International Journal of Speleology	47 (2)	173-187	Tampa, FL (USA)	May 2018

Available online at scholarcommons.usf.edu/ijs

International Journal of Speleology

Official Journal of Union Internationale de Spéléologie

Sulfur Cave (Romania), an extreme environment with microbial mats in a CO_2-H_2S/O_2 gas chemocline dominated by mycobacteria

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Abstract: Sulfur Cave (Puturosu Mountain, Romania) is an extreme environment, unique for displaying life in a gas chemocline. The lower part of the cave is filled with CO_2, CH_4, and H_2S of mofettic origin, while the upper part contains air that floats above the heavier volcanic gasses. S° and H_2SO_4 (from sulfur-oxidation) cover the cave wall at and below the CO_2-H_2S:O_2 gas/gas interface. On the cave wall, near the interface the pH is <1 and unusual microbial biofilms occur on the rock's surface. We provide context information on the geology, mineralogy, chemistry and biology to better understand this unique environment. We have used X-ray diffraction, optical microscopy, scanning electron microscopy with EDAX capabilities, stable isotope analysis and 16S and 18S rDNA amplicon sequencing. The most common taxa in the microbial biofilms are Mycobacteria, *Acidithiobacillus* and Ferroplasmaceae. Liquid water in this system originates solely from condensation of water vapor onto the cave walls making inflow of organic carbon from outside unlikely. The most likely primary source of energy for this microbial community is sulfur oxidation with H_2S and S° as main reductants and atmospheric O_2 as the main oxidant. Ferric iron from the rock surface is another potential oxidant. In Sulfur Cave, gaseous CO_2 (from mofettic emission) maintains the stability of the gas chemocline. Sulfur Cave biofilms can help the search for extreme life in the subsurface, near volcanic systems on Earth and Mars. The Sulfur Cave example shows that a habitable environment can be established underground in gas chemoclines near CO_2-dominated gas discharge zones, where it can have a steady supply of water and energy.

Keywords: Sulfur Cave, mofette, gas chemocline, biofilm, sulfide, astrobiology

Received 6 December 2017; Revised 11 April 2018; Accepted 13 April 2018

Citation: Sarbu S.M., Aerts J.W., Flot J.-F., Van Spanning R.J.M., Baciu C., Ionescu A., Kis B.M., Incze R., Siko-Barabasi S., Para Z., Hegyeli B., Atudorei N.-V., Barr C., Nealson K.H., Forray F.L., Lascu C., Fleming E.J., Bitter W. and Popa R., 2018. Sulfur Cave (Romania), an extreme environment with microbial mats in a CO_2-H_2S/O_2 gas chemocline dominated by mycobacteria. International Journal of Speleology, 47 (2), 173-187. Tampa, FL (USA) ISSN 0392-6672
https://doi.org/10.5038/1827-806X.47.2.2164

INTRODUCTION

Adopting a strategy to search for life on Mars or other extraterrestrial bodies is always fraught with uncertainties such as: (i) the best habitat to find life; (ii) the type of metabolism; and (iii) resemblance with life on Earth. Mars, in particular, is enigmatic in these regards, with a highly oxidized surface, a very thin (≈17 mbars) atmosphere with little nitrogen (1.9%), very limited levels of oxygen (0.15%) and water vapor (mean 9.6 pr. precipitable microns), and 96% CO_2 (Mahaffy et al., 2013; Sindoni et al., 2011).

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Due to profound differences in geological history, climate, atmospheric composition and electromagnetic shielding from cosmic radiation (among other things), there are no terrestrial environments that fully mimic the present conditions on Mars. However, a multitude of Mars-analog environments have been used for studying the effects of certain Mars-like conditions (i.e., temperature, aridness, radiation) on microbial life and biosignatures (Aerts et al., 2014; Amils et al., 2007; Navarro-González et al., 2003). Here we report on a terrestrial cave environment in Sulfur Cave (also known as "Peştera de la Turia", Romania) that resembles several extreme conditions expected to exist, or to have existed, in the regolith' subsurface near fumarole fields on Mars. Sulfur Cave is accessible for human exploration, but the target environment for this study is located in the deepest section of the cave and shielded from sunlight. Microbial communities colonize the cave walls at the level of a gas/gas interface between volcanic gasses of mofettic origin (and more abundant in the lower sections of the cave) and atmospheric air that floats above the heavier volcanic gases. Condensation of water vapor onto the cave wall is the only source of liquid water available for life.

The redox interface in Sulfur Cave may resemble an environmental niche for microbial life associated with it that could have been present on Mars in the distant past. Similar environments may even be present on Mars today in fumarole fields. The lower gas layer in Sulfur Cave shows similarities to the Martian atmosphere in terms of the relative abundance of CO_2, N_2 and O_2 (Althaus et al., 2000; Vaselli et al. 2002). An extinct near-surface fumarole field was found at the Gusev crater on Mars (Yen et al., 2008), and cave-like structures have been detected from orbit (Cushing et al., 2007). Learning what we can from similar terrestrial environments could offer valuable insights for future missions to Mars. Knowledge about the properties of the environment and about the structure of the bacterial community in Sulfur Cave may well contribute to better understanding of potential life forms in similar harsh environments on Earth and Mars.

History and geographic settings

Sulfur Cave is located on the Ciomadul volcanic edifice, at the southeastern end of the Calimani-Gurghiu-Harghita volcanic chain, in the East Carpathian Mountains (Romania) (Fig. 1). Ciomadul consists of central lava domes hosting the Mohos and Sfânta Ana explosive craters, and is surrounded by peripheral lava domes: Haramul Mic, Bálványos, Puturosu, and Dealul Mare. Puturosu Mountain (Mt). (Büdös Hegy - *in Hungarian*), was first mentioned (under the name Bydushyg) in a 1349 document (Szabó, 1872). Several other documents dated 1580, 1591, and 1614 indicate that mining of sulfur and alum had occurred in and around caves on this mountain (Fridvaldszky, 1767; Orbán, 1869). Sulfidic and carbon dioxide poisoning risks later halted the mining of sulfur (Timon, 1733). With a total area of 5,993 ha and an average altitude of 914 m, the Ciomadul-Bálványos (ROSCI0037) site of Community Importance (SCI) is now part of the Natura 2000 EU-wide network of nature protection areas. The *Vinca Minor Association* (Sfântu Gheorghe, Romania) is the custodian of this protected area.

Geology

The Ciomadul lava dome complex is composed of about 8-14 km^3 of eruptive rocks (Szakács et al., 2015) and it is developed on the folded and thrusted Lower Cretaceous flysch units. Volcanism began at this site about 1 Ma, while the Ciomadul volcano has been built up in the past 200 ky. The youngest active stage (from 57 to 32 ky) of the volcanic activity was predominantly explosive and involved several events of lava dome collapses, accompanied by volcanian and sub-plinian eruptions (Harangi et al., 2015 a,b; Karátson et al., 2016). Most of the lava domes have preserved their original structure well, but some (e.g., Bálványos and Puturosu domes) exhibit signs of intensive erosion and alteration (Szakács et al., 2015). The volcanic products are predominantly potassium-rich dacites (Szakács et al., 1993; Vinkler et al., 2007). Petrogenetic as well as zircon dating studies indicate the existence of long-lasting (up to 350 ky), low-temperature (700-750°C) silicic crystal mush body, which was periodically remobilized by injection of hot basaltic magmas, rapidly triggering volcanic eruptions (Kis et al., 2014).

