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Investigation of clay sediments and bedrock morphology in caves with seismic traveltime tomography: an application at Alepotrypa Cave (Diros, Greece)

Lazaros Polymenakos
Consultant-Independent Researcher, Athens, Greece

Abstract: The deposition of unconsolidated clay sediments in caves, in relation to the buried morphology of the karstic conduit, are important parameters for the study of cave evolution. We introduce the application of an active seismic imaging technique to investigate the clay deposits and bedrock morphology in caves. Seismic traveltime tomography, applied for the first time in cave studies, can assist with the interpretation of cave geomorphology. Utilizing the P-wave velocity contrast between the clay sediments and the surrounding rock mass, we map the buried rock surface and significant sediment interfaces and provide an estimate of the sediment thickness and volume. Our study focuses on the Alepotrypa Cave located in Diros (Peloponnese, Greece), revealing important information for the evolution of the cave. The proposed technique could be applied in caves with significant clay deposits, in order to constrain the clay volume and reconstruct the buried floor shape of the cave. The technique exploits fully the ground morphology and access points in a cave, so it is suitable for a detailed three-dimensional exploration of cave deposits and the underlying cave morphology.

Keywords: seismic traveltime tomography, P-wave velocity, sediment thickness, bedrock surface, Alepotrypa Cave, Greece

INTRODUCTION

Sedimentary deposits in caves are important for the study of cave evolution and past environmental conditions related to human occupation. As a result, cave sediments have been the focus of many speleogenesis studies (e.g., Ford & Ewers, 1978; Palmer, 2007; Farrant & Smart, 2011). Cave deposits are primarily of clastic type (clays, terra rossa), followed by sediments of chemical or biological origin. Depending on the environmental and depositional conditions, the clay deposits may reach several meters in thickness (Perroux, 2005). These deposits cover the floor of caves, limiting speleogenesis studies to the observation of morphology on ceiling and cave walls, which cannot reveal the dynamics of the cave such as collapse phases or clay evacuation during cave evolution history.

A non-destructive means of studying the sedimentary geology of a cave would provide significant information otherwise inaccessible, while preserving the delicate environmental conditions of the cave. This paper proposes the use of an active geophysical imaging technique that can be applied for studying clay deposits and cave buried morphology complementing other methods of cave speleogenetic exploration.

Geophysical methods commonly used in exploration have potential application to geoenvironmental investigations in karstic terrains and caves (e.g., Chalikakis et al., 2011; Nehmé et al., 2013). Ground penetrating radar (GPR) and electrical resistivity tomography (ERT) can be used to trace caves (e.g., Chamberlain et al., 2000; Gibson et al., 2004; El-Qady et al., 2005; Valois et al., 2010; Gosar & Čeru, 2016), search for discontinuities in limestone rocks and determine soil depth in buried karst terrains (e.g., Zhou et al., 2000; Harvitch & Valenta, 2011; Hamdan et al., 2012) and determine soil thickness in archaeological sites (see e.g., Griffiths & Barker, 1994; Drahor et al., 2008), mines and caves (Kauffman, 2002). Seismic methods can be used for geological mapping and estimating the depth of soft cover layers (e.g., Ibs-Von Seht & Wohlenberg, 1999; Polymenakos & Papamarinopoulos, 2007; Coulouma et al., 2012;
Giocoli et al., 2015) and for the detection of caves (e.g., Surányi et al., 2010). Considering the application of geophysical techniques inside a cave, GPR techniques can provide detailed information on cave sediment stratigraphy, however severe limitations can arise when applied on surficial wet clay layers of a cave, because of the strong signal attenuation in these types of soils (Jessop, 1995). The use of ERT inside caves is capable of providing information related to cave sediment stratigraphy, but its use is hindered due to limited layout deployment and edge effects that can dominate the electrical signals near cave walls (Kauffman, 2002; Nehmé et al., 2013). Passive seismological imaging (PSI; Nehmé et al., 2013) can overcome cave limitations on spatial and depth coverage, but it is subject to inherent limitations in horizontal and vertical resolution.

This paper proposes the use of seismic traveltime tomography as a technique suitable for cave imaging for several reasons: (i) it is a non-destructive procedure adapted to variant relief landscape that can be applied to all sizes of caves without the need for trenching or drilling; (ii) it is an active-source seismic technique that can overcome limits on efficiency and spatial coverage posed by the cave environment on other active-source techniques such as ERT or GPR, and passive-source techniques such as PSI; (iii) this seismic technique is extensively used to discriminate between geological materials that present a velocity contrast such as sediments lying over or in-between rocks, as in the case of clay sediments overlying limestone bedrock in a cave; (iv) the tomography technique provides the most robust, spatially continuous and detailed mapping of seismic wave velocities, in two or three dimensions at various scales, overcoming the limitations of velocity estimation by seismic refraction (Barton, 2007) with increased resolution (Polymenakos & Tweeton, 2015). Typical applications of seismic traveltime tomography include mapping of empty or sediment filled fractures and soft or hard geological materials in underground mines and facilities (e.g., Jackson et al., 1995; Ajo-Franklin et al., 2013), and estimating the geometry and depth of cover sediments in landslides (e.g., Lanz et al., 1998; Samyn et al., 2012; Adamczyk et al., 2013) and archaeological sites (e.g., Witten et al., 1995; Polymenakos et al., 2004; Polymenakos & Papamarinopoulos, 2005; 2007).

Seismic traveltime tomography is used to map clay thickness and bedrock morphology of Alepotrypa Cave. Use of other geophysical techniques for possible validation or complement of the tomography results was inefficient because of hindering local conditions (severe effects of the clay sediments on GPR signal attenuation; strong spatial limitations and surface obstructions on efficient ERT layout deployment and signal penetration). The results will improve observation of the bottom parts of the cave and provide information related to stages of speleogenesis.

Cave description

Alepotrypa Cave is located in Southern Mainland Greece, on the western shore of Mani Peninsula in Peloponnese (Fig. 1). It was discovered in 1958 (Petrochellou, 1992). It is located 15 m above sea level, is 280 m long and has an elongated shape with numerous chambers and passageways. It can be divided in two main areas, the largest of which dimensions 130 x 50 m and has a lake of fresh water (Fig. 2). The cave is one of several karst formations which abound along the western Mani peninsula as a result of post-Miocene tectonic processes that greatly contributed to karstification and cave morphology (Bassiakos, 1993). Post-glacial environmental and geological conditions contributed to an accumulation of paleontological and paleoanthropological finds in the caves and caverns of Mani area, usually within cohesive limestone breccia with terra-rossa as bond material (Giannopoulos, 2000). The cave contained a large Neolithic settlement with thick cultural levels and a continuous human occupation from 8000 BP until abandonment of the site around 5200 BP (Papathanassopoulos, 1996; 2011; Papathanasiou, 2001; Tomkins, 2009). A multidisciplinary research project has been conducted at the site by members of the Diros Project (Parkinson et al., 2016).

