environments worldwide, mainly in
29 karstic caves (Cañaveras *et al.*, 2006) but also in volcanic caves (Polyak and Provencio,
30 2006) and soils (Millière *et al.*, 2011a,b; Bindschedler *et al.*, 2014). The term moonmilk
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indicates a texture and not a rigorously defined mineralogy; it comprises microcrystalline aggregates predominantly of carbonate minerals with soft and spongy-like texture, which may have different origins and mineral composition (Baskar *et al.*, 2011). Calcite, hydromagnesite, aragonite, vaterite and huntite were reported as the main carbonate constituents of moonmilk (Cuezva *et al.*, 2003). Nitrates and sulfates were identified as minor compounds (Martinez-Arkarazo *et al.*, 2007). Under the microscope, calcite-rich moonmilk consists of aggregates of needle-shaped crystals, the so-called needle fibre calcite (NFC) introduced by Stoops (1976). Its origin is a controversial issue that has generated debate during the last decade. Moonmilk formation has been ascribed either to physicochemical processes, biotic processes, or even a combination of both (Verrechia and Verrechia, 1994; Cañaveras *et al.*, 2006; Cacchio *et al.*, 2014).

From a geological perspective, mineral precipitation is commonly considered to be an abiotic process. However, it is well known that microorganisms dwelling in caves may mediate the precipitation of secondary minerals by modifying the chemical conditions of the surrounding microenvironment or by producing organic polymers which induce the nucleation and growth of minerals (Benzerara *et al.*, 2011; Cuezva *et al.*, 2012). In fact, a considerable number of soil bacteria have the ability of precipitating calcium carbonate (Cañaveras *et al.*, 1999, 2006; Groth *et al.*, 2001). Actinobacteria were considered one of the major components of the microbial community associated with moonmilk deposits and capable of forming the typical NFC (Cuezva *et al.*, 2012; Maciejewska *et al.*, 2015). However, fungal structures and NFC also share some morphological traits, and recent studies have attributed a fungal origin to moonmilk deposits (Bindschedler *et al.*, 2012, 2014).

In this study, a multidisciplinary approach was performed to characterize the moonmilk deposits and associated microbial communities found in the granitic Paranhos spring water gallery (Porto, NW Portugal). To our knowledge, this study is the first record of moonmilk in an urban subsurface granitic environment. We report data for their mineralogical and microbiological composition that provide insights into the origin of these secondary mineral deposits.

MATERIALS AND METHODS

Studied site and sampling

The 16th century Paranhos-Salgueiros spring water galleries are ancient granitic water mines dug under the highly-urbanized Porto city (NW Portugal) to transport groundwater to public fountains through a stone gutter pipe (Chaminé *et al.*, 2010, 2014; Freitas *et al.*, 2014) (Fig. 1A).

The bedrock of Porto city (NW Portugal) is mainly granite, involving different hydrogeological compartments such as overlying sediments, weathered rock, a weathered-fissured zone, and fractured bedrock (Afonso *et al.*, 2007a,b, 2010, 2016). This bedrock consists mainly of moderately deformed two-mica granite, with K-feldspar and oligoclase-albite plagioclase, medium- to fine-grained, greyish in colour, changing to yellowish when weathered (Almeida, 2006; Afonso *et al.*, 2010; Almeida and Begonha 2015). Throughout the centuries, a network of underground galleries was excavated in this granite under the urban area of Porto city to provide water supply for the population (Afonso *et al.*, 2010, 2016; Chaminé *et al.*, 2010; Freitas *et al.*, 2014). These water galleries were the most important water supply for centuries, and currently comprise valuable geosites for hydrogeochemical and geomicrobiological studies on urban subsurface granitic environments (Miller *et al.*, 2012a,b; Afonso *et al.*, 2016).

The Paranhos underground gallery has 3.2 km extension, a maximum depth of 20 m below ground level and is divided in three sections, each one with three entrance gates (Chaminé *et al.*, 2010). It is devoid of light sources and has stable environmental conditions throughout the year, with air temperature around 18°C and relative humidity between 95 and 100% (Miller *et al.*, 2012a). Today, the Paranhos-Salgueiros spring water is no longer used for public purposes due to groundwater quality degradation and vulnerability of the granite galleries to contamination (Fernandez-Cortes *et al.*, 2014; Afonso *et al.*, 2016). Previous hydrogeochemical studies showed a nitrate and sulfate-enriched composition for the Paranhos groundwater probably resulting from urban drainage, sewer leakage and agricultural activities (Afonso *et al.*, 2007a,b, 2010). According to Sanz-Rubio *et al.* (2013), this environment is characterized by relatively high levels of CO₂ with very low $\delta^{13}\text{C}$ isotopic signal values and low levels of CH₄ compared with the external atmosphere. The CO₂ present in the internal atmosphere has a soil-derived origin.

Abundant white deposits of moonmilk forming coatings up to 2 cm thick were observed on the walls and ceiling of the Paranhos spring water gallery (Fig. 1B-C). These deposits develop both on granite walls and granite joints filled with mortars or concrete from repairing works carried out in the 17th century and later (Fig. 1B-C).

A sampled area inside the Paranhos spring water gallery presenting abundant white moonmilk deposits and located at 250 m from one of the secondary entrances of the tunnel (S. Dinis street) at 6m depth was selected (Fig. 1A). Two set of representative samples (for geochemical and microbiological analyses) were taken aseptically from an area of 1 m² by scraping the white deposits coating the granite surfaces and gathering it into sterile tubes. Special care was taken in order to minimize damage of the NFC structure. Samples for molecular biology were stored at -80°C until laboratory analyses.

Samples for mineralogical, geochemical and microscopy analyses were stored in the dark at +4 °C until required.

Morphological, geochemical and mineralogical characterization

The moonmilk deposits were examined using an Olympus SZ51 stereomicroscope (Olympus, Tokyo, Japan) for description of the texture and hand-picking subsamples of homogeneous materials for further analyses. To assess the potential presence of non-carbonate mineral phases, a subsample was treated with 1M HCl, rinsed with deionized water, dried at 50°C for 24 h and inspected under the stereomicroscope.

The morphology and elemental composition of the moonmilk samples were studied using field emission scanning electron microscopy (FESEM) combined with energy dispersive X-ray spectroscopy (EDS). Air-dried moonmilk samples were directly mounted on sample stubs, sputter coated with gold-palladium, and subsequently examined in a Jeol JSM-7001F microscope (Jeol, Tokyo, Japan) equipped with an Oxford EDS detector (Oxford Microbeam, Oxford, UK). FESEM examinations were operated using the secondary electron (SE) detection mode with an acceleration voltage of 15 kV. The moonmilk samples were also observed in a FEI's Teneo FESEM (FEI Company, Eindhoven, The Netherlands) after drying at 50°C for 24 h. Samples were sputter coated with gold and observed using the SE detector and an acceleration voltage of 2 kV.

X-ray micro-computed tomography (micro-CT) was used to assess the three-dimensional (3-D) microstructural and compositional heterogeneities of the moonmilk deposits. Digital radiographs were acquired with a micro-CT SkyScan 1172 scanner (Bruker, Massachusetts, USA) using an X-ray cone incident on a rotating specimen. Due to sample variable size and composition the experimental conditions were

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optimized for each specimen using a constant source power (10W). A second run was needed to reduce sample dehydration effect in the acquisition process. The following operating conditions were used: source voltage of 100 kV, current of 100 μ A, a pixel size resolution ranging from 11.5 to 17.8 μ m, and an averaging of five radiographs per each position. Downstream 0.5 mm aluminum filtration was used to increase beam penetration into the samples to prevent “beam hardening”, a nonlinear X-ray absorption effect. The acquisition was performed by rotating the specimen over 180° with variable rotational step. Slice reconstructions were obtained with the NRecon 1.6.3 routine, and volumetric visualization was achieved with DataView and CTvox programs.