Hydrology, water chemistry and gas emissions

The aquifers of this area are marked by the dominant presence of CO_2 as free and dissolved gas (Althaus et al., 2000; Vaselli et al., 2002; Italiano et al., 2017). The chemical composition of the mineral springs found along the creeks and major tectonic

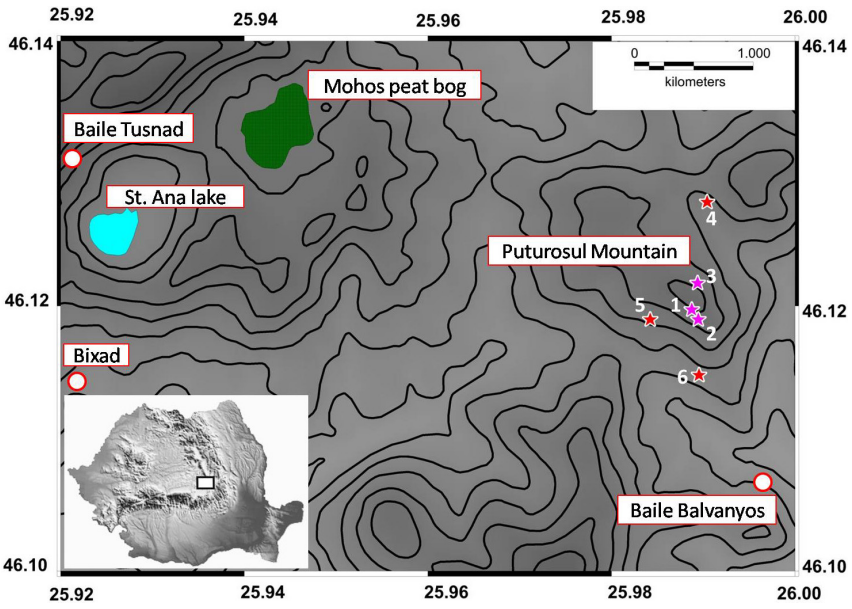

Fig. 1. The location of Puturosu Mountain (white line) in the Ciomadul volcanic edifice area (dotted line) in Romania (lower left inset). The numbered stars indicate important hydrological and geological features, some of them discussed in this paper. (1) Sulfur Cave. (2) Alum Cave. (3) Killer Cave. (4) Boffogó Peat Bog. (5) Slope Emission. (6) Apor Baths.

lines is highly variable, namely slightly acidic (pH 5-6): Na-K-Cl-HCO_3, Ca-Mg-HCO_3, to strongly acidic (pH ~1.6) Ca-SO_4 type of waters (Jánosi et al., 2011). The origin of the water is meteoric with a recharge area typical for mountain regions, as shown by their stable isotopic compositions (Fórizs et al., 2010). CO_2-bubbling peat bogs can be found North-East (Buffogó Peat Bog, Hegyeli, 2008) and South of Puturosu Mt. (Zsombor-Valley, Jánosi et al., 2011). The youngest structures of Ciomadul volcanic area consist of the twin-craters hosting the St. Ana Lake and the Mohos Peat Bog. St. Ana Lake is the only existing volcanic lake within the East Carpathians, with a surface of 22 ha and the maximum depth up to 7 m (Magyari et al., 2006, 2009). Stable isotope data ($\delta^{13}C_{CO_2}$ with a value of -5‰) suggests that magmatic degassing occurs at the bottom of the lake (Túri et al., 2016).

The term mofette is used to indicate gas emission sites on the Ciomadul Mt., but other terms such as fumarole and solfatara, although not suitable, have been used in the past to describe the diversity of gas emissions. The term mofette is generally reserved for low-temperature gas emissions mainly composed of dry CO_2, while the terms fumarole and solfatara are generally used to indicate high temperature and acidic fluid emissions that are directly related to recent volcanic activity (Martini, 1996).

In the Ciomadul area, emanations of CO_2 and H_2S in various proportions, are in the form of gas bubbling, mofettes, and naturally sparkling mineral water springs, often associated with rock alterations. The total output of CO_2 within the area of Ciomadul is 8.7×10^6 kg y^{-1} (Kis et al., 2017), consistent with other quiescent volcanoes worldwide. When emissions occur on a slope, the vegetation downhill from the vents is killed by toxic gases. The temperature of the gas when released from the rock fissures and vents is not higher than 10°C. Some CO_2-filled depressions such as the "Birds' Cemetery" located close to the "Killer Cave" (Fig. 1), are suffocation traps for insects, birds and mammals. Native sulfur occurs in some fissures and cavities that convey the CO_2-rich gas to the atmosphere. Historically, the mofettes from Puturosu Mt. have been used for therapy and recreational purposes (Incze et al., 2016). In larger caverns, the gas level is indicated by the deposition of a layer of elemental sulfur on the walls. The chemical composition of the gas emissions in Sulfur Cave was first determined by L. Ilosvay as early as 1885. Modern gas geochemical data from a few sites (Bálványos, Sulfur Cave) were previously reported by Althaus et al. (2000), Vaselli et al. (2002) and Frunzeti (2013) (Table 1) with the CO_2 content of the gases ranging between 95.63 and 98.26%. Other gas components were also reported: N_2 (0.89 to 1.97%), CH_4 (0.65 to 2.35%), O_2 (0.02 to 0.04%), H_2S up to 0.012% and noble gases (Vaselli et al., 2002), including radon (Szabó & Szabó-Sellenyi, 1981).

Table 1. Composition of gases from Sulfur Cave (% by volume; n.m. = not measured).

CO_2	N_2	CH_4	O_2	H_2S	H_2	He	Ne	Ar	Source of data
98	0.97	0.80	0.06	n.m.	0.25	0.0011	n.m	0.004	Althaus et al. 2000
98.26	0.90	0.78	0.04	0.012	0.00004	0.0004	n.m.	0.02	Vaselli et al. 2002
96.70	2.11	1.19	n.m	n.m.	n.m.	0.0027	0.000005	0.00001	Frunzeti, 2013

THE CAVES OF PUTUROSU MOUNTAIN

Sulfur Cave is the most important cave for this study, but several similar caves are located on Puturosu Mt. and will also be shortly described below, to provide insight in the regional environment.

Sulfur Cave, also known as *Peştera de la Turia*, *Peştera Sulfuroasă* / *Puturoasă* (in Romanian) and *Büdös-barlang* (in Hungarian), is located at 1,044 m altitude (N46.119764; E25.948640). It is one of the most famous mofettes in Europe, with a daily gas outflow of up to 5.26×10^3 kg/day (Kis et al., 2017). Ilosvay (1885) published a detailed description of Sulfur Cave. The main gallery is 14 m long, and about 7 m of it accessible to tourists (Fig. 2). The cave is characterized by the presence of a two-layer atmosphere. Continuous gas emissions from vents located on the cave floor fill the lower sections with a CO_2-rich gas (up to 98.26%, Vaselli et al., 2002, Table 1) denser than air (Fleischer, 1876). Sulfur deposits cover the cave floor and the cave walls in the lower sections of the cave, but they are absent from the upper part of the cave (Fig. 3a) which is exposed to atmospheric air. The zone where the two gas phases meet is a gas/gas redox interface (gas chemocline) that is well defined and relatively steady (within cm scale range) due to density differences between the two gas phases (Fig. 3b).

Small Cave, at 1,029 m altitude, (N46.119982; E25.947987) is in close proximity of Sulfur Cave. It is smaller and emits less gas. The cave walls in the lower sections of the cave are also covered with a thin layer of sulfur.

Alum Cave, also known as *Peştera cu Alaun* (in Romanian) and *Timsós-barlang* (in Hungarian) is at 1,039 m altitude (N46.119018; E25.949432), southeast of Sulfur Cave. The alum (K-Al-sulfate) deposits from the walls of this cave were mined in the past and were analyzed by various researchers (Ilosvay, 1885).

Killer Cave also know as *Peştera Ucigaşă* (in Romanian) and *Gyilkos-barlang* (in Hungarian) is located at 1,066 m altitude (N46.121795; E25.949343) and used to be a sulfur mine in the past. The name comes from the fact that CO_2 gas fills the entrance of the cave almost completely, making this cave a death trap, though inside the cave there is a high point located above the gaseous interface. The walls of Killer Cave are not covered by sulfur, except for its deeper sections, thus there is no warning for the toxic area. Bats often use the upper sections of the cave, which are filled with air, but sometimes fly under the redox interface, and fall victim to the toxic gas.