The cave is formed in Upper Cretaceous-Eocene semi-crystalline limestones of the Kriti-Mani (Plattenkalk) Unit (Bassiakos, 1993), a medium-to-thick bedded, heavily jointed and karstified carbonate formation with an overall thickness of 300 m. This rock formation is exposed at the walls and roof of the cave dome (Fig. 3a). The speleogenesis of Alepotrypa Cave is not studied in detail so far. However, the primary cause would have been chemical weathering of limestone with the assistance of fresh and brackish water circulation within the cave (a reminder of which is the lake at the eastern end of the cave) as suggested for the neighboring Glyfada Cave-lake (e.g., Giannopoulos, 2000). Rock joints and faults in the rock mass are considered to have greatly contributed to the directional development and shape of the numerous cave sub-areas: the directions of passageways in Alepotrypa Cave are consistent with the principal directions of rock joints in the area.
Seismic traveltime tomography investigations in Alepotrypa Cave, Greece

International Journal of Speleology, 46 (1), 1-12. Tampa, FL (USA) January 2017

Fig. 2. Floor plan of Alepotrypa Cave (modified after Petrocheilou, 1992). The tomography survey area is shown at far left.

Fig. 3. a) Typical interior morphology of Alepotrypa Cave with rock walls, stalagmites and clay sediments; b) Clay/ash interval observed in an excavation pit (for location see Fig. 4). Photos taken by the author in July 2014.

NNW-SSE and E-W (Bassiakos, 1993; see also Fig. 2). The cave morphology would have also been affected by local alterations between crystalline limestone and limestone breccia, the latter appearing as a result of tectonic activity and being more susceptible to erosion. A recent factor affecting speleogenesis could be related to human intervention when transforming it into a show cave, which happened immediately after its discovery. Within the cave there are sediment pouches, the shallow part of which (to a least depth of 5 m from the interior surface) is formed of a dark reddish-brown clay alternating with thin layers of ash (Fig. 3b), deposited by humans (Papathanassopoulos, 2011). This clayey formation is underlain by an unknown sedimentary sequence down to the limestone bedrock. Research on the cave sediments is limited and is presently confined to chemical (Boyd, 2015) and human (Karkanas, 2016) superficial sediment deposition. Questions related to the deposition and depth of clay sediment and the morphology of the underlying bedrock remain open.

The geophysical investigation presented in this paper attempts to address the questions related to the clay deposition and the buried bedrock morphology, providing new information for the interpretation of cave evolution.

SEISMIC TOMOGRAPHY SURVEY

Alepotrypa Cave has large surfaces of clay deposits (exceeding 2000 m$^2$) in the Entrance and Main Chambers (Fig. 2). The question we attempt to answer is to estimate the total thickness and volume of clay deposits in the Entrance Chamber, and image the sediment stratigraphy and the buried bedrock morphology. We address the above question with use of seismic traveltime tomography.
Seismic tomography

Seismic tomography aims at reconstructing the internal characteristics of a medium by exploiting the seismic energy that has traveled through it, from a set of seismic source-receiver pairs that encloses the medium. The reconstruction is made by the tomographic inversion of the measured traveltimes obtained from the set of source-receiver pairs to produce detailed maps of seismic velocity of the medium’s interior. The inversion process iteratively updates an initial velocity model, defined by data analysis and geological information, after comparison of the measured traveltimes with those calculated from the model. The region between source and receiver positions is divided into cells and modifications of the initial model that minimize the differences between calculated and observed traveltimes are sought in every iteration. The inversion result is the velocity pattern that minimizes the total differences, typically root-mean-square, between calculated and measured traveltimes (e.g., Tien-when & Inderwiesen, 1994; Nolet, 2008).

Tomographic studies correlate P-wave velocity (derived from inversion of first-arrival traveltimes) to the bulk material quality and subsequently to the degree of weathering and fracturing of a rock mass (Barton, 2007). Velocity decreases significantly in fractured and weathered rocks and unconsolidated sediments in contrast to consolidated sediments and intact rocks (Jongmans & Garambois, 2007). As a result, vertical and lateral changes of velocity can discriminate the overlying sediment cover from the hard rock substratum (Lanz et al., 1998; Polymenakos & Papamarinopoulos, 2007; Samyn et al., 2012) and highlight the alternation of steeply dipping fracture areas and rocks (Heincke et al., 2006).

In this study, we invert first-arrival traveltimes to obtain P-wave velocity maps for an evaluation of the clay sediment stratigraphy, the depth to bedrock and the bedrock morphology.

Data acquisition and traveltimes

The seismic tomography imaging at Alepotrypa Cave was made in 2014 (Polymenakos, 2014) in an area extending to about 1200 m² in the Entrance Chamber, where an excavation pit is also located (Fig. 4). The survey area extends to the rock walls of the cave and is covered by clayey soil. Surface elevation ranges from about 12 to 19 m above sea level, with the highest slope to the east, a lower slope to the west and the lowest slope to the north and south.

The tomographic layout consisted of 12 receivers and 60 sources. Receivers were located along the west side of the survey area, near the entrance of the cave. Receivers were not placed in the east side of the survey area, because of surface obstructions that precluded a receiver separation adequate for the scope of the tomographic survey. Sources were positioned throughout the survey area, to overcome the limitations of receiver placement and provide sufficient ray coverage of the entire survey area. Source and receiver locations were separated by 2 to 5 m and arranged to achieve an average spatial coverage of 1 m (corresponding to an average angular coverage of 5°), sufficient for resolving geological features related to cave speleogenesis, while keeping the field logistics

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Fig. 4. The tomography survey area in the Entrance Chamber of Alepotrypa Cave. The tomography field layout and elevations (in meters above sea level) are shown. Coordinates are local easting/northing topographic coordinates.
at a reasonable level. Source locations extended over the maximum possible elevation difference, necessary to reach the largest possible exploration depth. In addition, sources were located so as to avoid damage to stalagmites and other important surface features. The elevation difference across the survey area (about 7 m in the E-W direction) allowed investigation of traveltime variations as a function of depth. This provided for a quasi-3D imaging of sedimentary structure and the bedrock morphology. The positions of receivers and sources were measured with centimeter precision in the XYZ coordinates with digital surveying instruments. The source was a 2 kg sledgehammer, hitting on a plate made of synthetic material (acetal) that provided improved ground coupling, high-frequency energy transmission, and portability, with less weight. The receivers were 10 Hz vertical geophones, positioned on metal bases for better coupling with variant ground surface conditions (firm soil, concrete) and avoid drilling in the delicate cave environment. Data were recorded with a 12 channel Geometrics seismograph, using a sampling window of 192 ms and a sampling rate of 0.125 ms. Stacking and appropriate filters were used for signal enhancement. A total of 720 high quality wave records were acquired.