The mineralogical composition of the moonmilk samples was determined by X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FT-IR). The X-ray diffractograms were performed using a XPERT-PRO (PANalytical, Almelo, The Netherlands) diffractometer with CuK α radiation and a X'Celerator detector. The measurement conditions were: 40 kV and 35 mA, 0.002 °2 θ step size and 20 s of counting time. The “High Score Plus” analytical software and PDF2 database were used. FTIR spectra were recorded with a Perkin Elmer Spectrum 65 spectrometer for samples dried at 50°C. Powdered samples were dispersed in KBr pellets and IR spectra were recorded for the 4000 cm⁻¹ to 400 cm⁻¹ region, with 1 cm⁻¹ resolution. Each data point was the result of the accumulation of 20 scans.

The stable carbon and oxygen isotope analysis of three replicate moonmilk samples was performed at the Institute of Earth Surface Dynamics of the University of Lausanne (Switzerland) using a Thermo Fisher Scientific (Bremen, Germany) Gas Bench II carbonate preparation device connected to a Thermo Fisher Delta Plus XL isotope ratio mass spectrometer, which was operated in the continuous He flow mode. The CO₂ extraction was done by reaction with anhydrous phosphoric acid at 70°C. The stable

carbon and oxygen isotope ratios are reported in the delta (δ) notation as the per mil (‰) deviation relative to the Vienna Pee Dee belemnite standard (VPDB). The standardization of the $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values relative to the international VPDB scale was done by calibration of the reference gases and working standards with IAEA standards. Analytical reproducibility (1σ), monitored by replicate analyses of the international calcite standard NBS-19 and the laboratory standards Carrara Marble was not greater than $\pm 0.05\text{‰}$ for $\delta^{13}\text{C}$ and $\pm 0.1\text{‰}$ for $\delta^{18}\text{O}$.

Molecular analysis of microbial communities

The microbial communities associated with the moonmilk deposits from the Paranhos gallery were characterized by molecular biology techniques. DNA was extracted using FastDNA SPIN for Soil Kit (MP Biomedicals, Illkirch, France), following the manufacturer's instructions.

The prokaryotic 16S rRNA gene was amplified by PCR using primers 616F (5'-AGAGTTTGTATYMTGGCTCAG-3'; Snaird *et al.*, 1997) and 1510R (5'-GGTTACCTTGTTACGACTT-3'; Lane, 1991) for bacteria, and 109F (5'-ACKGCTCAGTAACACGT-3') and 915R (5'-GTGCTCCCCCGCCAATTCCT-3'; Großkopf *et al.*, 1998) for archaea. The eukaryotic internal transcribed spacer regions (ITS1, 5.8S rDNA and ITS2), mainly those belonging to fungi, were PCR-amplified using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White *et al.*, 1990). PCR reactions were performed in 50 μL volumes, containing 5 μL of 10x PCR buffer, 2 μL of 50 mM MgCl_2 , 5 μL of 2 mM dNTP mix (Invitrogen, Carlsbad, CA, USA), 0.5 μL of 50 μM of each primer (Macrogen, Seoul, Korea), 10-20 ng of the extracted DNA as template, 1.25 units of Taq DNA polymerase (Bioline, GC Biotech, The Netherlands), and the rest of volume

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of sterile ultrapure water. Reactions were performed in duplicate, and negative controls (containing no DNA) were included in each PCR trial. PCR amplifications were performed in a BioRad iCycler thermal cycler (BioRad, Hercules, CA, USA) using the following cycling parameters: 2 min of initial denaturing step at 95°C, followed by 35 cycles of denaturing (95°C for 15 s), annealing (55°C for 15 s for 16S rRNA, 50°C for 15 s for ITS regions) and extension (72°C for 2 min), with an additional extension step at 72°C for 10 min at the end. To evaluate the PCR results, all products were electrophoresed on 1% (w/v) agarose gels, stained with SYBR Green I (Roche Diagnostics, Mannheim, Germany) and visualized under UV light. The positive PCR products were purified using the JetQuick PCR Purification Spin Kit (Genomed, Löhne, Germany) according to the manufacturer’s instructions and stored at –20°C for later analysis.

The purified PCR products were ligated into the pGEM-T Easy Vector (Promega, Milan, Italy) and then transformed into One Shot Max Efficiency DH5α-T1 Chemically Competent *Escherichia coli* (Invitrogen, Carlsbad, USA). Transformants were plating onto Luria Bertani (LB) agar plates supplemented with ampicillin (100 µg/mL), IPTG (0.5 mM) and X-Gal (80 µg/mL), and incubated at 37°C overnight. From each clone library, a total of 100 white colonies were randomly picked onto a new plate of the same medium and incubated at 37°C overnight to obtain enough biomass for subsequent analysis and storage at –80°C. Selected clones were PCR-screened directly for the presence of inserts using the T7-promoter and pUC/M13-reverse primers; 1 µl of colonies suspended in sterile ultrapure water was used as template. Positive PCR products of clones with inserts were sent to Macrogen Inc. (Amsterdam, The Netherlands) for purification and sequencing using the same primer set. Sanger

sequencing was chosen over high-throughput methods for obtaining near-full length 16S rRNA gene sequences for detailed phylogenetic analyses.

All sequences from the pool of inserts were manually checked, and low quality and very short sequences were removed. The presence of chimeras was tested by Bellerophon program (Huber *et al.*, 2004) using a variety of window sizes (200-400 bp) and corrections. Putative chimeric sequences were excluded from further analysis. The sequences were aligned using MUSCLE (Edgar, 2004) and the respective distance matrices were generated by PHYLIP (Felsenstein, 1993). The resulting matrices were used as input for DOTUR (Schloss and Handelsman, 2005) to assign sequences to Operational Taxonomic Units (OTUs) using the furthest neighbour algorithm at evolutionary distances of 3%, 5% and 20%, and to generate the respective rarefaction curves. One clone for each OTU defined at 3% of distance, a rough approximation to species level, was chosen as representative and its sequence was submitted to the European Nucleotide Archive (ENA, EMBL-EBI) under the accession numbers HG008768-HG008780 and HG008829-HG008892.. To approximate the phylogenetic affiliation of the OTUs, representative sequences were compared to the non-redundant databases using BLASTn algorithm (Altschul *et al.*, 1990) from the National Center for Biotechnology Information (NCBI), and the EzBioCloud's Identify Service (Yoon *et al.*, 2017).