Vertical Cave is situated at 1,072 m altitude (N46.121703; E25.949398) and is located close

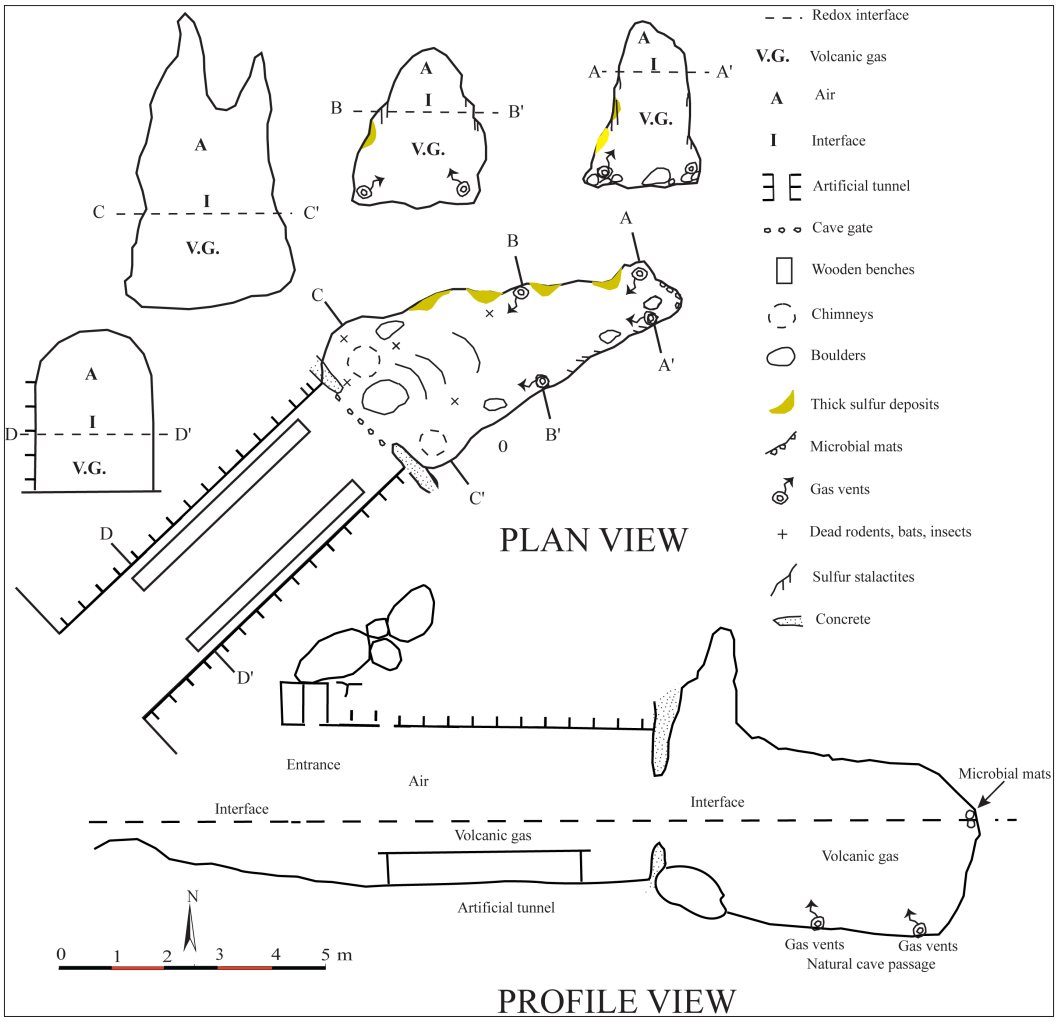

Fig. 2. Map of Sulfur Cave from Puturosu Mountain (Romania).

to Killer Cave. It is filled with CO_2 rich gasses, its entrance is relatively narrow, its depth appears to be approximately 7 m, and its lower sections are yet to be explored.

MINERALOGY

The most important minerals detected previously in Sulfur Cave are Alum-(K) (potassium alum; $KAl(SO_4)_2{\cdot}12H_2O$, cubic) and sulfur (Brem, 1955; Nedopaca, 1982; Onac, 2003; Szakáll et al., 2006; Szakáll et al., 2010). Sulfur (S_8, predominantly orthorhombic) was reported in both Sulfur Cave and Alum Cave (Fridvaldszky, 1767; Orbán, 1869), but also in Killer Cave, Small Cave, and Vertical Cave. Sulfur is likely an oxidation product from the emitted H_2S present in the volcanic gasses. Other minerals described from this region are alunogen, celestine, cristobalite, gypsum, halotrichite, pickeringite and tamarugite.

Koch (1884) reported the presence of alum in several acidic springs near Sulfur Cave and Alum Cave. Alunogen ($Al_2(SO_4)_3(H_2O)_{12}{\cdot}5H_2O$, triclinic) forms transparent flakes of sub-millimeter size or lose aggregates (Szakáll et al., 2010). Celestine ($SrSO_4$, orthorhombic) was identified as very small (0.1 mm) prismatic colorless crystals in Alum Cave (Szakáll et al., 2006). Cristobalite (SiO_2 tetragonal) is present as transparent glossy crusts surrounding rocks in Alum Cave (Szakáll et al., 2006). Although it should be a common mineral in sulfuric acid environments, the presence of gypsum ($Ca(SO_4){\cdot}2H_2O$, monoclinic) in these caves was only reported relatively recently (Szakáll et al., 2006, 2009). In Alum Cave fibrous gypsum is present as aggregates of acicular crystals (Szakáll et al., 2006, 2009). Halotrichite ($Fe^{2+}Al_2(SO_4)_4{\cdot}22H_2O$, monoclinic) was found in Alum Cave (Szakáll et al., 2006; Kristály & Szakáll, 2013) and we assumed that it is what Fridvaldszky (1767) had reported as "alumine" from Puturosu Mt. Pickeringite ($MgAl_2(SO_4)_4{\cdot}22H_2O$, monoclinic) was described from Alum Cave as silky aggregates (Szakáll et al., 2010). Tamarugite, $NaAl(SO_4)_2{\cdot}6H_2O$, monoclinic is found in Alum Cave as micrometer-size tabular crystals (Szakáll et al., 2006, 2009).

MATERIALS AND METHODS

Mineralogy

Samples of mineral deposits from Sulfur Cave were analyzed by X-ray powder diffraction analysis using a Bruker D8 Advance powder diffractometer with Bragg-Brentano geometry. The diffractometer used a cobalt anode ($CoK\alpha_1$ with λ = 1.78897 Å and $CoK\alpha_2$ with λ = 1.79285 Å) and the Kβ line was filtered with a 0.01-mm iron foil. The instrument was operated at 35 kV and 40 mA, and the diffracted X-rays were registered by a one-dimensional LynxEye detector.

Fig. 3. Images from Sulfur Cave (Romania). a) The floor and the lower part of the walls in the deep section of the cave are covered with yellow sulfur deposits. Their upper limit indicates the approximate position of the gas/gas redox interface (gas chemocline). Microbial communities colonize the cave walls at the level of the redox interface; b) Smoke released in the entrance area of the cave helps visualize the location of the gas/gas interface. The location of the gas/gas interface is also indicated by the abrupt ending of sulfur deposits on the cave wall. Oxygen is present above the interface, but absent below it, where CO_2 is approximately 96%.

The scanning 2θ angle range was between 5 and 64°, with a step size of 0.02° (2θ) and a measuring time of 0.5-2 s per step. The alignment of the diffractometer's goniometer was verified using the NIST SRM1976a (corundum) standard. The DifracEva software (Bruker Corporation) was used for mineral phase identification using the International Centre for Diffraction Data Powder Diffraction Files (ICDD PDF) database.

XRD bulk analysis

Semi-Quantitative X-ray diffraction (XRD) analyses were conducted to determine the composition and relative abundance of the bulk sediments. The samples were dried at 70°C for 48 hours, ground in a bead-mill to a particle size < 1-5 µm and measured using a Bruker D8 Advance diffractometer equipped with an X-ray Cu source at the Centre de Diffractométrie of the University Lyon 1, France. Disoriented measurements were made over a 2Theta range of 3° to 70°. XRD patterns were analyzed using the Bruker DIFFRAC.SUITE EVA software. Mineralogical fits were performed by comparing D-spacing values to those of minerals listed in the International Center for Diffraction Data database and the Crystallography Open Database (Kabekkodu et al., 2002; Gražulis et al., 2009). Basic mineralogy and crystallinity were derived from the analyses. Mineral abundance was determined as weight percent (wt. %) using the Rietveld method, with a 10 to 20% accuracy.