First arrival picking was made with TomTime software (Tweeton, 2016), taking into consideration the waveform characteristics, the specific source/receiver distance and the estimated average velocity. Representative seismic recordings with time picks are shown in Fig. 5a. Frequency spectra are shown in Fig. 5b (dominant frequencies range from 70 to 300 Hz). The typical error in time picking is estimated at 1 ms. Times and straight-ray velocities versus source-receiver distances are shown in Fig. 5c. Traveltimes range from 1 to 33 ms. The time range widens with increasing distance, indicating that the raypaths sample the bedrock and a sediment cover with significant differences in consolidation and/or lithology. Straight-ray velocities range from 400 to 2500 m/s and increase with raypath length indicating that a greater fraction of the raypath was in the more competent materials at depth where the attenuation

Fig. 5. Tomographic data. a) Seismic waveforms with picks of first arrival times for a near field (left) and far field (right) seismic source. Solid dots indicate time picks. Source labels refer to locations in Fig. 4; b) Fourier frequency spectra of the near and far field source records of Fig. 5a; c) Time-distance plot (left) and straight-ray velocity-distance plot (right) of the tomography dataset.
was smaller. Higher velocities at short distances indicate the influence of shallow bedrock. Based on recent observations on the actual detection ability of seismic traveltime tomography (e.g., Li & Duric, 2013; Polymenakos & Tweeton, 2015), the average resolution is estimated to be better than 2 m (1.1 m), or less than 25% of the Fresnel zone width (7.5 m) calculated for the highest dominant frequency (300 Hz), an average source-receiver distance of 18 m and an average straight line velocity of 930 m/s.

**Data inversion**

First arrival times were inverted into a three-dimensional distribution of P-wave velocity, from which interpretations related to subsurface lithology and bedrock morphology were made. Traveltime inversion was performed with the SIRT algorithm (Dines & Lytle, 1979) using GeoTomCG software (Tweeton, 2015), that allows both straight and curved raypath calculation in two or three dimensions and can obtain the most stable results and smoother images which are not critically deteriorated by noise, in contrast to other algorithms (Lehmann, 2007).

After initial testing and removal of traveltime outliers, 645 traveltimes were available for tomographic inversion. Inversion was performed on an initial model designed with a three-dimensional rectangular grid having cell dimensions of 2 m in either direction, approximately the smallest source-to-source and receiver-to-receiver separation, allowing for adequate ray coverage of the grid cells. Based on the tomography data, velocities in the initial model range from 330 to 2600 m/s, increasing gradually towards lower elevations. Tests with straight and curved rays resulted in a final inversion with curved ray iterations allowing P-wave velocity to range between 300 and 3500 m/s. The most physically realistic result with fewer velocity artifacts was after 5 curved ray iterations with RMS residual of 2.4 ms. Tests with different starting models and increased number of iterations showed that an improvement of the RMS residual did not lead to better imaging but only increased velocity smearing and unrealistic velocity contrasts (artifacts). Fig. 6a shows the good overall convergence between observed and calculated traveltimes. Fig. 6b shows that the survey area is adequately and fully covered by calculated raypaths. Raypath coverage and trajectories reflect strong velocity contrasts between parts of the survey area, that resulted in relatively lower coverage and decreased detail in the velocity model at the deeper part of the eastern side of the survey area (see Fig. 8 and relevant discussion below). The placement of additional receivers that might increase ray coverage in this area was not possible due to access limitations.

**INTERPRETATION AND DISCUSSION**

**P-wave velocity**

P-wave velocities show a wide range, from 400 to 3000 m/s (Fig. 7a, c). As elevation decreases, higher velocities gradually prevail over larger parts of the survey area. Considering the velocity variations, two primary limits can be set, at 1000 m/s and 1900 m/s respectively. These velocities separate areas with low velocity gradient (Fig. 7b, 7c). The higher primary velocity limit is interpreted as separating two major lithological formations, that of the sediments (velocity range 400-1900 m/s) and the rocky bedrock (velocity range 1900-3000 m/s). The lower velocity range corresponds to soil material with variable stiffness,
Fig. 7. Interpreted velocities. a) and b) Horizontal velocity maps resulting from inversion of traveltime data, with velocity limits emphasizing possible lateral variations within the sedimentary pack at higher elevations (a), and between the sedimentary pack and the rocky substrate at lower elevations b); c) Vertical velocity map emphasizing possible vertical variations through the sediments and the substrate. A relation of the velocity pattern with part of the shallow geological structure is also shown. For a detailed discussion see the P-wave velocity section.

whereas the higher velocity range corresponds to medium-to-hard rock with velocities typical for limestone (Schön, 2015). Values higher than 2600 m/s are interpreted to correlate with crystalline limestone and are consistent with the lower limit of the regional velocity range estimated for the limestones and marbles of the Kriti-Mani unit (3500 to 4500 m/s; IGME, 1989). The velocity pattern shows no effect of groundwater, suggesting its absence (or, very limited presence) within the depths explored with tomography. The lower primary velocity limit is interpreted as separating sediments of different stiffness that could be related to different lithology: velocities up to 1000 m/s correspond to a low stiffness soil unit (sed-1), while values in the range 1000-1900 m/s suggest a stiff soil unit (sed-2), with the higher values (1300-1900 m/s) being typical for very stiff clay sediments (Schön, 2015). A further differentiation can be implied by setting a secondary low-velocity limit at 600 m/s, separating the sed-1 unit into two sub-units which show low velocity gradient (sed-1a, sed-1b; Fig. 7a). Likewise, a high-velocity secondary limit can be set at 1500 m/s, separating the sed-2 unit into sed-2a and sed-2b sub-units (Fig. 7b).

From a comparison of the vertical velocity pattern with the sedimentary sequence in the excavation pit (Fig. 7c), the 600 m/s sub-limit corresponds approximately to a transition from the superficial fine-grained clay/ash interval to the underlying sediments. This correspondence is tentative, since excavation data are not conclusive and the depth span of the excavated sediments is quite shorter than that explored with seismic tomography. It indicates, however, a relation to lithological changes. P-wave velocity is primarily related to the stiffness/strength of geological materials which is affected by parameters such as porosity/fracture density, saturation/groundwater, and grain size (Barton, 2007), that are closely related to lithology. Karstification and weathering of the bedrock surface may affect P-wave velocity at the sediment-bedrock interface, however at a much lesser degree than lithological changes (e.g., Hamdan et al., 2012; Schmelzbach et al., 2015). Furthermore, tomographic P-wave velocity models have been proved capable of reconstructing sharp lithological boundaries better than other velocity models obtained with refraction, surface waves, etc. (e.g., Barton, 2007; Gance et al., 2012). Thus, we assume that the above velocity limits relate accurately to major lithological changes and provide us with a means to follow possible lithological variations throughout the survey area, as discussed below.
Fig. 8. Velocity maps resulting from traveltime inversion. a) Horizontal maps; b) Vertical maps along Y direction, i.e. across the cave; c) Vertical maps along X direction, i.e. along the cave. The X or Y location of each map is shown on the map. Graphical locations of vertical maps are shown in Z = 16.85 (Fig. 8a, top left). Color scale as in Fig. 7.
**Morphology of buried rock surface**