RESULTS

Morphological features

The moonmilk deposits observed in the Paranhos spring water gallery consisted of white, soft and cotton-like nodular aggregates (Fig. 1B-D). The stereomicroscopic observations revealed fine fibre crystals (Fig. 2A,B), as well as fragments of the

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3 weathered granite and mortar entrapped in the moonmilk deposits (Fig. 2C). After the
4 removal of the carbonated fraction, some granite mineral particles, including quartz,
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7 feldspars and mica, were clearly recognized (Fig. 2D).
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10 The FESEM-EDS revealed aggregates of microcrystalline calcite fibres with distinct
11 morphological features (Fig. 3). The moonmilk samples were composed of intertwined
12 clusters of fibre calcite crystals with different shapes and size (Fig. 3A). These
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14 aggregates included mineralized fungal-like hyphae structures coated with NFC (Fig.
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17 3B). Three main needle calcite morphologies were observed even in the same moonmilk
18 sample (Fig. 3C), comprising: (i) long needle-shaped crystals ($> 10\ \mu\text{m}$ long and $< 1\ \mu\text{m}$
19 wide) with smooth surfaces, including single or paired rods in the external part of the
20 samples (Fig. 3D); (ii) very thin filaments or nanofibers intertwining around the needle-
21 fibre crystals (Fig. 3E,F), and (iii) simple needle-fibres and polycrystalline crystals with
22 serrated edges (epitaxial growth), more abundant in the inner part of the moonmilk
23 samples (Fig. 3G-I).
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34 In addition, the FESEM-EDS examinations revealed organic-like filaments with $1\ \mu\text{m}$
35 diameter and serrated-edged outlines (Fig. 3J), resembling those described by Cailleau
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38 *et al.* (2009). The likely biogenic origin of the needle-shaped calcite fibres was also
39 evidenced by the presence of a slime matrix of extracellular polymeric substances (EPS)
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42 embedded within the NFC (Fig. 3K,L).
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45 The micro-CT images provided information on the spatial distribution of mineralogical
46 phases and internal density of the moonmilk samples (Fig. 4). The black areas
47 corresponded to voids and the bright-colored areas to denser material. Multiple growth
48 layers and variations in density were observed in the moonmilk samples. The
49 reconstructed X-ray radiographs revealed poorly consolidated growth layers ($\sim 100\ \mu\text{m}$
50 thickness each) near the contact zone with the granite substrate (initial stage of
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speleothem formation, < 1mm). Towards the outermost part of the sample (intermediate area), the internal structure was more heterogeneous in density, with large voids surrounded by dense material aggregates, implying an increase in porosity (Fig. 4).

Geochemical and mineralogical composition

The XRD analysis showed that calcite was the dominant mineral in the moonmilk deposits (Fig. 5). The XRD pattern displayed sharp calcite peaks with little background absorption, which is in good agreement with the Joint Committee on Powder Diffraction Standards (JCPDS) card number 5-586 (calcite). In addition, minor muscovite and alkaline feldspars from the granite substrate were also detected, reinforcing the stereomicroscopy observations.

FTIR analysis of the moonmilk samples in the range 400–4000 cm^{-1} were in line with the XRD data. Calcite was identified by the diagnostic peaks at 1428, 874 and 713 cm^{-1} , with additional peaks at 1795 and 2507 cm^{-1} (Fig. 6). A broad band centred at 3416 cm^{-1} was due to symmetric and antisymmetric stretching vibration of water bound. The remaining low intensity bands were attributed to granite minerals, such as minor phyllosilicates including muscovite (Fig. 6).

The stable isotope composition of the moonmilk deposit samples ranged between -6.05 and -5.92 ‰ (-5.99 ± 0.07 ‰, $n = 3$) $\delta^{13}\text{C}$ and -5.19 to -4.97 ‰ (-5.05 ± 0.12 ‰, $n = 3$) $\delta^{18}\text{O}$ values.

Microbial communities

To characterize the microbial communities associated with moonmilk, clone libraries were constructed for Bacteria, Archaea and Fungi. A total of 155 high-quality sequences from 207 sequenced clones were used for the final analysis. The distribution of different

phyla among the clone libraries is shown in Figure 7. Most of the bacterial clones analysed corresponded to the phylum Actinobacteria (52 %) (Fig. 7A), mainly represented by the genus *Pseudonocardia*. Nearly all OTUs belonging to *Pseudonocardia* had as closest cultured relative the species *Pseudonocardia hispaniensis* (Table S1). A significant fraction of clones was related to Proteobacteria (44 %), represented by Alphaproteobacteria (23 %), Gammaproteobacteria (14 %), Betaproteobacteria (5 %) and Deltaproteobacteria (2 %) (Fig. 7A). Among Alphaproteobacteria, clones were identified as *Reyranella soli* and members of the genera *Hyphomicrobium* and *Pedomicrobium*. Within Gammaproteobacteria, the dominant genera were *Panacagrimonas* and *Pseudofulvimonas* (Table S1). Betaproteobacteria, Deltaproteobacteria, Acidobacteria, Bacteroidetes and Firmicutes were also retrieved from the clone sequences of Paranhos moonmilk samples (Fig. 7A), but with similarities under 90% with their closest cultured relative, except for the acidobacterium *Aridibacter kavangonensis* (Table S1).

The Archaea found in the moonmilk samples showed a high number of clones without possible affiliation due to the very low percentage of similarity (less than 80%) with their closest cultured matches when compared using EzBioCloud database (Table S1). From the archaeal clones retrieved, 77 % were affiliated to Thaumarchaeota (Fig. 7B), from which only two clones could be identified with 99% similarity as ‘*Candidatus Nitrosoarchaeum koreensis*’ (Table S1).

Twenty-five clones were retrieved from the ITS clone library (Fig. 7C), comprising 13 OTUs at 4% of evolutionary distance, which were represented by Ascomycota (72%) and Basidiomycota (4%). Several phylotypes (24%) showed less than 80% of similarity when compared using EzBioCloud database, and thus they were unassigned to any known lineage within Eukaryota (Fig. 7C). Clones assigned to uncultured

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3 *Cladosporium* represented 40% within the Ascomycota clones (Table S2), followed by
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5 uncultured *Lewia* (12 %), *Gymnascella* (8 %), *Epicoccum* (4%) and *Verticillium* (4%).

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7 Rarefaction curves were performed for estimating the biodiversity in the moonmilk
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9 samples. Species richness for microbial communities failed to reach a saturation phase
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11 (Fig. S1), which suggests that further sequencing efforts would be required to detect
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13 additional phylotypes. However, enough clones were studied to reliably characterize the
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15 major taxonomic groups. The rarefaction curve for the bacterial community showed
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17 high diversity at the species level (Fig. S1A). At 3% of evolutionary distance, the 90
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19 clones analysed were classified into 55 OTUs. For the archaeal community, despite the
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21 lower number of clones retrieved (40), the rarefaction curve tended towards a plateau
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23 with only nine OTUs at the species level (Fig. S1B). For the fungal community, the
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25 rarefaction curve followed the same pattern as bacteria due to the sparse number of
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27 clones analysed (25 clones).
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34 DISCUSSION

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36 This study revealed that the white cotton-like nodular deposits coating extensive areas
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38 of the granitic walls in the Paranhos spring water gallery are calcite moonmilk. The
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40 band positions of the XRD pattern and FTIR spectrum precisely matched the reference
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42 values of calcite (Gunasekaran *et al.*, 2006; JCPDS card number 5-586). Besides calcite,
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44 all recognized phases were consistent with the composition of the granitic bedrock with
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46 some degree of weathering (Afonso *et al.*, 2010; Almeida and Begonha, 2015). The
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48 morphological characterization by means of FESEM and micro-CT analyses revealed
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50 that the moonmilk deposits were composed of NFC with distinguishable morphologies,
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52 which were also reported and tentatively classified by several authors (Verrecchia and
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54 Verrecchia, 1994; Cañaveras *et al.* 2006; Cailleau *et al.*, 2009; Curry *et al.*, 2009;
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Millière *et al.*, 2011a). Cañaveras *et al.* (2006) and Cailleau *et al.* (2009) attributed the distinct NFC morphologies and distribution to different phases of moonmilk formation. According to these authors, simple needles or rods are initially formed inside calcified microbial filaments (like those observed in Figure 3J), which are then released and evolve giving rise to a wide range of needle morphologies and fibre growth favored by physicochemical processes. The polycrystalline crystals with epitaxial growth are formed in a more evolved stage in the high porous water-rich inner area (Cañaveras *et al.*, 2006). The complex internal structure revealed by three-dimensional micro-CT images suggested a similar evolution in the formation of the moonmilk deposits from the Paranhos gallery. The three main NFC morphologies (Fig. 3) and material density distribution (Fig. 4) observed in the cross sections evidence different phases of moonmilk formation as in the case of Altamira limestone cave, Spain (Cañaveras *et al.*, 2006).