Scanning Electron Microscopy

Samples of biofilm and microbially-colonized rocks were collected from the walls of Sulfur Cave in September 2016 and stored at -20°C for subsequent SEM analyses. Biofilm samples were attached and allowed to air dry on 0.2 µm membrane filters, and subsequently fixed for 24 hours at 4°C in a 2.5% glutaraldehyde solution. All samples were then subjected to an ethanol dehydration series with a final wash concentration of 100% ethanol. After dehydration, samples underwent critical-point drying (Autosamdri 815, Tousimis, Rockville, MD) and sputter coated with ~3 nm of Pd (Sputter Coater 108, Cressington, Watford, UK). SEM images were obtained using a JEOL 7001F FEG SEM instrument.

Stable isotope analyses

Stable isotope analyses were performed at the Center for Stable Isotopes of the University of New Mexico. Carbon isotope ratios in gas samples were measured by headspace analysis using a Thermo Fisher Scientific Gasbench II coupled to a Delta Plus Isotope Ratio Mass Spectrometer. Calibration was performed using CO_2 resulting from the reaction of a carbonate standard (NBS 19) with phosphoric acid at 50°C. Nitrogen, carbon and sulfur isotope ratios were measured by continuous flow isotope ratio mass spectrometry using a Costech ECS 4010 Elemental Analyzer coupled to a Thermo Fisher Scientific Delta V Advantage mass spectrometer via a CONFLO IV interface. Sulfur isotope measurements were performed using the method of Fry et al. (2002). Isotope ratios are reported using the standard delta (δ) notation relative to V-AIR for nitrogen, the Vienna Pee Dee belemnite (V-PDB) for carbon and the Canyon Diablo troilite (CDT) for sulfur. Average analytical precision, based on routine analysis of a laboratory standard was better than 0.1‰ (1 σ) for both $\delta^{15}N$ and $\delta^{13}C$ and 0.2‰ for $\delta^{34}S$.

Microbial sampling and fungi cultures

Microbial samples for DNA analysis were collected from Sulfur Cave during two field trips in May and September of 2016. During the May fieldtrip we have collected samples from the cave wall at the gas/gas interface using sterile tubes and spatulas. These samples (Interface 1–3) were shipped to the Vrije Universiteit in Amsterdam and stored at -20°C or 4°C until further processing. During the September fieldtrip, samples (Interface 4) were collected from the oxic/anoxic biofilm as well as from directly above and directly below the interface biofilm. These samples were stored in 96% ethanol during transport and stored at -20°C until further processing. To isolate fungi we have inoculated three different growth media: agar plates with LB, GYM and HDM. The same type of fungi (from the genus *Acidomyces*) was obtained on all culture media.

DNA extraction and quantification

DNA was extracted using the MO BIO powersoil extraction kit (MoBio, Carslbad, USA) following the

manufacturer's instructions. Procedural blanks were incorporated alongside the samples during the extraction process. Concentrations of the extracts were determined using a Quant-iT high-sensitivity DNA assay kit and a Qubit® fluorometer (Invitrogen, Carlsbad, USA). DNA extracts were stored at -20°C until further processing.

DNA sequencing and sequence processing

The samples analyzed in this study were sequenced in parallel with over a hundred other unrelated samples resulting in a total of ±14 million reads using the Illumina sequencing platform. The four interface samples combined are composed of ~400.000 reads (varying from 32.000 to 194.000 reads). Pre-processing of the samples, was done separately in order to minimize the possibility of cross contamination. *Mycobacterium* sequences were not detected in other unrelated samples, and thus are not an artifact. PCR reactions were performed in triplicate using Phusion Green Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Sweden). We targeted the V3-V4 region of the 16S rRNA gene, using the V3 forward primer S-D-Bact-0341-b-S-17, 5'-CCTACGGGNGGCWGCAG-3 (Herlemann et al., 2011), and the V4 reverse primer S-D-Bact-0785-a-A-21, 5'-GACTACHVGGGTATCTAATCC-3' (Muyzer et al., 1993), giving rise to ~430 bp long dsDNA fragments. For fungal amplicons we have used the primers Euk528 (575–590) 5'-CGGTAATTCCAGCTCC-3' and Euk690R (896–916) 5'-ATCCAAGAATTTCACCTCTGA-3', giving rise to ~302 bp long dsDNA fragments. The primers were dual barcoded and were compatible with Illumina sequencing platforms as described previously (Caporaso et al., 2011). Performance of the PCR reaction was checked by running incorporated positive and negative controls from each triplicate plate on 1.5% (w/v) agarose gels. Triplicate PCR products were combined and each combined triplicate sample was purified using SPRI beads (Agencourt® AMPure® XP, Beckman Coulter, CA, USA). The DNA concentration in the purified samples was determined as described above. Samples were diluted to identical concentrations of 2 ng/µl prior to pooling the diluted PCR products together in equal volumes (10 µl) in one composite sample (including positive and negative controls). Samples were also taken from above and below the redox interface in Sulfur Cave, but did not reach sufficient product after amplification and thus were added undiluted to the pool. The number of reads of these samples was similar to those of the procedural blanks and negative controls, reflecting the low prokaryotic DNA content in these samples.

The composite samples were paired-end sequenced at the Vrije Universiteit Amsterdam Medical Center (Amsterdam, The Netherlands) on a MiSeq Desktop Sequencer with a 600-cycle MiSeq Reagent Kit v3 (Illumina) according to manufacturer's instructions. High-throughput sequencing raw data were demultiplexed using bcl2fastq software version 1.8.4 (Illumina) and primers were trimmed using Cutadapt (Martin, 2011). Demultiplexed samples were further processed using a modified version of the Brazilian Microbiome Project 16S profiling analysis pipeline (Pylro et al., 2014). Paired-end reads were joined using PANDAseq (Masella et al., 2012) allowing for a minimum overlap of 10 nucleotides between the forward and reverse reads, a minimum sequence length of 285 and no mismatches in the primer region were allowed. PANDASeq addresses mismatches in overlapping regions by selecting the nucleotide with the best sequencer-assigned quality score. Because PANDAseq incorporates a base quality filter during read assembling, the threshold for consecutive high quality bases per read was set to zero. Metadata and demultiplexed samples were merged using add_qiime_labels.py (Caporaso et al., 2010) and sequence headers were changed using bmp-Qiime2Uparse.pl (Pylro et al., 2014). UPARSE was used to dereplicate, filter chimeras, discard OTUs detected less than 2 times and OTU clustering at 97% similarity (Edgar, 2010, 2013). The OTU taxonomy was assigned using the UCLUST algorithm (Edgar, 2010) on QIIME (Caporaso et al., 2010) using SILVA compatible taxonomy mapping files (Silva database release 128) (Quast et al., 2013, Yilmaz et al., 2014) and aligned using align_seqs.py in QIIME (Caporaso et al., 2009). Taxonomy was manually curated and refined up to genus level based on 97% similarity of reference sequences. The reference tree was calculated using FastTree 2 (Price et al., 2010). We generated a BIOM file using make_otu_table.py on QIIME (Caporaso et al., 2010). Prior to further analysis we produced an OTU table and a taxonomy table using BIOM scripts (McDonald et al., 2012). The OTUs detected in negative controls and procedural blanks were manually removed from the dataset.

RESULTS

Mineralogy

In Sulfur Cave, sulfur deposits are always present below the oxic:anoxic interface as a continuous layer covering the cave walls and the cave floors, while above the CO_2-H_2S:O_2 gas/gas interface the S° deposits are absent (Fig. 3a). There are also no sulfur deposits around the gas vents located on the cave floor most probably for lack of oxygen. In some areas of the cave, walls are covered by 5-20-cm-thick sulfur deposits (Fig. 4a, b), while in other area these deposits are thin (< 1 mm). Water films and water droplets found on the cave walls in S°-rich areas near the interface are very acidic (pH ≈ 0.5 to 1).