A sequence of horizontal velocity maps (Fig. 8a) shows that the bedrock gradually abounds in the survey area with decreasing elevation, with the shallowest end in the western, northern and southwestern part and the deepest end in the eastern part. Examination of vertical velocity maps (Fig. 8b, 8c) allows us to visualize the original cross-section shape of the buried karstic conduit in the Entrance Chamber. Vertical maps across the cave (Fig. 8b) show that the conduit floor, from shallow and rough in the west, becomes narrow at its edges and deep in the center, describing a smooth but irregular V shape in the east, with a steeper southern slope and a gentler north slope. Vertical maps along the cave (Fig. 8c) show a considerable elevation drop of the conduit floor in the central and eastern part of the cave. The lowest bedrock elevation is estimated at 6.0-6.5 m near the eastern end of the survey area (Fig. 8b; map at X = 3622, Fig. 8c; map at Y = 5652). Decreased ray coverage did not allow a detailed imaging of the sediment-bedrock interface in the deepest eastern part of the model (Fig.8a; central eastern part of maps at Z = 8.85 and 6.85, Fig.8b and 8c; central bottom parts of maps at X = 3618 and 3622, and lower right parts of maps at Y = 5648 and 5652). The isolated high-velocity features at higher elevations in the western part of the survey area (Fig. 8a; maps at Z = 14.85 and 12.85), have the form of relief-relics, or pinnacles, of the bedrock when seen on vertical velocity maps (Fig. 8b; map at X = 3602, Fig. 8c; map at Y = 5652). These features are interpreted as bases of large stalagmites on the bedrock surface. One such stalagmite, actually present on the ground surface at location X = 3598, Y = 5654 (Fig. 4), corresponds to the larger high velocity anomaly seen in the horizontal velocity maps at Z = 14.85 and 12.85 m. Indications of possible locally increased karstification in the bedrock (karst pockets) may be expressed through locally reduced values in the interpreted rock velocity range, in particular in the 1900-2200 m/s range (Fig. 8a; maps at Z = 12.85 to 6.85, Fig. 8b; maps at X = 3602 and 3606; Fig. 8c; map at Y = 5652).

The bedrock morphology is reconstructed in detail by the bedrock surface calculated from the interpreted horizontal velocity maps (Fig. 9). The buried rock surface follows a distinctive, semi-circular shape, with the concave side oriented to the east-northeast (Fig. 9a). This funnel-like shape of the bedrock surface implies principally a karstic origin, possibly related to a sinkhole. However, the elevation drop, being larger on the south flank than on the north flank of the bedrock surface, suggests that the southern flank could have been shaped by the influence of a possible rock fracture running WNW-ESE (Fig. 9b). The calculated bedrock surface emphasizes the elevation differences across the survey area and the abundance of relief-relics (Fig. 9b).

**Sediment deposition, thickness, and volume**

Considering the main differentiation within the sediments delineated by the 1000 m/s value, the upper less stiff material (sed-1) covers much of the survey area at the highest elevation (Fig. 8a; map at Z = 16.85), while it is confined to the central and eastern part at lower elevations until entirely replaced by the stiffer material (sed-2) below Z = 8. As suggested by the vertical velocity maps (Fig. 8b and 8c), the deposition of sed-1 and sed-2 units follows the underlying bedrock morphology. However, significant differentiations appear in the deposition of the lower (sed-2) unit, as suggested by local increase in depth (Fig. 8b; maps at X = 3618 and 3622, Fig. 8c; maps at Y = 5648 and 5652). The interfaces of interpreted sedimentary units were used for estimating their thicknesses. For the total sedimentary pack, thickness ranges from 2 up to and 12 m, increasing towards the eastern part of the survey area where it reaches its maximum, eastward of the excavation pit (Fig. 10a). A similar depositional pattern is suggested for the upper (sed-1) and the topmost soil units (sed-1a, interpreted as corresponding to the clay/ash interval), with thicknesses ranging from 0.5 to 9 m and from 0.2 m to 4 m, respectively, reaching their maxima in the eastern part of the survey area (Fig. 10b and 10c). The thicknesses of sediment units are considerably larger in the center than on the sides of the survey area with decreasing elevation, with the shallowest end in the western, northern and southwestern part and the deepest end in the eastern part.
area. This suggests that the sediment deposition in the Entrance Chamber is related to the original shape of the buried karstic conduit. On the other hand, the areas with a total thickness of less than 2 m in the western edge of the survey area may relate to clay evacuation areas due to depositional dynamics (c.f. Nehme et al., 2013). However, human intervention may also be an important factor in the discharging of clay volume.

The interpreted sediment and bedrock surfaces combined with the ground surface allowed us to calculate the volume of the main sediment units with use of the Simpson’s rule provided with Surfer software (Golden Software, 2011), applied on a 1x1 m grid. This amounts to about 4000 m$^3$ for the total sediment pack, about 2000 m$^3$ for the upper (sed-1) unit and 900 m$^3$ for the topmost (sed-1a) unit related to the clay/ash interval.

Calculated thicknesses and volumes are accurate within the limits of spatial coverage and resolution of the tomographic experiment, taking into account possible local effects of factors other than lithology on P-wave velocity, and effects on the detail of the velocity model due to reduced ray coverage; as with all geophysical methods, use of independent information (geophysical, geological, etc.), if available, may constrain traveltime inversion and reduce possible uncertainties. Nevertheless, the information on sediment thicknesses and volumes can be significant for studying speleogenesis when expanded to the total volume of sediments in all chambers of Alepotrypa Cave.

CONCLUSIONS

The seismic tomography imaging of Alepotrypa Cave succeeded in mapping the bedrock surface and revealing the shape of the original conduit of the cave, currently covered with up to 12 m of clay sediments. Relief-relics (pinnacles; bases of large stalagmites) and possible tectonic influences in the shaping of cave buried morphology were inferred from variations of the bedrock surface. The sediments overlying the bedrock amount to a total volume of 4000 m$^3$ and show significant differentiations in stiffness that may be also related to differing lithology.

Our results show that seismic tomography can be a very effective exploration tool in the interior of a cave, for mapping the bedrock underlying the surface sediments and large-scale lithological and stiffness variations within the sediments. The method can also be used for mapping the shallow underwater cave chambers and non-destructively assessing rock quality in caves to serve in rock stabilization studies.

ACKNOWLEDGEMENTS

The author wishes to acknowledge the Director of the Diros Project Dr. Giorgos Papathanassopoulos (Honorary Director of Antiquities) and Co-Directors Dr. Anastasia Papathanasiou (Ephorate of Paleoanthropology and Speleology of Southern Greece), William Parkinson (American Field Museum), and Michael Galaty (Mississippi State University), for supporting the tomography survey at Alepotrypa Cave. The Diros Project is conducted through the Directorate of Paleoanthropology and Speleology of Southern Greece, and financially supported by the Wenner-Gren Foundation for Anthropological Research, the Institute for Aegean Prehistory, the Leon-Levy Foundation, and the Archaeological Institute for America-Cotsen Excavation Grant.

Ms. Danielle Riebe (University of Illinois at Chicago) and Ms. Rebecca Seifried (University of Illinois at Chicago) are acknowledged for the topographical work at the tomography site. Mr. Apostolis Giagakis is acknowledged for his assistance in fieldwork.