The questions that arise are related to the source of Ca^{2+} ions and the origin of the abundant moonmilk in this subsurface granitic environment. Possible influence in the source of Ca^{2+} in the Paranhos gallery could include the $\text{HCO}_3\text{-Ca}$ and $\text{SO}_4\text{-Ca}$ water types that percolate through the rock discontinuities and flow along the granitic gutter pipe (Afonso *et al.*, 2010). The repairing works on the Paranhos granitic gutter pipe and masonry carried out in the 17th century and later could have also contributed to an increase of Ca^{2+} liberated during water-concrete interactions (Jacques *et al.*, 2013). The meteoric seepage waters may dissolve the Ca-bearing cement phases of the repairing materials (e.g. concretes and mortars) applied on the granite joints, promoting calcite supersaturation and subsequent precipitation.

The average oxygen isotope composition ($\delta^{18}\text{O}$ value) of the moonmilk samples was -5.105 ± 0.12 , which is in agreement with the data reported by Afonso *et al.* (2010) for

the Paranhos groundwater. The authors found that this groundwater has a homogeneous isotopic composition, with mean $\delta^{18}\text{O}$ values of -5.3‰ for the groundwater running along the granitic gutter pipe and -6‰ for the water in rock discontinuities, corresponding mostly to meteoric waters resulting into a direct infiltration through the fractured granitic rock. Of note is that moonmilk collected from Aven d'Orgnac limestone Cave in France showed $\delta^{18}\text{O}$ values of -5.4‰ and -5.1‰ , similar to values of modern speleothems that formed under equilibrium conditions in that region (Duplessis *et al.*, 1969).

Regarding the stable carbon-isotope composition, Lacelle *et al.* (2004) reported that the $\delta^{13}\text{C}$ values of actively growing moonmilk in the marble cave Caverne de l'Ours (Quebec, Canada) were in the range of -7.5 to -4.4‰ , with some of the values near -6‰ , similar to those obtained for the Paranhos moonmilk ($-5.99 \pm 0.07\text{‰}$). For Bajnóczi and Kovács-Kis (2006) the stable isotope composition of pedogenic needle-fiber calcite ($\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values of -7.1‰ and -7.3‰ , respectively) indicated significant incorporation of organically derived CO_2 and probably this pointed to a biological origin in needle genesis. Both $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values of Paranhos moonmilk samples matched well within those measured for pedogenic NFC from Benasque region, central Pyrenees, Spain (Millière *et al.*, 2011b). Similarly, $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values were observed by Richter *et al.* (2008) in unconsolidated (recent) moonmilk from Tunnel-Cave in Devonian massive limestone (Germany). The authors attributed an inorganic origin for the NFC based on the isotopic signatures, but linked its formation to microbially induced calcite precipitation based on the morphology of the fibres and electron backscatter diffraction observations. They explained that biologically induced mineralization, in contrast to biologically controlled mineralization, has little or no effect on isotope fractionation (Richter *et al.*, 2008).

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The $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values of the Paranhos moonmilk suggest a meteoric origin of the moonmilk calcite, with carbon derived from atmospheric CO_2 and soil respiration, and oxygen from rainwater. The infiltrating waters within the granite tunnel had some contribution from dissolved inorganic carbon, which is strongly dependent on soil respiration (e.g., plant root, bacterial and fungal respiration) and/or dissolution of carbonate minerals (Millière *et al.*, 2011a,b; Hasinger *et al.*, 2015). The HCO_3^- contribution from dissolution of carbonate phases in mortars cannot be excluded. Lime mortars contain Ca-carbonate aggregates formed during the carbonation process, i.e., the transformation of portlandite into calcite with atmospheric CO_2 (Cultrone *et al.*, 2005).

Percolating water entering into subsurface environments may introduce not only inorganic compounds and contaminants but also organic matter and surficial microorganisms from the overlying layers (D'Angeli *et al.*, 2017; Ruggieri *et al.*, 2017). The input of nutrients can thus be relevant for the development of microbial colonization and associated microbe-mineral interactions within the Paranhos spring water gallery. To assess microbial communities concealed within the Paranhos moonmilk samples, phylogenetic affiliations of the 16S rDNA and ITS clone libraries were determined. Most of the bacterial clones were represented by Actinobacteria (52 %), a phylum frequently found in numerous caves (Groth and Saiz-Jimenez, 1999; Laiz *et al.*, 1999; Porca *et al.*, 2012; Northup *et al.*, 2003; Riquelme *et al.*, 2015), and associated with the formation of moonmilk deposits from Altamira Cave (Cañaveras *et al.*, 1999, 2006). *Pseudonocardia* was the predominant genus in the Paranhos moonmilk samples, which was also found in several caves (Stomeo *et al.*, 2008) and Etruscan tombs (Diaz-Herraiz *et al.*, 2013, 2014). The species *P. hispaniensis* represented 89% of the sequences affiliated to Actinobacteria and 47 % of all bacterial clones retrieved from

the moonmilk deposits. Considering that the Paranhos water gallery showed a high vulnerability to contamination, as recently reported by Fernandez-Cortes *et al.* (2014) and Afonso *et al.* (2016), the copiousness of *P. hispaniensis* or closed relatives (clones with 93% to 98% similarity) is not surprising. The considerable number of anthropogenic contamination sources and urban surface activities around Paranhos-Salgueiros galleries, such as garages, petrol stations and sanitary sewer systems (Afonso *et al.*, 2008, 2016), influences the diversity and distribution of microbial communities inside the Paranhos gallery. Indeed, within the 107 potential contamination sources inventoried by Afonso *et al.* (2007b, 2016) in the surroundings of the Paranhos-Salgueiros galleries, garages represent 26% of these sources including a petrol station above the sampling site (Afonso *et al.*, 2007b, 2016; Garcia-Sanchez *et al.*, 2014). This is compatible with the presence of *P. hispaniensis* in the moonmilk samples, whose type species was isolated from activated sludge collected in an oil refinery plant (Cuesta *et al.*, 2013). Hence, the high number of potential oil contamination sources of the Paranhos gallery is highly suggestive of hydrocarbon degradation by this bacterium. In addition, the abundance of *Pseudomonas putida* in the enrichment cultures from black ferromanganese deposits collected in the Paranhos gallery (Garcia-Sanchez *et al.*, 2014) supports this hypothesis, because *Pseudomonas* species are able to grow in crude oil and to degrade petroleum compounds (Mulet *et al.*, 2011).

Proteobacteria was the second well-represented phylum (43% within bacteria) in the Paranhos moonmilk. Engel *et al.* (2013) found that most of the bacterial clones (>50 %) were affiliated to Proteobacteria in moonmilk samples from two Italian caves, but the distribution of clones among classes was different for the moonmilk samples of the Paranhos gallery. In fact, the percentage of clones belonging to Betaproteobacteria in the Paranhos moonmilk was 5%, whereas in the two Italian caves were 22 and 15%.