The sulfur from Sulfur Cave was shown to be of various types. The most common type is an earthy aggregate of a powdery deposit with micron-sized yellow or pale yellow fine acicular crystals. Some crystals are tens of microns long (Fig. 4c, d) with a crystallite size of 860 Å (based on analysis using the program DifracEva). Twinning behavior ([001], 90°) of sulfur crystals is common in the sulfur deposit situated below the gas/gas redox interface from Sulfur Cave (Fig. 4e, f) and less frequent at the gas/gas interface where mostly orthorhombic crystals are

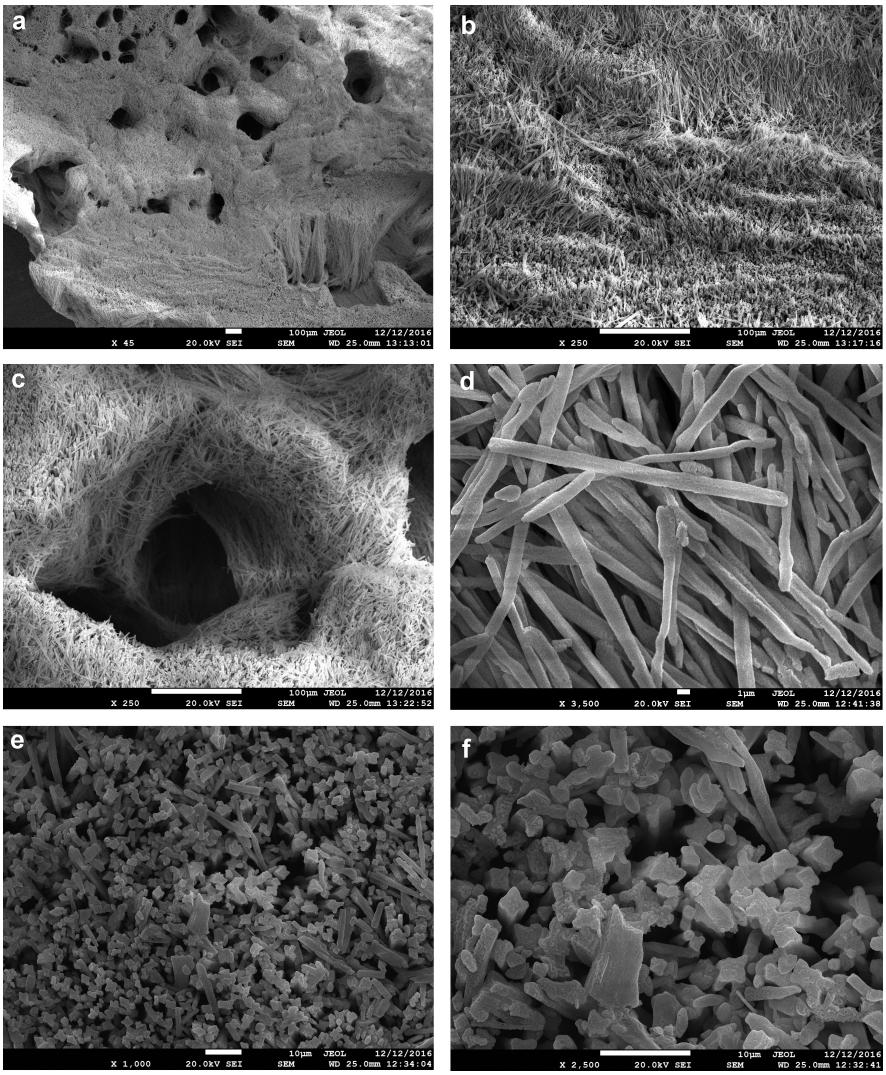

Fig. 4. SEM images of acicular sulfur deposits in Sulfur Cave (Romania). a) General view of a thick sulfur deposit observed from above at 45x magnification; b) detail of fibrous crystals from (a) at 250x magnification; c) details of (a) showing sulfur fibers distributed around holes and tubes at 250x magnification; d) detail of micron-sized sulfur fibers from (c) at 3,500x magnification; e) deposit from Sulfur Cave showing frequent twinning of sulfur crystals, at 1,000x magnification; f) detailed view of twinning sulfur crystals from (e) at 2,500x magnification.

seen. We used the WinXMorph program (Kaminsky, 2005; 2007) to model this twinning (Fig. 5). EDAX-SEM analysis confirmed that the thick yellow deposits from Sulfur Cave (Fig. 4) are dominated by sulfur. Also, X-ray diffraction analysis has confirmed that the thick wall deposits from Sulfur Cave (Fig. 6) are in fact sulfur crystals with a crystallinity index of 82%. Based on Rietveld analysis of the diffraction data using the FullProf Suite (Rodríguez-Carvajal, 1993; Roisnel and Rodriguez-Carvajal, 2000) and MAUD (Lutterotti et al., 2007) programs, the unit cell parameters for the sulfur crystals are: a = 10.462 Å; b = 12.865 Å; c = 24.497 Å; α = 90°; β = 90°; and γ = 90°, almost identical to those published by Rettig and Trotter (1987).

Besides sulfur, a very small amount of gypsum is present as transparent sub-millimeter-sized crystals on the surface of the sulfur deposit. The presence of this mineral was also confirmed by X-ray diffraction analysis.

Gas chemical composition and gas flux

The published gas analyses from Sulfur Cave are listed in Table 1. The CO_2 concentration is in the range of 96.7 to 98.2%, followed by other gases, here including N_2, CH_4, H_2, and H_2S. Noble gases (He, Ne, and Ar) also occur in low amounts. The differences in the gas composition are confined to a narrow range; they can be due to natural variations or to some extent, to the different sampling/analytical methods that have been used by the respective authors. Compared to the works of Frunzeti (2013), Vaselli et al. (2002), and Althaus et al. (2000) the data for carbon dioxide show similar values, while the other gases show small differences, which could be due to sampling strategy. Higher values were detected for N_2 and CH_4 by Frunzeti 2013, than the values reported by Althaus et al. (2000), and Vaselli et al. (2002).

Based on the measurements of Kis et al. 2017, the total output from Sulfur Cave is approximated to 1.92×10^6 kg y^{-1} CO_2. The Sulfur Cave together with other high emission sites from the neighboring area show a total output derived from soil degassing and focused emissions of 8.70×10^6 kg y^{-1} CO_2. The highest CO_2 gas fluxes at Ciomadul were found at the periphery of the youngest volcanic complex, at the intersection of the older lava domes, Puturosu and Bálványos (500-600 ky). The gas emissions are assumed to be controlled by tectonic features, like fractures or faults (Kis et al., 2017). Nevertheless, the locations of the strongest outgassing do not coincide with the youngest eruption centers of Ciomadul, but they are in a peripheral occurrence, like Puturosu Mt., where the caves are located. Compared to other locations through Europe, one may observe that the CO_2 emissions from Ciomadul complex are in the same order of magnitude as in the case of other European volcanic structures of similar age (Kis et al., 2017; Caracausi et al., 2015).

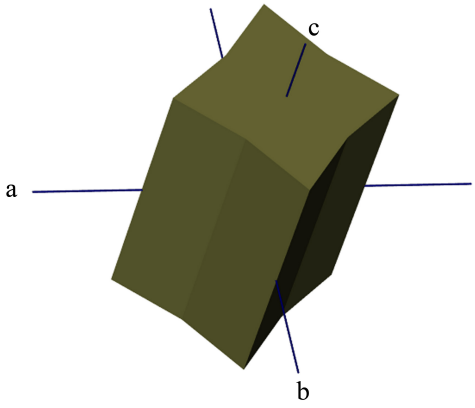

Fig. 5. Model of twinning in a sulfur crystal ([001], 90°) using the WinXMorph software. SEM images of twinning sulfur crystals from Sulfur Cave are shown in Fig 4f.

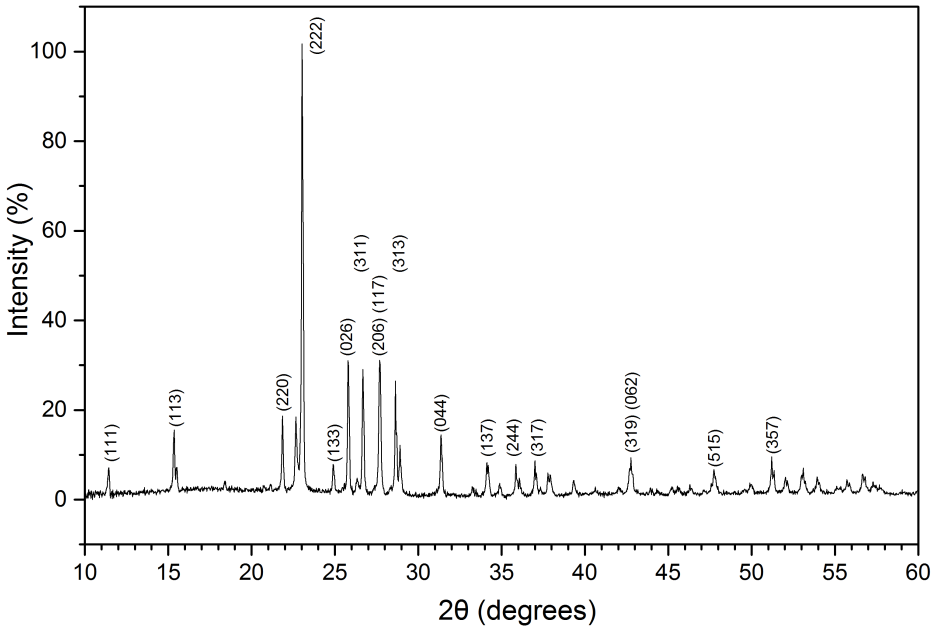

Fig. 6. XRD pattern of a sample collected from the sulfur deposits below the interface in Sulfur Cave. The sample has a crystallinity index of 82%.