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Population density, habitat dynamic and aerial survival of relict cave bivalves from genus Congeria in the Dinaric karst

Olga Jovanović Glavaš¹, Branko Jalžić², and Helena Bilandžija³*

¹Department of Biology, University Josip Juraj Strossmayer, Cara Hadrijana 8/A, 31000 Osijek, Croatia
²Croatian Biospeleological Society, Demetrova 1, 10000 Zagreb, Croatia
³Department of Molecular Biology, Rudor Bosković Institute, Bijenička 54, 10000 Zagreb, Croatia

Abstract: Caves are some of the least-known ecosystems on Earth and long-term ecological studies and population size estimates are very rare. Genus Congeria is a Tertiary relict that comprises three species from Dinaric karst area; C. kusceri, C. jalzici and C. mulaomerovicic, each with very limited distribution. They are the only known cave bivalves and in contrast to many other cave species, they form populations with high densities. We estimated that the population of C. kusceri in Jama u Predolcu is between 72,454 and 72,906 individuals. The highest density occurred between one and three meters depth, and reached maximum of 1,625 individuals per square meter. Here we also present the results of a two-year water temperature and water level survey in several C. kusceri and C. jalzici localities. Data loggers showed that C. jalzici occurs in colder caves and higher water level oscillations then C. kusceri. From our data, it is obvious that the natural hydrological conditions in Congeria habitats were seriously altered. This and a series of other threats led to Congeria species being highly endangered. During low water levels, a part of the bivalve population becomes exposed to air in most localities. Uniquely, they are active during that period and are able to survive those conditions for more than 2 months.

Keywords: cave animals, Dreissenidae, Bivalvia, temperature, water level, aerial survival, population size

Received 7 July 2016; Revised 9 December 2016; Accepted 16 December 2016

Citation: Jovanović Glavaš O., Jalžić B. and Bilandžija H., 2017. Population density, habitat dynamic and aerial survival of relict cave bivalves from genus Congeria in the Dinaric karst. International Journal of Speleology, 46 (1), 13-22. Tampa, FL (USA) ISSN 0392-6672 https://doi.org/10.5038/1827-806X.46.1.2020

INTRODUCTION

Biodiversity in subterranean realm is generally low compared to surface habitats of the same region. This is presumably due to the very specific and often harsh environmental conditions such as the lack of light and low nutrient availability (Gibert & Deharveng, 2002). In that context, some of the functional species groups are mostly absent in the subterranean ecosystems, i.e., primary producers and primary consumers (herbivores; Mohr & Poulson, 1966).

Unlike surface habitats where low biodiversity is often coupled to high population densities, in caves population sizes do not seem to be large, at least judging from the time and effort researchers require to collect cave fauna (Trajano, 2001). There are exceptions, and certain species can form large aggregations in some caves (e.g., Fenolio & Graening, 2009). One of those exceptions are cave bivalves of the genus Congeria which can cover most or all of available surfaces in some localities (H.B., B.J. pers. observ.). However, the total number of individual bivalves in any of the sites has never been assessed and is not known.

Within the last decade and with the application of molecular phylogenetic methods it became apparent that subterranean diversity is often underestimated, and the existence of cryptic species is a very common phenomenon in the subterranean habitats. As a result, the majority of groundwater species and/or lineages have very narrow distributions, regularly not exceeding 200 km (Trontelj et al., 2009). One of these species is Congeria kusceri, Bole, 1962, which was considered to be the only known troglobiotic bivalve species until recently. Today we know that different populations of previously described C. kusceri represent three distinct species (Bilandžija et al., 2013), including Congeria jalzici, Morton & Bilandžija, 2013 and Congeria mulaomerovicic, Morton & Bilandžija, 2013.

All three species are found in only 15 cave localities in the Dinaric Karst region; 1 in Slovenia, 6 in Croatia, and 8 in Bosnia and Herzegovina (Jalžić, 1998; Jalžić,
2001; Bilanđžija et al., 2013). Their distributions do not overlap and the range of each individual species is actually very small. The most widespread is *C. kusceri* found in 8 localities within the Neretva River basin, in Croatia and Bosnia and Herzegovina. The second widespread is *C. jalzici*, known from 4 localities; 1 isolated site in Slovenia and 3 in Croatia, within Lika River basin. *Congeria mulaomerovici* is known from only 3 localities in the Sana River basin in Bosnia and Herzegovina. The most research has been done on *C. kusceri* (e.g., biology and anatomy, Morton et al., 1998; phylogeny, Stepień et al., 2001; life history, Morton & Puljas, 2013; growth and longevity, Puljas et al., 2014), as some of the caves it inhabits are easily accessible and available for research throughout the year.

*Congeria* is one of the three extant genera in the family Dreissenidae. Phylogenetic analyses place *Congeria* in close relationship with *Mytilopsis*, while nominal genus *Dreissena* is positioned in a separate clade (Stepień et al., 2001; Bilanđžija et al., 2013). All three species of *Congeria* are considered Tertiary relicts that begun to diverge at the end of Miocene. The related genera *Mytilopsis* and *Dreissena* share similarities in their life history traits such as a life span of two years, a high reproductive rate and rapid growth (Morton, 1969; Morton, 1989; Pathy & Mackie, 1992). In addition, both genera contain highly invasive species on a global scale (i.e., *Dreissena polymorpha*, (Pallas, 1771), *Dreissena bugensis*, Andrusov, 1897, *Mytilopsis leucophaeata*, (Conrad, 1831)). Their reproductive strategy has an advantage for invading new territories and probably explains their success in introduced areas (Borcherding, 1991; Ram et al., 2011). Further, they are most commonly found between 2 and 12 m of depth and can tolerate a wide range of water salinities and temperatures (Erben et al., 1995; Stanczykowska, 1977).

On the contrary, representatives of genus *Congeria* have developed some specific and unique features presumably due to their adaptation to subterranean environment. Similarly to other stygobiont species, *Congeria kusceri* has a long life span of more than 50 years (Puljas et al., 2014) and reaches sexual maturity at the age of 10 years. It has an annual reproductive cycle, produces just a few offspring, and broods early larvae in ctenidia and juveniles in mantle pouches (Morton & Puljas, 2013). These features contribute to the low recovery potential of these species, making cave bivalves very susceptible to environmental changes.

*Congeria kusceri* is listed as Vulnerable (VU) in the European Red List of Non-marine Mollusks based on IUCN criteria (Cuttelod et al., 2011). Since *C. jalzici* and *C. mulaomerovici* have been described only recently (Morton & Bilanđžija, 2013), their IUCN status has not yet been evaluated on the international level. However, both *C. jalzici* and *C. kusceri* in Croatia are listed as Critically Endangered (CR) (Bilanđžija & Jalžić, 2009). They are distributed in areas heavily affected by man-made changes: building of hydroelectric power plants and redirection of water courses left many sinkholes (including *Congeria* sites) without any influx of water. Unfortunately, human interventions are still ongoing and future plans include another dam in the Lika River basin that will affect at least two but more likely all three *C. jalzici* localities in Croatia and a series of dams in the upper Neretva River basin in Bosnia and Herzegovina that may have an effect on most or all of the *C. kusceri* sites.