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Moreover, Bacteroidetes represented 17 and 15% in these caves, contrasting with the 1% from the Paranhos samples. In general, the biodiversity in the Italian moonmilk was much higher (12 major taxonomic groups, including 2 candidate divisions) than in the Paranhos gallery, with only 5 phyla. At the genus level, bacteria were even taxonomically distinct when compared with the Paranhos moonmilk, suggesting that not only the mineralogy of the host rock but also environmental factors and contamination level influence moonmilk microbial communities.

Within the Proteobacteria phylum, Alphaproteobacteria (23%) was the most abundant class, represented by members of the genera *Hyphomicrobium* and *Pedomicrobium*. These are known to oxidize manganese and were previously detected in black ferromanganese coatings from the Paranhos gallery (Miller *et al.*, 2012b), Etruscan tombs (Diaz-Herraiz *et al.*, 2013, 2014) and moonmilk from Cataract Cave (Curry *et al.*, 2009). Another species conclusively identified was *Reyranella soli*, originally isolated from a Korean forest soil (Kim *et al.*, 2013). *Reyranella massiliensis*, a species close to *R. soli*, was found on the paintings of an Etruscan tomb (Diaz-Herraiz *et al.*, 2013) and represented 18% of all alphaproteobacteria detected. The presence of *R. massiliensis* in subterranean environments could be related to the abundance of amoebae, from which is an endosymbiont (Pagnier *et al.*, 2011), as also found for some other genera, including *Legionella* in Lascaux Cave (Garcia-Sanchez *et al.*, 2013).

Most of the Gammaproteobacteria clones (14%) were predominately related to the Xanthomonadales order, represented by the genera *Pseudofulvimonas* and *Panacagrimonas*. Both genera were recently described and have unique species: *Pseudofulvimonas gallinarii* and *Panacagrimonas perspica*. These species have been originally detected in soils, rhizosphere and air (Im *et al.*, 2010; Castro-Silva *et al.*, 2013; Yang *et al.*, 2016). Of note is that most of the closest uncultured relatives of the

sequenced clones were recovered from contaminated environments, such as wastewaters and hydrocarbon-contaminated soil (Table S1). This is consistent with the contamination sources around the Paranhos gallery, reinforcing our hypothesis that the microbial diversity from this site is affected by the infiltration of water and substances derived from sewer pipeline leakages and oil compounds.

Within Archaea, Thaumarchaeota was the unique phylum retrieved (77%), which is dominated by ammonia-oxidizing microorganisms found in marine and terrestrial environments (Schleper and Nicol, 2010), including caves (Gonzalez *et al.*, 2006). Ammonia, a pollutant often found in agricultural soils and wastewater, has long been demonstrated to be converted into nitrate by both autotrophic bacteria and archaea in soil ecosystems (Leininger *et al.*, 2006; Schauss *et al.*, 2009). Reitschuler *et al.* (2015) reported that Archaea comprised 59% of the total microbial community found in moonmilk deposits from an Alpine cave with a mean annual air temperature of 4.2°C. Yet, the archaeal community had limited diversity, and undescribed Euryarchaeota dominated over Thaumarchaeota (closely related to *Nitrosopumilus maritimus*). The detection of high nitrate contents in seepage waters from the Paranhos gallery (Fernandez-Cortes *et al.*, 2014), is consistent with the presence of wastewater pipeline leakage and the dominance of Thaumarchaeota within the archaeal 16S rRNA gene library.

Phylogenetic affiliations of the ITS clones were mainly ascribed to the phylum Ascomycota (72%) from which 40% corresponded to uncultured *Cladosporium*. Spores from members of this fungal genus are widespread in cave air (Docampo *et al.*, 2011; Fernandez-Cortes *et al.*, 2011; Garcia-Anton *et al.*, 2014) and are well-known petroleum degraders (Pérez-Armendáriz *et al.*, 2010). Due to similar traits shared between NFC and fungal filaments (like hyphae and rhizomorphs), their potential

relationship has been proposed by Bindschedler *et al.* (2012). Conversely, Cañaveras *et al.* (2006) attributed the formation of NFC to active actinobacteria, as no fungal DNA was detected in the moonmilk from Altamira Cave (Spain). Engel *et al.* (2013) reported that rare fungal hyphae were observed by scanning electron microscopy in moonmilk from Italian caves. For Reitschuler *et al.* (2015), the fungal contribution to moonmilk precipitation was negligible. In contrast, Cailleau *et al.* (2009) showed that the simplest morphology of NFC is formed inside fungal-like filaments (a single biogenic rod), which is then released and gradually evolve to more complex needles through abiotic processes. Similarly, Richter *et al.* (2008) evidenced the complex interplay of microbially induced mineralization and inorganic factors in the formation of cave moonmilk. The authors suggested that the growth of NFC was initiated from nuclei embedded in EPS or fungal tubes, whereas serrated-edged and outgrowths on fibres resulted from physico-chemical processes related to the oxidation of organic matter. Here we evidenced the gradual evolution of moonmilk formation as proposed by Cañaveras *et al.* (2006), and corroborated the complex nature of NFC, which combines biological and physico-chemical processes, as suggested by Richter *et al.* (2008).

CONCLUSIONS

This multiproxy study on abundant moonmilk in the Paranhos subsurface granitic environment revealed a meteoric origin of the fibre calcite crystals, with an important contribution of atmospheric CO₂, soil respiration and HCO₃⁻ from dissolution of carbonate phases in mortars of the repairing materials introduced in the granite joints. The wide range of NFC morphologies and material density suggested distinct stages of moonmilk formation, with initial needles formed inside microbial filaments and polycrystalline needles as the result of epitaxial growth formed under abiotic conditions.

The microbial communities thriving in the moonmilk deposits from Paranhos spring water gallery in Porto city (NW Portugal) showed to be different from other moonmilk deposits previously described in karstic caves. About 50% of the bacterial clones were closely related to *Pseudonocardia hispaniensis*, which is consistent with the contamination sources in the surroundings of the Paranhos gallery. The remaining clones were distributed among Proteobacteria, Acidobacteria, Bacteroidetes and Firmicutes, but the species retrieved showed abundances of 3% or lower. Uncultured Thaumarchaeota and unclassified archaeon were representatives of the Archaea domain and uncultured *Cladosporium* of Fungi. Thus, this survey provides new insights into calcite moonmilk recorded in a subsurface granitic environment and remarks the need to investigate urban underground environments in detail to understand the interactions between microbes and moonmilk.

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CAPTIONS FOR FIGURES AND TABLES

Figure 1. A) Paranhos-Salgueiros galleries alignment in the highly-urbanized Porto city (NW Portugal); B) Moonmilk deposits coating the granite wall and granite joints filled with mortar or concrete. C) Detailed view of the cotton-like moonmilk deposits forming nodular aggregates.

Figure 2. Stereomicroscopy images of moonmilk samples. A) white moonmilk deposits. B) detail of needle-like particles of moonmilk. C) mortar and weathered granite substrate. D) sand and granite mineral particles after carbonate removal with HCl.