Stable isotopes

The $\delta^{13}C$ values of the CO_2 in the gas samples collected from caves in the Ciomadul area range between -3.4 and -2.5‰ and are summarized in Table 2. The sampling location for measuring the $\delta^{13}C$ values for the CO_2 is the bottom of the cave at the vents indicated on the cave map profile, where no significant contamination is expected to occur from outside air.

The nitrogen and carbon elemental and isotopic composition of the microbial mat samples collected from the cave walls are reported in Table 3 and vary between -2.8 and 1.5‰ for $\delta^{15}N$ and between -26.8 and -31.9‰ for $\delta^{13}C$, with most of the $\delta^{13}C$ values (7 out of 8) in a very narrow range at around -31.2‰. The C/N ratios range between 10 and 15. The sulfur isotopic composition of two samples collected from the walls of Sulfur Cave in association with microbial mats display $\delta^{34}S$ values of -7.28 and -5.55‰.

Microbial communities in Sulfur Cave

Samples for microbial analysis were collected from the deepest sections of the cave at the interface level (Fig. 7a, b).

The 16S rDNA reads of four samples (collected in 2016 and 2017) from the gas/gas interface biofilm from Sulfur Cave were analyzed to genus level (97% similarity) and the relative abundances of the dominant phylotypes in each sample are summarized in Fig. 8 and Supplemental Table 1. For the four samples a total of 407,426 reads were obtained (after removal of contaminating sequences that were present in the negative controls and extraction blanks). The overall microbial diversity in the samples is low, with less than 50 genera detected and a total of 62 OTUs. Among them, only a few are abundant, which can be explained by the unusual and extreme conditions of the Sulfur Cave environment such as local anaerobiosis, high CO_2 levels, extremely low pH

Table 2. $\delta^{13}C$ values of the CO_2 gas from caves in the Ciomadul area.

Location	Collection date	$\delta^{13}C$ (‰)	%CO_2	Source of data
Sulfur Cave	September 10, 2016	-2.8	n/a	this study
Sulfur Cave	February 23, 2014	-3.2	96.52	this study
Sulfur Cave	n/a	-3.2	96.8	Vaselli et al. (2002)
Sulfur Cave	September 10, 2016	-2.5	n/a	this study
Alum Cave	February 23, 2014	-3.4	96.77	this study
Killer Cave	February 23, 2014	-3.2	94.46	this study

Table 3. Nitrogen and carbon elemental and isotopic composition of eight replicates from the microbial biofilm at the redox gas/gas interface in Sulfur Cave.

Sample	%N	$\delta^{15}N$ (‰)	%C	$\delta^{13}C$ (‰)	C/N
Wall-Biofilm 1	1.9	0.7	22.1	-31.2	11.6
Wall-Biofilm 2	1.0	-2.8	15.7	-30.6	15.1
Wall-Biofilm 3	2.9	1.6	31.7	-31.9	10.8
Wall-Biofilm 4	2.3	0.8	22.1	-31.3	9.8
Wall-Biofilm 5	2.0	1.00	28.0	-30.3	13.9
Wall-Biofilm 6	3.3	-0.8	34.9	-26.8	10.6
Wall-Biofilm 7	3.3	0.5	41.5	-31.5	12.5
Wall-Biofilm 8	3.2	0.5	39.7	-31.5	12.3

and absence of sunlight. For fungi identification we have isolated colonies on organotrophic plates and then sequenced PCR-amplified DNA using fungal 18S rDNA primers. We have also repeated this sequencing on DNA extracted from the microbial community at the gas/gas interface. Images of fungal isolates and hyphae were taken by optical microscopy.

The microbial community making up the biofilm on the Sulfur Cave wall at the gas/gas interface is dominated by species from the genus *Mycobacterium*, which contributes between 70 and almost 100% of the reads found in the biofilm samples. While limited biodiversity is seen in the interface samples, the overwhelming dominance of the *Mycobacterium* taxa may suggest that a new habitat has been occupied by this group. Most of the other sequences detected belong to known acid-tolerant/acidophilic microbial species, expected to be found in such environment, including some capable of autotrophic growth by oxidizing iron and/or sulfur compounds (i.e., Ferroplasmaceae and *Acidithiobacillus*) (Kelly & Wood, 2000; Dopson et al., 2004; Golyshina & Timmis, 2005). Members of the *Acidithiobacillus* genus are known to produce sulfuric acid as a result of sulfur oxidation (Kempner, 1966) and may therefore contribute to the acidification of the water film on the cave wall and the deposition of sulfur on the acicular crystals (Fig. 4).

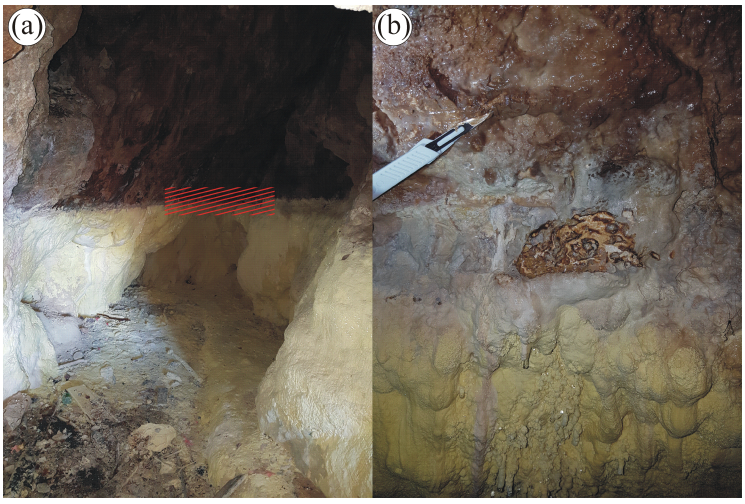

Fig. 7. Sampling location and morphology of the microbial biofilms in Sulfur Cave. a) Samples were collected at the interface in the deeper sections of the cave (approximate location highlighted in red); b) A close-up of a biofilm at the interface after a sample was collected.

Acid-fast staining (which is highly selective for Mycobacteria) was performed on biofilm samples from the gas/gas interface in order to eliminate the suspicion that the high abundance of Mycobacteria may be a PCR amplification bias. Optical microscopy observations of the acid-fast stained samples confirmed that *Mycobacterium* species dominate the microbial community in terms of biomass (Fig. 9). The morphology of the dominant cells in the biofilm (Fig. 10) also fits with that of Mycobacteria. Lastly, the Mycobacteria putative cells from Sulfur Cave also shown Mycobacteria-characteristic ridges left after cell division (image not shown).