*Congeria* species are also included in Annexes II and IV of the Habitat Directive (94/93/ECC). As a part of preparation for admittance of Croatia to the European Union (EU) and the obligations to comply with EU’s nature conservation legislation, especially related to implementation of Habitats Directive, we conducted research on *Congeria* including searches for new localities, visits to all known sites, studies of population ecology and assessments of threats. Here we report some of the results of those multiannual studies.

In the first part we focus on the *C. kusceri* population from Jama u Predolcu cave, located in the town of Metković, Croatia with the goal to determine the population size. In the second part we describe the results of a two-year water temperature and levels surveys in 3 caves inhabited by *C. kusceri*: Jama u Predolcu, Pukotina u tunelu Požeče-Blažo, and Žira, as well as in a single *C. jalzici* locality: Markov Ponor (Fig. 1). We also report on the ability of these species to survive long periods living outside of the water. Our research gives us insights into the basic habitat characteristics of these unique species and is valuable from the species conservation perspective. Since *C. jalzici* and *C. kusceri* are under a great threat from both past and future pressures it is important to establish reference points for environmental parameters that can serve for planning future interventions as well as subsequent monitoring of *Congeria* populations.

**MATERIALS AND METHODS**

**Density survey**

We estimated the density of the *Congeria kusceri* population in Jama u Predolcu cave, in the town of Metković, Croatia. This 71 m long and 20 m deep cave (Fig. 2) is composed of three chambers: the Entrance Hall, the Speleothem Passage and the Water Passage. A lake at the bottom of the Entrance Hall is divided into the Shallow and Deep Lake by a ridge. The Entrance Hall is illuminated but not much sunlight reaches the level of the lakes which are 10 m below the entrance. The Speleothem Passage leads to the Water Passage which ends in a Third Lake. This site was chosen because the cave and subterranean lakes are easily accessible throughout the year.

The population size was estimated based on the census of 10% of the surface in the Shallow and Deep Lake, and 20% of the surface in the Third Lake. Since the bottom of all three lakes is covered in mud and sediment and does not provide suitable habitat for *C. kusceri*, counting was performed only on lake walls. Counting lasted over two weeks (in June and September 2012) and was performed by four or five divers. Counting was carried out in squares of...
Population density, habitat dynamic and aerial survival of cave Congeria

International Journal of Speleology, 46 (1), 13-22. Tampa, FL (USA) January 2017

Fig. 1. Congeria kusceri and C. jalzici localities were included in our study. Lika river basin: 1) Markov Ponor; Neretva river basin: 2) Pukotina u tunelu polje Jezero – Peračko Blato, 3) Jama u Predolcu, and 4) Žira.

40 x 40 cm, which were divided in four 20 x 20 cm. The squares were placed diagonally to the next square from the surface to the bottom, i.e., upper left corner of the new counting square would be positioned according to placement of the lower right corner of the preceding square. The starting points for each of the diagonal were 4 m apart in the Shallow and Deep Lake, and 2 m apart in the Third Lake.

Data obtained by census of sample plots were used as a basis for population size estimation. The estimation was calculated following Hanson (1967) since both of the presumptions for this method were met; i.e., the animals were sampled at random, and all the animals existing on each plot were counted only once. The method is based on the mean number of animals recorded per each plot, and the variance of the mean, which is used to calculate the 95% confidence limit. The results are then extrapolated to the whole surface of lake walls.

In order to examine a possible correlation between the number of bivalves and depth, correlation coefficient was calculated for each lake separately. Data were grouped by meters of depth for each of the lakes (0 m to maximum 9 m in the Deep Lake) and examined with nonparametric Spearman’s rank order correlation test, with significance level of P < 0.05. Analyses were performed using STATISTICA software (StatSoft Inc., 2014).

Water level and temperature data

We placed data loggers (HOBO 250-Foot Depth Water Level Data Logger, Onset, USA) in three C. kusceri and one C. jalzici cave. All three C. kusceri caves are in the Neretva River catchment. The Žira and Jama u Predolcu caves are part of the Trebišnjica river basin, a left tributary of the Neretva River, whereas the Pukotina u tunelu Polje Jezero-Peračko Blato Cave is located on the right bank of the Neretva River. The Žira Cave is a sinkhole of the Trebišnjica River and is located downstream of the Jama u Predolcu Cave. The Jama u Predolcu receives water mostly from the Trebišnjica River but its water level is also influenced by the Neretva River since it is situated directly in the Neretva river valley.

Data loggers recorded water level and temperature every 45 minutes. Data logger was placed in the Shallow Lake of Jama u Predolcu Cave, and recorded data from 23 June 2010 until 19 September 2012, in total for the period of 816 days. In the
Pukotina u tunelu Polje Jezero-Paračko Blato data was collected between 18 September 2010 and 14 September 2012, for a total of 726 days. A third data logger was placed in Žira Cave in the period from 3 September 2011 until 8 September 2012 (370 days).

Water level and temperature data for *C. jalzici* were monitored in Markov Ponor, a sinkhole in Lika River Basin, located over 250 km northwest from the Neretva River and *C. kusceri* sites. Data was recorder from 8 September 2010 until 21 September 2012, for a period of 744 days. On 18 October 2011 the data logger was moved to another more secluded place, since it was detached from the original spot and was found lying in the rocks 2 m away.

**RESULTS**

**Density survey**

We counted a total of 7,412 individuals in all three lakes of Jama u Predolcu cave. The highest number was registered in the Shallow Lake, 4,111 individuals, 3,010 individuals in the Deep Lake, and 291 individuals in the Third Lake. Based on these results, we estimated the number of individuals for all tree lakes; the population ranges between 40,995 and 41,240 individuals in the Shallow Lake, between 30,024 and 30,185 individuals in the Deep Lake, and from 1,435 to 1,481 individuals in the Third Lake. Altogether, the population size for the Jama u Predolcu Cave ranges between 72,454 and 72,906 individuals.

The density of individuals varied considerably, both among and within lakes, and depended on the microhabitat conditions and depth in each of the lakes. The highest total density was recorded in the Shallow Lake, where mean density was 211 ± 305 individuals per square meter (ind/m²). The recorded mean density was 103 ± 201 ind/m² in the Deep Lake, and 59 ± 111 ind/m² in the Third Lake.

Our analyses showed that there is a correlation between the depth and the number of individuals, in all three lakes, although it was not significant in the Third Lake (Third Lake $P > 0.05$, $r = -0.62$; Shallow Lake $P < 0.05$, $r = -0.75$; Deep Lake $P < 0.01$, $r = -0.84$). The highest density of individuals was observed between 1 and 3 m of depth (Fig. 3); in the Shallow Lake the average density between 1 and 3 m was 267 ± 333 ind/m² with maximal value of 1625 ind/m², in the Deep Lake the average was 192 ± 286 ind/m² and in the Third Lake 107 ± 153 ind/m². Such high standard deviations are a result of the fact that bivalves are very patchily distributed on cave walls; in certain areas they form large congregations whereas in other, such as smooth surfaces, they are not present at all.