Figure 3. FESEM images of moonmilk samples from Paranhos gallery showing different morphologies of the needle-shaped crystals. A) Overview of the intertwined needle fibre crystals. Bar = 10 μm . B) mineralized fungal-like hyphae structures coated with NFC. Bar =10 μm . C) Three main NFC morphologies observed in the same moonmilk sample: (1) single or paired rods with smooth surfaces; (2) very thin filaments or nanofibers on the needle-fibre crystals; (3) Polycrystalline crystals with serrated edges. Bar = 1 μm . D) Detailed view of simple rods with smooth surfaces. Bar = 1 μm . E-F) Thin filaments or nanofibers (arrows). Bars = 2 μm . G) simple needle-fibre with serrated edges (arrow). Bar = 2 μm . H-I) Needle-fibre crystals with serrated edges. Bars = 5 μm . J) Organic-like filaments with 1 μm diameter and serrated-edged outlines. Bar = 2 μm . K-L) slime matrix of EPS embedded within the fibre crystals (arrows). Bars = 1 and 4 μm , respectively.

Figure 4. Micro-CT visualizations of moonmilk samples from the Paranhos gallery cut perpendicular to the granite substrate. Arrows indicate growth direction.

Figure 5. Representative X-ray diffractogram of moonmilk from Paranhos spring water gallery showing the characteristic peaks of calcite and muscovite (M). Remain small peaks correspond to granite related minerals (quartz, biotite and alkaline feldspars).

Figure 6. Infrared spectrum depicting the characteristic bands of calcite.

Figure 7. Phylum-level taxonomy distribution of the microbial communities in the Paranhos moonmilk samples: A) bacterial 16S rRNA gene community. Phylum Proteobacteria is divided in class-level taxonomies. B) archaeal 16S rRNA gene community, and C) Fungal (ITS regions) community.

Figure 1. A) Paranhos-Salgueiros galleries alignment in the highly-urbanized Porto city (NW Portugal); B) Moonmilk deposits coating the granite wall and granite joints filled with mortar or concrete. C) Detailed view of the cotton-like moonmilk deposits forming nodular aggregates.

94x88mm (300 x 300 DPI)

Figure 2. Stereomicroscopy images of moonmilk samples. A) white moonmilk deposits. B) detail of needle-like particles of moonmilk. C) mortar and weathered granite substrate. D) sand and granite mineral particles after carbonate removal with HCl.

197x151mm (299 x 299 DPI)

Figure 3. FESEM images of moonmilk samples from Paranhos gallery showing different morphologies of the needle-shaped crystals. A) Overview of the intertwined needle fibre crystals. Bar = 10 µm. B) mineralized fungal-like hyphae structures coated with NFC. Bar =10 µm. C) Three main NFC morphologies observed in the same moonmilk sample: (1) single or paired rods with smooth surfaces; (2) very thin filaments or nanofibers on the needle-fibre crystals; (3) Polycrystalline crystals with serrated edges. Bar = 1 µm. D) Detailed view of simple rods with smooth surfaces. Bar = 1 µm. E-F) Thin filaments or nanofibers (arrows). Bars = 2 µm. G) simple needle-fibre with serrated edges (arrow). Bar = 2 µm. H-I) Needle-fibre crystals with serrated edges. Bars = 5 µm. J) Organic-like filaments with 1 µm diameter and serrated-edged outlines. Bar = 2 µm. K-L) slime matrix of EPS embedded within the fibre crystals (arrows). Bars = 1 and 4 µm, respectively.

221x205mm (299 x 299 DPI)

Figure 4. Micro-CT visualizations of moonmilk samples from the Paranhos gallery cut perpendicular to the granite substrate. Arrows indicate growth direction.

199x102mm (300 x 300 DPI)

Figure 5. Representative X-ray diffractogram of moonmilk from Paranhos spring water gallery showing the characteristic peaks of calcite and muscovite (M). Remain small peaks correspond to granite related minerals (quartz, biotite and alkaline feldspars).

228x115mm (72 x 72 DPI)

Figure 6. Infrared spectrum depicting the characteristic bands of calcite.

135x101mm (72 x 72 DPI)

Figure 7. Phylum-level taxonomy distribution of the microbial communities in the Paranhos moonmilk samples: A) bacterial 16S rRNA gene community. Phylum Proteobacteria is divided in class-level taxonomies. B) archaeal 16S rRNA gene community, and C) Fungal (ITS regions) community.

298x144mm (299 x 299 DPI)

SUPPORTING INFORMATION

Origin of abundant moonmilk deposits in a subsurface granitic environment

A.Z. Miller^{1*}, A.M. Garcia-Sanchez¹, P.M. Martin-Sanchez², M.F.C. Pereira³, J.E. Spangenberg⁴,
V. Jurado¹, A. Dionísio³, M.J. Afonso⁵, H.I. Chaminé⁵, B. Hermosin¹, C. Saiz-Jimenez¹

Figure S1. Rarefaction curves for 16S rDNA clone libraries of: Bacteria (a) and Archaea (b) from moonmilk collected in the Paranhos spring water gallery.

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Table S1. Phylogenetic affiliations of the OTUs obtained for bacterial and archaeal communities from moonmilk deposits collected in the Paranhos spring water gallery.

Representative clone (accession number) ^a	No. of clones	Closest relative from NCBI (accession number)	Isolation source	Similarity %	Closest cultured relative from EzBioCloud (accession number)	Similarity %
Domain Bacteria	90					
Actinobacteria	47					
6FB-A4 (HG008829)	22	<i>Pseudonocardia hispaniensis</i> (FR695486)	Industrial wastewater treatment plant, Huelva, Spain	96	<i>Pseudonocardia hispaniensis</i> (FR695486)	98
6FB-F2 (HG008830)	9	Uncultured bacterium (JX098342)	Mineral soils Atacama desert	95	<i>Pseudonocardia hispaniensis</i> (FR695486)	97
6FB-B1 (HG008831)	2	<i>Actinobispora</i> sp. QAI43 (EU274329)	Soil, Kanas Lake, Xinjiang, China	97	<i>Pseudonocardia hispaniensis</i> (FR695486)	98
6FB-C9 (HG008832)	1	<i>Pseudonocardia hispaniensis</i> (FR695486)	Industrial wastewater treatment plant, Huelva, Spain	93	<i>Pseudonocardia hispaniensis</i> (FR695486)	95
6FB-G4 (HG008833)	1	<i>Pseudonocardia hispaniensis</i> (FR695486)	Industrial wastewater treatment plant, Huelva, Spain	93	<i>Pseudonocardia hispaniensis</i> (FR695486)	95
6FB-A11 (HG008834)	1	Uncultured bacterium (JX098342)	Mineral soils Atacama desert	93	<i>Pseudonocardia hispaniensis</i> (FR695486)	94
6FB-D7 (HG008835)	1	Uncultured bacterium (JX098323)	Mineral soils Atacama desert	91	<i>Pseudonocardia hispaniensis</i> (FR695486)	93
6FB-D8 (HG008836)	1	Uncultured bacterium (JX098798)	Mineral soils Atacama desert	95	<i>Pseudonocardia hispaniensis</i> (FR695486)	94
6FB-C5 (HG008837)	1	Uncultured bacterium (GQ495403)	Hnausahraun lava flow, Iceland	97	<i>Pseudonocardia hispaniensis</i> (FR695486)	97
6FB-H3 (HG008838)	1	Uncultured bacterium (HM445437)	Yellow microbial mat on the walls of a lava tube, Terceira, Azores, Portugal	94	<i>Pseudonocardia hispaniensis</i> (FR695486)	94
6FB-A1 (HG008839)	1	<i>Actinobispora</i> sp. QAI43 (EU274329)	Soil, Kanas Lake, Xinjiang, China	93	<i>Pseudonocardia hispaniensis</i> (FR695486)	95
6FB-A2 (HG008840)	1	Uncultured bacterium (GQ065789)	Skin, occiput of Homo sapiens	93	<i>Pseudonocardia benzenivorans</i> (AJ556156)	92
6FB-A9 (HG008841)	1	Uncultured beta proteobacterium (HE860775)	Rhizosphere soil, Norway	87	<i>Pseudonocardia seranimata</i> (FJ817379)	88
6FB-D1 (HG008842)	1	Uncultured bacterium (JX098342)	Mineral soils Atacama desert	95	<i>Pseudonocardia hispaniensis</i> (FR695486)	97
6FB-B7 (HG008843)	1	Uncultured actinobacterium (JN178925)	Speleothem surface, Kartchner Caverns, AZ, USA	92	<i>Aciditerrimonas ferrireducens</i> (AB517669)	88
6FB-H6 (HG008844)	1	<i>Mycobacterium</i> sp. (DQ249999)	Rock surface, Kartchner Caverns, AZ, USA	97	<i>Mycobacterium fallax</i> (AF480600)	93
6FB-C7 (HG008845)	1	Uncultured bacterium (EF020175)	Rhizosphere of <i>Populus tremuloides</i> , Rhinelander, WI, USA	87	<i>Saccharomonospora marina</i> (CM001439)	83
Alphaproteobacteria	21					
6FB-A6 (HG008846)	2	Uncultured bacterium (JN592703)	Yellow microbial mat on the wall of a lava tube, Terceira, Azores, Portugal	99	<i>Hyphomicrobium vulgare</i> (Y14302)	93
6FB-A12 (HG008847)	2	Uncultured bacterium (HQ121112)	Loamy sand from tomato orchard, Escondido, CA, USA	99	<i>Azospirillum rugosum</i> (AM419042)	90
6FB-D2 (HG008848)	1	Uncultured bacterium (HQ121112)	Loamy sand from tomato orchard, Escondido, CA, USA	98	<i>Elstera litoralis</i> (EU678309)	89
6FB-D3 (HG008849)	1	Uncultured bacterium (JN592703)	Yellow microbial mat on the walls, Gruta do Natal, Terceira, Azores, Portugal	97	<i>Pedomicrobium manganicum</i> (X97691)	96
6FB-E3 (HG008850)	1	Uncultured bacterium (EU133559)	Soil from a mixed grass prairie, OK, USA	90	“ <i>Candidatus</i> Sphaeronema italicum” (AY428765)	87