Microbial samples were collected in 2017 from above and below the interface. These samples contained low biomass and low numbers of reads comparable to the negative controls and extraction blanks (sample from above the interface had only 30 reads and the sample taken from below the interface had 470 reads after removal of contaminating sequences). Although the number of reads in these samples was low, the dominant phylotypes belonged to *Mycobacterium* (above the interface), *Acidithiobacillus* (below the interface), and *Acidomyces* (at the interface, Fig. 11). The main lesson we draw from the large

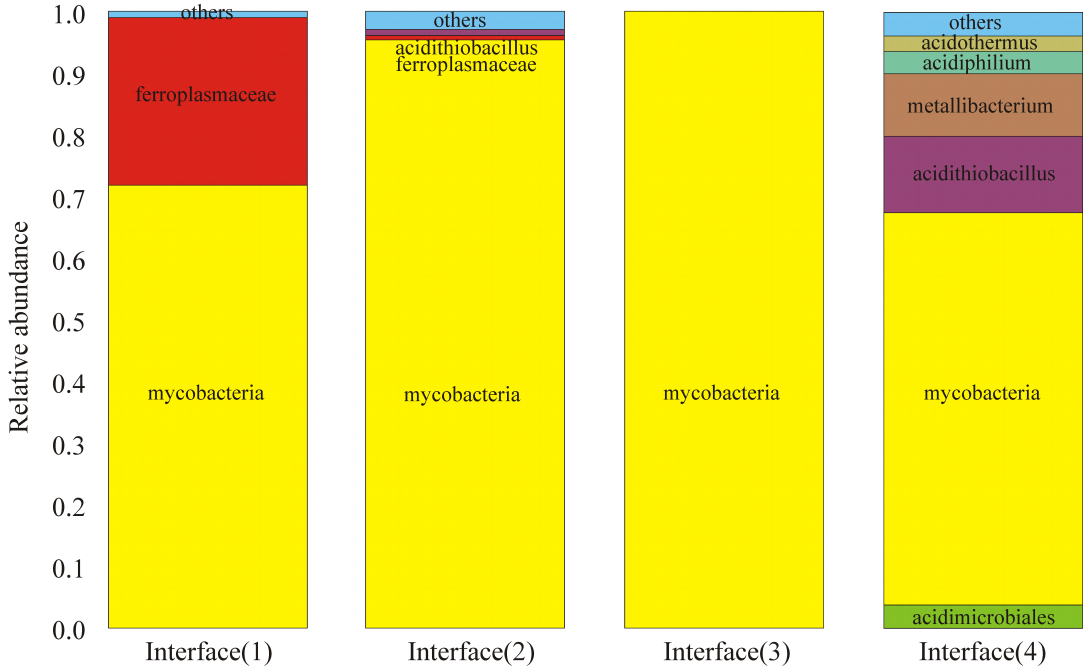

Fig. 8. Relative abundances of the dominant phylotypes in the biofilm samples grouped by genus, family or order in six samples from Sulfur Cave. Three independent samples of biofilm situated on the cave wall at the gas/gas interface have been collected in May 2016. These are called “Interface (1)”, “Interface (2)”, and “Interface (3)”. Another biofilm sample called “Interface (4)” was collected from the same in September 2016. Only the most abundant phylotypes are shown. The abundance of all phylotypes from Sulfur Cave is given in Supplemental Table 1.

difference in reads between the samples from the interface and those taken from above and from below it, is that the biomass in the cave is found predominantly at the level of the redox interface.

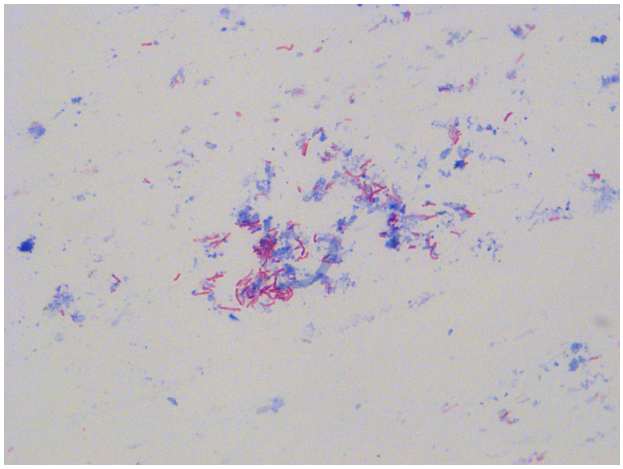

Fig. 9. Optical microscopy images of a biofilm sample from the interface that was subjected to acid-fast staining. Acid-fast positive cells stain pink.

DISCUSSIONS AND CONCLUSIONS

Mineralogy

Twinning on {101}, {011}, and {110} is generally rare in sulfur crystals. However, twinning ([001], 90°) is very abundant in some microenvironments from Sulfur Cave (Fig. 4e, f, and Fig. 5) and this occurrence requires further studies. Based on XRD data, these sulfur crystals are the low temperature (α) form of S_8 from the orthorhombic system. The minerals described by Szakáll et al. (2006) and later by Kristály and Szakáll (2013) have been analyzed by XRD and SEM, yet several questions remain unanswered about how the geochemical or potentially biological processes responsible for the formation of the various minerals observed are controlled by pH, redox conditions, gas concentrations, cave ventilation or potentially biological activity.

Stable isotopes

The relatively high $\delta^{13}C$ values for the CO_2 gas samples from Sulfur Cave (-2.8 and -2.5‰) are similar with the values measured in other caves from the Ciomadul area (Table 1; Vaselli et al., 2002; Althaus et al., 2000; Frunzeti, 2013). Vaselli et al. (2002) reported carbon isotope values of CO_2 gas derived from natural springs and dry volcanic moffetes from the inner part of the Eastern Carpathians, including the Ciomadul area, and showed that most of the $\delta^{13}C$ values lie within a relatively narrow interval ranging between -2.1 and -4.7‰. They suggest a deep crustal origin of the gases from a mixture of mantle derived CO_2 and CO_2 resulting from alteration and/or metamorphism of marine carbonates. Our carbon isotope results are within the range reported by these authors, as well as other studies in similar geologic settings (see review in Fischer & Chiodini, 2015). Similar isotopic compositions were reported for other volcanic and non-volcanic regions worldwide (Tedesco et al., 2010; Barry et al., 2013, Ruzié et al., 2013, Oppenheimer et al., 2014; Rizzo et al., 2014; Mason et al., 2017 and references therein), giving a mean global volcanic carbon isotopic composition ranging between -3.8 and -4.6‰ (Mason et al., 2017). Considering the neighboring areas within the Carpathian Region, the Pannonian Basin, Cornides, (1993) reported an average $\delta^{13}C_{CO2}$ composition of -5‰, inferring "magmatic" origin, Palcsu et al. (2014) reported -3.3 to -2.1‰ from wells from the Pannonian Basin suggesting interaction between a magmatic and a crustal component, Bräuer et al. (2016) reported values of -3.5 to -7.5‰ for the westernmost Pannonian Basin, suggesting evidence for active litospheric mantle degassing. Our data show slight ^{13}C-enrichment comparable to the regional carbon isotopic compositions, suggesting a deep origin of the gases derived from a mixture of mantle CO_2 and CO_2 resulting from alteration and/or metamorphism of marine carbonates.

Assuming a -30‰ fractionation between the inorganic carbon and the microbial mat (Sarbu et al., 1996), the stable isotope values for carbon and nitrogen in the microbial biofilms from the cave walls at the level of the redox interface (Table 2) are consistent with the CO_2-rich gases vented in Sulfur Cave serving as a main carbon source. The C/N ratios resemble those reported from biofilms consisting of chemoautotrophic microbes, found in other sulfidic cave environments (Engel et al., 2004).

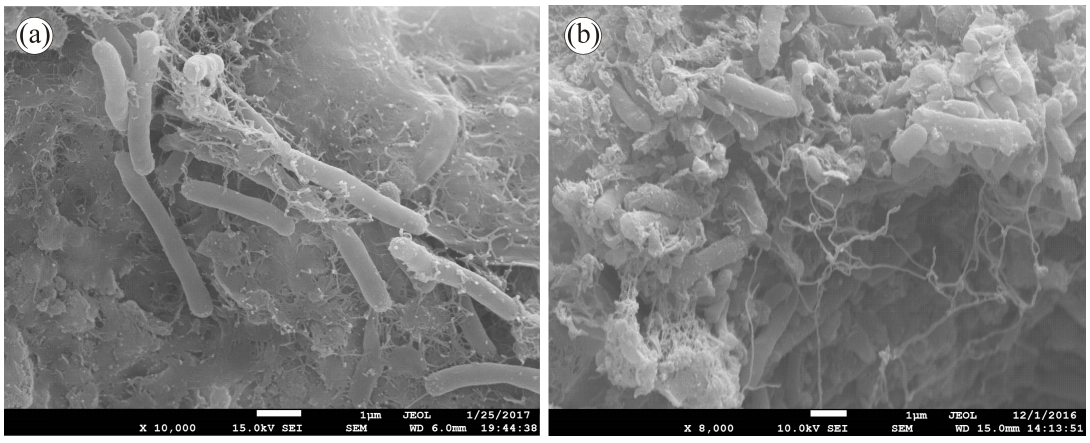

Fig. 10. SEM images of microorganisms growing in Sulfur Cave at the gas/gas redox interface; a) Cells colonizing mineral surfaces coated with elemental sulfur; b) Cross section of a microbial mat from the rock walls of Sulfur Cave at the redox interface.