**Water level and temperature data**

In Jama u Predolcu Cave, the data logger collected 25,975 records of temperature and water level in the period of 816 days. The data are shown in Table 1 and Fig. 4. There are several abrupt peaks in water level, short in both the size (max 2 m) and duration. The fact that natural hydrological conditions are interrupted is obvious from the irregular pattern of peak occurrence (October and November 2010, and February and April 2012) including no high peaks in 2011. The temperature data is more in accordance with expected values; peak temperatures of above 18°C were recorded in summer months each year and lower values (around 13°C) in winter months.

In the Pukotina u tunelu Polje Jezero-Paračko Blato the data logger recorded 23,253 measurements in the period of 726 days. The data are shown in Table 1 and Fig. 5. A period of low water levels started in June of both 2011 and 2012. Water levels began rising in late autumn, followed by oscillations during the whole winter and spring with several high peaks throughout that period. Temperature data shows that the lowest temperatures coincide with highest water levels and vice versa. For example, the highest water level peak recorded in November/December 2010 coincided with the lowest recorded temperature (7.18°C).

In Žira Cave the data logger recorded 11,879 measurements in a period of 370 days (Table 1 and Fig. 6). A flat line in the water level data is present because the data logger was above the water for most of the time. Consequently, the details for water level changes during the low water levels are missing. High peaks (> 0.5 m change) occurred in December 2011, and February and April 2012. The temperature in the cave was very stable throughout the recording period, with a total variation of only 1.08°C.

In Markov Ponor sinkhole the data logger recorded a total of 23,695 measurements during 744 days (Table 1 and Fig. 7). However, due to the detachment of the data logger, on 18 October 2011 it had to be moved to a more protected spot where the chances that water current would detach it again were minimized. Therefore, during first period from 08 September 2010 until 17 October 2011, 12,910 measurements were recorded, and from 18 October 2011 until 21 September 2012, 10,761 measurements. Other than just a few peaks, the water level was about the same.
Table 1. Data logger measurements on three sites, Jama u Predolcu, tunnel between Polje Jezero-Peračko Blato, and Žira. N: number of measurements, T max: highest recorded temperature, T min: lowest recorded temperature, T mean ± SD: average temperature at the site in the measured period ± standard deviation, $H_2O$ max: highest water level recorded at the site, $H_2O$ min: lowest water level recorded at the site, $\Delta$ depth: difference between the highest and lowest water level, $H_2O$ mean ± SD: mean water level ± standard deviation.

<table>
<thead>
<tr>
<th>Site</th>
<th>N</th>
<th>T max °C</th>
<th>Date max</th>
<th>T min °C</th>
<th>Date min</th>
<th>T mean ± SD °C</th>
<th>$H_2O$ max (m)</th>
<th>Date max</th>
<th>$H_2O$ min (m)</th>
<th>Date min</th>
<th>$\Delta$ depth (m)</th>
<th>$H_2O$ mean ± SD (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jama u Predolcu</td>
<td>25 975</td>
<td>19.38</td>
<td>9.8.2012</td>
<td>11.63</td>
<td>6.2.2012</td>
<td>15.38 ± 2.00</td>
<td>3.01</td>
<td>23.11.2010</td>
<td>0.97</td>
<td>1.12.2011</td>
<td>2.04</td>
<td>1.51 ± 0.26</td>
</tr>
<tr>
<td>Žira Cave</td>
<td>11 879</td>
<td>11.14</td>
<td>12.5.2012</td>
<td>10.06</td>
<td>4.2.2012</td>
<td>10.95 ± 0.18</td>
<td>2.35</td>
<td>17.4.2012</td>
<td>-0.26</td>
<td>3.2.2012</td>
<td>2.61</td>
<td>-0.07 ± 0.34</td>
</tr>
</tbody>
</table>

Fig. 4. Water level (blue) and temperature (black) in Jama u Predolcu from 23 June 2010 until 19 September 2012.

Fig. 5. Water level (blue) and temperature (black) in Pukotina u tunelu Polje Jezero- Peračko Blato from 18 September 2010 until 14 September 2012.

and followed a predictive pattern. Starting in June 2011, the water level dropped, reaching the lowest point in early to mid-August; in late autumn it increased several meters and then showed only slight oscillations from December to June. The highest change in water level was recorded on 31 November 2010 when water level had risen for 40 m in 90 minutes, and in the next four hours it reached 50.63 m (Fig. 7). The total difference of water level in Markov Ponor was slightly above 60 m. The data logger was outside of the water two times, from mid October to mid December 2011 and beginning of August to the end of September 2012. Both periods are characterized by more substantial
temperature variations as it would be expected from the air compared to water temperature patterns. The temperatures in Markov Ponor varied between 5°C and 6°C throughout most of the research period. Both the maximum and minimum recorded temperatures occurred within just two months (October to December 2010) and coincided with high peaks in the water level data. First, at the end of October 2010 a rise of approximately 20 m in water level caused a steep temperature increase to 10.45°C, and in the second half of December 2010, the 60 m water level rise caused a decrease of temperature almost to 2°C. A second substantial drop in the temperature, to less than 3°C occurred in late autumn 2011 and was caused by the water level drop and the data logger not being submersed.

Aerial survival

We have observed *Congeria* individuals from all three species living on air-exposed cave walls for a part of the year. The proportion of the population that gets exposed to air is small but it happens in most localities: for *C. kusceri* it is Žira, Pukotina u tunelu Polje Jezero-Peračko Blato, Jasena, Gradnica, Doljašnica, and Plitica; for *C. jalzici* it is Markov Ponor and Dankov Ponor; and for *C. mulaomerovici* Suvaja and Dabarska pećina. Only in five localities bivalves do not get exposed to air and these localities are mostly springs with direct outlet towards the surface where, as far as we know, there is no dry rock surface in the cave itself.

During the period of low water levels in Markov Ponor, the period of air exposure that bivalves were able to survive lasted for more than two months. Often, patches of bivalves living outside of the water were found in passages that were far away (tens of meters) from remaining lakes and ponds which excludes the possibility that they migrate in and out of the water. Interestingly, we would invariably find individuals with their shells open and inhalant and exhalant syphons extruded (Fig. 8).

![Fig. 8. An individual with open shells found outside of the water in Markov Ponor (photo by H. Bilandžija).](image-url)
DISCUSSION

The densities of individuals in extreme environments, such as caves, are usually limited due to the low food availability. This applies to the majority of stygobiont species; for example, cave populations of fishes are generally smaller than of those living in epigean habitats (Trajano, 1997; Bichuette & Trajano, 2015). There are exceptions like the sulpicient, nutrient rich caves where the animals live in high density populations (e.g., Jourdan et al., 2014). We have observed Congeria bivalves forming dense populations during our field surveys. Results of the population size study show that C. kusceri can live in populations with up to 1625 individuals per square meter. The reason for such high density could be related to a filter feeding life style of this species. There are two more sedimentary filter feeders in the Dinaric Karst, the cave sponge Eunaplia subterranea Sket & Velikonja, 1984 and the cave tube worm Manfugia cavatica, Absonol & Hrabe, 1930 which co-occurs with Congeria at all sites. Both the sponge and the tube worm can form large colonies and live in dense populations (H.B., B.J. pers. observ.). In addition, Stygobromus emarginatus (Hubricht, 1943), a non-sedimentary filter feeding amphipod, also has high population sizes (Knapp & Fong, 1999). Filter feeding therefore seems to be a good feeding strategy that can sustain large populations of subterranean animals.