Table S1. Phylogenetic affiliations of the OTUs obtained for bacterial and archaeal communities from moonmilk deposits collected in the Paranhos spring water gallery.

6FB-F12 (HG008851)	1	Uncultured bacterium (EU133369)	Soil from a mixed grass prairie, OK, USA	97	“ <i>Candidatus</i> Sphaeronema italicum” (AY428765)	88
6FB-G6 (HG008852)	1	Uncultured bacterium (JN616189)	Yellow microbial mat on the walls, Gruta dos Principiantes Lava Tube, Terceira, Azores, Portugal	95	<i>Bauldia consociata</i> (FJ560750)	91
6FB-G8 (HG008853)	1	Uncultured alpha proteobacterium (FJ535079)	Biofilm on the wall, Pajsarjeva Cave, Ljubljana, Slovenia	93	<i>Thermovum composti</i> (AB563785)	91
6FB-H7 (HG008854)	1	Uncultured bacterium (JF266548)	White microbial mat on the walls, Gruta das Torres Lava Tube, Pico, Azores, Portugal	97	<i>Hyphomicrobium zavarzinii</i> (Y14305)	96
6FB-H8 (HG008855)	1	Uncultured bacterium (EU488001)	Siliciclastic sediment from Thalassia sea grass bed, Dog Island, Cedar Key, Florida	91	<i>Hyphomicrobium aestuarii</i> (Y14304)	88
6FB-H9 (HG008856)	1	Uncultured alpha proteobacterium (HE614773)	Rock biofilm from gold mine, Zloty Stok, Poland	96	<i>Hyphomicrobium sulfonivorans</i> (AF235089)	94
6FB-F3 (HG008857)	1	Uncultured bacterium (EU133255)	Soil from a mixed grass prairie, OK, USA	97	<i>Dongia mobilis</i> (FJ455532)	91
6FB-F7 (HG008858)	1	Uncultured bacterium (EU133588)	Soil from a mixed grass prairie, OK, USA	87	<i>Mesorhizobium loti</i> (AXAE01000021)	86
6FB-B9 (HG008859)	1	Uncultured bacterium (AB294313)	Deep coal seam groundwater, Hokkaido, Japan	98	<i>Blastochloris sulfovirdis</i> (D86514)	91
6FB-C12 (HG008860)	1	Uncultured bacterium (JQ675501)	Water sample from carbonate cave pools, Carlsbad Caverns, NM, USA	99	<i>Reyranella soli</i> (JX260424)	99
6FB-F9 (HG008861)	1	Uncultured bacterium (GU455159)	Anaerobic fermentation reactor with waste activated sludge	97	<i>Oligotropha carboxidovorans</i> (CP002826)	93
6FB-H11 (HG008862)	1	Uncultured bacterium (DQ125654)	Uranium contaminated soil, Oak Ridge, TN, USA	94	<i>Pseudolabrys taiwanensis</i> (DQ062742)	93
6FB-H2 (HG008863)	1	Alpha proteobacterium (AY429692)	TCE and cis-DCE contaminated groundwater, Sacramento, CA, USA	99	<i>Amaricoccus macauensis</i> (U88042)	91
6FB-D10 (HG008864)	1	Uncultured bacterium (KJ782898)	Petrochemical wastewater treatment plant sludge	96	<i>Parvibaculum hydrocarboniclasticum</i> (GU574708)	92
Gammaproteobacteria	12					
6FB-B4 (HG008865)	3	Uncultured bacterium (JQ407965)	Horizontal subsurface flow constructed wetland treating domestic wastewaters, Mahdia, Tunisia	98	<i>Pseudofulvimonas gallinarii</i> (FN298515)	96
6FB-E11 (HG008866)	2	Uncultured bacterium (HE602880)	Yellow spots on the walls, Altamira Cave, Santillana del Mar, Spain	99	<i>Panacagrimonas perspica</i> (AB257720)	97
6FB-F1 (HG008867)	1	Uncultured bacterium (JQ675464)	Water sample, Carlsbad Caverns, NM, USA	98	<i>Panacagrimonas perspica</i> (AB257720)	98
6FB-G11 (HG008868)	1	Uncultured bacterium (HM277149)	Skin, popliteal fossa, Homo sapiens	94	<i>Panacagrimonas perspica</i> (AB257720)	94
6FB-D4 (HG008869)	1	Uncultured Xanthomonadaceae (AM936440)	Aliphatic hydrocarbon-contaminated soil, France	82	<i>Panacagrimonas perspica</i> (AB257720)	81
6FB-E4 (HG008870)	1	Uncultured bacterium (HQ119992)	Loamy sand from tomato orchard, Escondido, CA, USA	96	<i>Pseudofulvimonas gallinarii</i> (FN298515)	92
6FB-A3 (HG008871)	1	Uncultured bacterium (JQ675502)	Water sample, Carlsbad Caverns, NM, USA	99	<i>Nevskia terrae</i> (GQ845011)	90
6FB-B8 (HG008872)	1	Uncultured bacterium (KJ615253)	GAC filters treating drinking water	98	<i>Ectothiorhodospira magna</i> (HM149323)	87
6FB-E5 (HG008873)	1	Uncultured bacterium (HM749729)	Yellow microbial mat on the walls, Gruta das Torres Lava Tube, Pico, Azores, Portugal	90	<i>Silanimonas mangrove</i> (HE573746)	87

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2 **Table S1.** Phylogenetic affiliations of the OTUs obtained for bacterial and archaeal communities from moonmilk deposits collected in the Paranhos

3 spring water gallery.