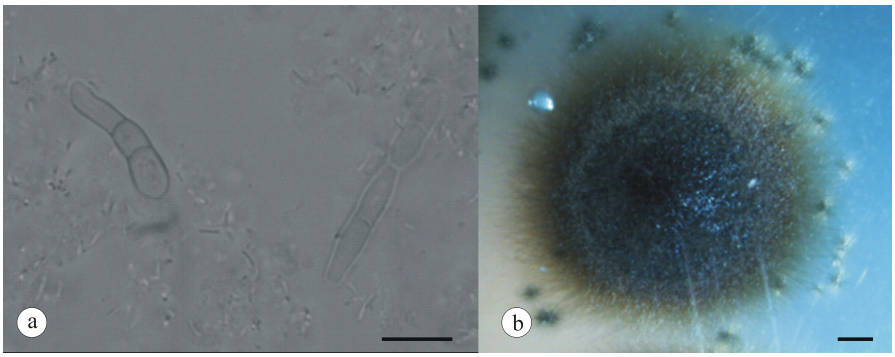

Fig. 11. Optical microscopy images of fungi from the microbial community situated at the CO_2-H_2S:O_2 gas/gas interface in Sulfur Cave. Image of hyphae (putatively *Acidomyces*) from the microbial community in Sulfur Cave (size bar = 10 µm). Colony of fungi from the genus *Acidomyces* isolated from the gas/gas interface community in Sulfur Cave (size bar = 10 mm).

The $\delta^{34}S$ values (-7.3 and -5.6‰) in the sulfur wall deposits are typical of reduced sulfur species with a possible volcanic origin. The sulfur isotopic composition of the H_2S gas on the Puturosu Mt. has not been measured.

Microbial communities

Stable isotope values (Table 1) indicate that the organic carbon within the microbial biofilm from Sulfur Cave is of autotrophic origin. Apart from *Mycobacterium*, the other phylotypes we have found, are known acidophiles: *Acidithiobacillus* (Bacteria), Ferroplasmaceae (Archaea), and *Acidomyces* (Fungi). Several of them can grow chemo(litho)autotrophically as well (i.e., *Acidithiobacillus*, Ferroplasmaceae), are (facultative) anaerobes (i.e., Ferroplasmaceae, *Acidothiobacillus, Metallibacterium*), and/or are capable of oxidizing sulfur compounds or iron in the presence of oxygen. In the absence of oxygen, alternative electron acceptors such as ferric iron (Fe^{3+}) or nitrate (NO_3^-) may be utilized by several community members (i.e., Ferroplasmaceae and *Acidithiobacillus*, respectively) (Baalsrud & Baalsrud, 1954; Golyshina & Timmis, 2005). Ferroplasmaceae, *Acidithiobacillus* and the fungus *Acidomyces* found in this system are known acidotolerants. Also, eukaryotic organisms can be found in this biofilm, most notably a fungus belonging to the genus *Acidomyces*, members from this genus can be isolated from extremely acidic soils and mine drainages and notably also from sulfur-containing soils. These fungi also show melanisation of their cell walls, which probably makes them more resistant to environmental stress.

The bacterium that dominates, by mass and number of sequences, the microbial biofilm growing on the walls of Sulfur Cave at the gas/gas redox interface is a member of the genus *Mycobacterium*. Mycobacteria are known for their potential to survive in extreme conditions. Their dominance in this community is surprising and suggests that they have adopted a life style in this particularly exceptional niche at pH less than 1, to survive and propagate not only with limited available carbon and free energy sources, but also at low humidity. One species of Mycobacteria was shown to grow chemolithoautotrophically by oxidizing elemental sulfur (Kusumi et al., 2011). Yet, because the vast majority of *Mycobacterium* species are heterotrophic, and in the absence of direct physiological observations, the role of this microorganism in the Sulfur Cave community cannot be speculated upon at this point.

Phylotypes belonging to the order of Rhizobiales and the family of Beijerinckiaceae were also found in the biofilm and could potentially be important in providing the community with a nitrogen source by N_2-fixation. Another interesting member of the Sulfur Cave microbial community was a species of the genus *Metallibacterium*. A recently described and widespread facultatively anaerobic, acid-tolerant, iron-reducing member of this genus (*M. scheffleri*) was shown to possess genes involved in sulfur oxidation and was capable to produce ammonium through protein (casein) consumption, thereby raising the pH in its proximate vicinity (Ziegler et al., 2013; Bartsch et al., 2017). Under acidic conditions ammonium is protonated to ammonia which can in turn serve as an easily accessible nitrogen source for other community members such as Mycobacteria.

The exact composition, function, metabolic properties and interactions between species of the Sulfur Cave microbial community remain to be elucidated. Presently, we speculate that Sulfur Cave contains an unusual community with a primary free energy transduction mode based on chemolitho-autotrophic growth. Free energy sources for the microbial community include sulfur deposits on the cave wall and the H_2S from the mofettic gas. Based on phylogenetic relatedness biomass formation in some of these species may occur via RuBisCO-based CO_2 fixation. Next to sulfur oxidation, iron oxidation may occur as well, by dedicated microorganisms such as Ferroplasmaceaea, as well as oxidation of H_2S and S° with ferric iron, if iron becomes available from the weathering of bedrock minerals. Given the presence of residual methane in the Sulfur Cave (above the atmospheric background) the presence of methanotrophic activity is also possible. Yet, so far we have not identified any 16S sequences indicative of classical methanotrophs. *In situ* carbon and nitrogen fixation by species in the biofilm is suggested by the sequence data, as well as by the stable isotope data (Sarbu et al., 1996). The bedrock minerals from Sulfur Cave may provide the microbial community with other essential nutrients and metals such as phosphorus, magnesium, and manganese that are not available in the gas phase.

Sulfur oxidation has been observed in caves, such as Movile (Sarbu et al., 1996), Frasassi (Sarbu et al., 2000) and Ayyalon (Por et al., 2013) but in all of these cases the H_2S is dissolved in water and, as such, flows into these caves. Establishing a rich microbial community inhabiting a CO_2-H_2S:O_2 gas/gas interface in an aphotic environment and with CO_2 and H_2S brought in by mofettic emissions is, so far, unique to Sulfur Cave.

The discovery of abundant biofilms in Sulfur Cave is, to our knowledge, the first report of a non-

aquatic gas/gas interface (i.e., with energy from a redox gas chemocline) in a volcanic zone, used by chemoautotrophic microorganisms to fix carbon and nitrogen underground.

Astrobiology

Our findings are particularly interesting in the context of the search for life on Mars, where possible volcanic cave skylights have been reported (Cushing et al., 2007). These could provide access to subsurface environments similar to the one reported here. As for water, arguments have been put forward for the stability of adsorbed water and thin liquid films on the Martian surface (Boxe et al., 2012). In terms of life on Mars, the discovery reported here may be used as evidence that an environment with geological energy inputs (from volcanic gases and mineral surfaces) could sequester water and become conducive to the establishment and maintenance of microbial communities. Although electron donors may be abundant in the subsurface, the limiting factor for life in certain environments might be the availability of water and electron acceptors, as in Sulfur Cave and in almost all sedimentary environments on our own planet (Nealson & Berelson, 2003; Nealson & Popa, 2005). The detection of potential electron acceptors in the Martian regolith such as nitrates, sulfates and abundant ferric iron (Gendrin et al., 2005; Stern et al., 2015) are therefore important findings when considering the potential for life on Mars.

ACKNOWLEDGEMENTS

We acknowledge help with logistics and field sampling from Vinca Minor Association (Sfântu Gheorghe, Romania), with field trip management from Grand Hotel (Bálványos), with field work and cave sampling from D. Pegulescu, L. Mark, and T. Brad, and for field photography from A. Levandis and A. Lascu. We thank the reviewers for their suggestions.

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