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6	Betaproteobacteria	4					
7	6FB-A5 (HG008874)	1	Uncultured bacterium (EU133779)	Soil from a mixed grass prairie, OK, USA	93	<i>Herbaspirillum seropedicae</i> (Y10146)	88
8	6FB-C4 (HG008875)	1	Uncultured bacterium (AJ295484)	Rhizoplane of <i>Brassica</i> napus, Braunschweig, Germany	96	<i>Herbaspirillum seropedicae</i> (Y10146)	89
9	6FB-C2 (HG008876)	1	Uncultured beta proteobacterium (HE860775)	Rhizosphere soil, Norway	91	<i>Rhizobacter dauci</i> (AB297965)	89
10	6FB-C3 (HG008877)	1	Uncultured bacterium (GQ397030)	Deglaciated soil, Mendenhall Glacier, Jeneau, AK, USA	98	<i>Cupriavidus gilardii</i> (EU024163)	91
11	Deltaproteobacteria	2					
12	6FB-A7 (HG008878)	1	Uncultured delta proteobacterium (JN178913)	Speleothem surface, Kartchner Caverns, AZ, USA	99	<i>Desulfosalsimonas propionica</i> (DQ067422)	86
13	6FB-E12 (HG008879)	1	Uncultured bacterium (HM445084)	Yellow microbial mat on the walls, Gruta das Balcoes Lava Tube, Terceira, Azores, Portugal	97	<i>Desulfuromonas michiganensis</i> (AF357915)	86
14							
15	Acidobacteria	2					
16							
17	6FB-E9 (HG008880)	1	Uncultured Acidobacteria (DQ139447)	Epilithic biofilms in Saint Callixtus Catacombs, Rome, Italy	100	<i>Aridibacter kavangonensis</i> (KF245633)	96
18							
19	6FB-H10 (HG008881)	1	Uncultured bacterium (EU132280)	Soil from a mixed grass prairie, OK, USA	99	<i>Aridibacter kavangonensis</i> (KF245633)	87
20	Bacteroidetes	1					
21	6FB-C1 (HG008882)	1	Uncultured bacterium (JQ769672)	Biological soil crust of copper mine tailings wastelands, Tongling, China	92	<i>Lewinella nigricans</i> (AB301615)	83
22							
23	Firmicutes	1					
24	6FB-C8 (HG008883)	1	Uncultured bacterium clone (EU048666)	Marine sediment, South Slope, South China Sea	90	<i>Oceanirhabdus sediminicola</i> (EU048666)	83
25							
26	Domain Archaea	40					
27	Thaumarchaeota	31					
28	6FA-H5 (HG008884)	11	Uncultured archaeon (KC437252)	Large-discharge carbonate spring water, Nevada, USA	99	“ <i>Candidatus</i> Cenarchaeum symbiosum” (DP000238)	90
29							
30	6FA-F3 (HG008885)	10	Uncultured crenarchaeote (JN820160)	Ferromanganese deposit, Carter Salt Peter Cave, TN, USA	99	“ <i>Candidatus</i> Cenarchaeum symbiosum” (DP000238)	92
31							
32	6FA-E5 (HG008886)	8	Uncultured archaeon (KC604545)	Groundwater, Mahomet aquifer, Illinois, USA	99	“ <i>Candidatus</i> Nitrosoarchaeum koreensis” (HQ331116)	93
33							
34	6FA-H1 (HG008887)	2	Uncultured archaeon (AB550808)	River water, Shiba, Japan	99	“ <i>Candidatus</i> Nitrosoarchaeum limnia” (CM001158)	99
35							
36	Unclassified archaeon^b	9					
37	6FA-C4 (HG008888)	3	Uncultured euryarchaeote (AB380105)	Upland rice field soils, Ibaraki, Japan	99	-	-
38	6FA-A4 (HG008889)	2	Uncultured Thermoplasmata archaeon (JN002476)	Serpentinized dunite	99	-	-
39							
40	6FA-C1 (HG008890)	2	Uncultured archaeon (KC955194)	Microbialite, Pavilion Lake, British Columbia, Canada	99	-	-
41	6FA-D4 (HG008891)	1	Uncultured euryarchaeote (JN820183)	Ferromanganese deposit, Carter Salt Peter Cave, TN, USA	90	-	-
42							
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Table S1. Phylogenetic affiliations of the OTUs obtained for bacterial and archaeal communities from moonmilk deposits collected in the Paranhos spring water gallery.

6FA-H8 (HG008892)	1	Uncultured archaeon (HG325813)	Red deposit on granite wall, Paranhos spring water tunnel, Porto, Portugal	99	-	-
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^a: Representative clone of each OTU at 97% identity for 16S rDNA analysis.

^b: OTUs with less than 80% of similarity with their closest cultured relative were not classified.

Table S2. Phylogenetic affiliations of the OTUs obtained for eukaryotic communities from moonmilk collected in the Paranhos spring water gallery.

Representative clone (accession number) ^a	No. of clones	Closest relative (accession number)	Isolation source	Similarity %
Domain Eukaryota	25			
Ascomycota	18			
6FF-C9 (HG008768)	10	Uncultured <i>Cladosporium</i> (KM062089)	Biotreated stone of Royal Chapel of Granada, Spain	99
6FF-A12 (HG008769)	3	Uncultured <i>Lewia</i> (HF947047)	Host phyllosphere, Magnesia, Greece	100
6FF-D7 (HG008770)	2	Uncultured <i>Gymnascella</i> (HE605247)	Black stain on the wall of Lascaux Cave, Montignac, France	99
6FF-A8 (HG008771)	1	<i>Epicoccum nigrum</i> (AB975291)	<i>Salicornia europaea</i> , Japan	99
6FF-F10 (HG008772)	1	Uncultured <i>Verticillium</i> (HE664132)	Air sample from Lascaux Cave, Montignac, France	99
6FF-D8 (HG008773)	1	Uncultured soil fungus (DQ420829)	Soil, Cedar Creek, Minnesota, USA	99
Basidiomycota	1			
6FF-D12 (HG008774)	1	<i>Serpula himantoides</i> (GU187545)	Brick wall in house, Louvain-la-Neuve, Belgium	99
Unclassified Eukaryota	6			
6FF-B9 (HG008775)	1	<i>Staurostrum orbiculare</i> (AJ829660)		88
6FF-C7 (HG008776)	1	Uncultured fungus (KC965193)	Soil, Banks Island, Canada	82
6FF-C11 (HG008777)	1	Uncultured ciliate (HQ219418)	Dimictic and eutrophic Aydat Lake, Massif Central, France	95
6FF-E12 (HG008778)	1	<i>Leucoagaricus</i> sp. (EU561513)	Fungus-gardening ant (<i>Trachymyrmex septentrionalis</i>)	86
6FF-G9 (HG008779)	1	Uncultured fungus (EF635712)	Bare soil, Austrian Central Alps, Austria	75
6FF-H12 (HG008780)	1	<i>Capsaspora owczarzaki</i> (AY363957)	Pulmonate snail (<i>Biomphalaria glabrata</i>), Brazil	89

^a: Representative clone of each OTU at 96% identity for ITS rDNA analysis.