High densities of cave bivalves suggest that subterranean waters they inhabit might be rich in organic matter. Changes in water levels, as indicated by our data, are very rapid and presumably can carry a rich supply of organic matter from the surface to the subterranean realm which would be the basis for sustaining large colonies of filter feeders. For example, C. jalzici lives in the sinkholes which are likely more nutrient rich due to the influx of organic matter with the water from the surface. Congeria kusceri lives in caves with various hydrological functions but it has been found only in the most downstream parts of the Neretva River basin. Water sinking and resurfacing in the upper parts of the catchment likely contributes to organic matter enrichment so that there are enough nutrients to sustain large colonies of bivalves (and in some sites cave tube worms too) in the downstream parts of the basin. Measurements of nutrient concentrations along river basins are needed to confirm this hypothesis but was outside the scope of this paper.

Other species in the family Dreissenidae also live in high density populations. Moreover, invasive species of dreissenid mussels are known to overgrow any sort of hard substrate (including other bivalves), and their densities in infested areas are commonly higher than few thousand ind/m² (Burlakova et al., 2006; Strayer & Malcolm, 2006; Nalepa et al., 2010). There are big differences in bivalve densities between different lakes in Jama u Predolcu. The low density in the Third Lake is potentially a consequence of the lower nutrient availability. Namely, sunlight reaches the surface of lakes in the Entrance hall and can potentially enable the growth of some photosynthetic organisms which can in turn form the basis of the food web. Further research is needed to test this hypothesis. The difference between bivalve densities in the Deep and Shallow lakes in the Entrance Hall can be explained by the micro relief. The Deep Lake has mostly simple and smooth cave walls, whereas the Shallow Lake has very rugged cave walls and therefore a greater surface for attachment of the animals.

Depth distribution of individuals shows resemblance in all three lakes. Congeria kusceri prefers depths between 1 and 3 m, with the peak at around 2 m depth in all three lakes (Fig. 3), which corresponds to some of the dreissenid species which also prefer shallow waters (e.g., D. polymorpha which is commonly found between one and 30 m; Stanczykowska, 1977; Mackie et al., 1989; Snyder et al., 1997).

Interestingly, Congeria bivalves are able to survive extended periods outside of the water, which is not a commonly encountered phenomenon. According to our data from Markov Ponor, these periods can last for more than 2 months. Remarkably, during those times we have observed C. jalzici individuals with their valves slightly open and exhalant and inhalant siphons ejected suggesting they are not dormant (Fig. 8). Some estuarine bivalves can survive outside the water, but for much shorter time periods (hours to several days; e.g., Hiroki, 1977). In addition, several freshwater mussels can survive up to one year buried in mud, but their shells are airtight closed (e.g., Velesunio ambiguous; Jones, 2007), and they lose up to 40 % of their body mass. Further, effects of desiccation depend upon temperature, and a cooler environment (like the one Congeria inhabits) means shorter survival time (Firth et al., 2011; Urian et al., 2011). In the closely related dreissenid species D. polymorpha and D. bugensis, emersion tolerance is lower than in other freshwater species, and at temperatures below 25°C and higher relative humidity, individuals are able to survive slightly more than 10 days (McMahon et al., 1993; Ussery & McMahon, 1995). So this trait in Congeria maybe evolved as a response to specific karst subterranean conditions where water level changes can be very severe. A big advantage in caves is that the air humidity is very high, often around 100%, so the threat of desiccation is minimized. However, the fact that bivalves have their shells open implies they may be extracting food or oxygen from the thin layer of water or just drops of water dripping over them from the cave ceiling. If so, this would be a unique adaptation in bivalves, and could help them to survive long periods outside of the water.

According to our data C. kusceri lives in a range of temperatures from 7.2 to 19.4°C. Previously reported temperatures are in the higher range; Morton et al. (1998) and Puljas et al. (2014) reported temperature of 13.5°C, whereas Jalžić (1998) temperatures between 14.5 and 19°C, probably because most of stygofauna research is done in the summer when water levels are low. The highest temperatures in C. kusceri localities were recorded in May 2012, except in Jama u Predolcu Cave where the peak was in August 2012. Low temperature extremes are always accompanied
by a water level rise meaning that an influx of cold water from the surface causes them. For instance, the lowest temperature in Zira and Jama u Predolcu caves was recorded at the same time, in February 2012, and was caused by the water level rise in the whole Trebišnjica river basin. In Pukotina u tunelu Polje Jezero-Peračko Blato and Markov Ponor the lowest temperature was recorded in December 2010 which coincides with the highest water level peak recorded in our study, suggesting that a heavy rainfall or other weather event filled up subterranean aquifers with cold water.

Temperature variations in Zira Cave were minimal (Δ = 1.08°C) because of hydrotechnological changes in Trebišnjica basin. The river was channelled into a concrete riverbed and the sinkholes were cut off from the water supply (except maybe during very high levels) leaving precipitation as the only source of water in the subterranean portion of the basin. This is also the cause of only 2 - 2.6 m water level change in both localities in Trebišnjica basin, Jama u Predolcu and Zira cave. Temperature variations were higher for the other two C. kusceri sites (Jama u Predolcu Δ = 7.75°C, Pukotina u tunelu Polje Jezero-Peračko Blato Δ = 10.27°C) as well as in C. jalzici site (Markov Ponor Δ = 8.37°C). However, both temperature and water levels can change very rapidly indicating that Congeria species are not highly sensitive to variations in environmental conditions.

The highest water level peaks tended to coincide in all four localities (November/December 2010) and are probably a consequence of the autumn with above average rainfall, especially in south Croatia. Another event in the Neretva river basin that is evident in all three C. kusceri sites happened in February/April 2012. The lowest water levels occurred in autumn 2011 but do not exactly match in different localities and are probably a result of a dry summer/autumn in the whole region. However, it is also obvious from water level data that natural hydrological conditions are very disturbed. In general, one would expect a trend of low water levels and high temperatures during the summer and the opposite during the winter, but there are many exceptions (e.g., high water levels from June to beginning of October 2011 in Jama u Predolcu or occurrence of peak temperatures in Pukotina u tunelu Polje Jezero-Peračko Blato in May of both 2011 and 2012). In addition to hydrotechnical changes in Trebišnjica basin (already explained above) another reason for these events could be the use of uncontrolled quantities of water for agricultural purposes, which has been observed in some tributaries of Neretva River (Bonacci et al., 2012). Lika river basin, which is characterized by extreme and very quick changes in its discharge, has been heavily impacted by human activities as well, and for the same purpose: electric energy production (Bonacci & Andrić, 2008). Markov Ponor has been cut off from the river and no water sinks into the cave, although excess water is released into the sinkhole during high water conditions to prevent flooding of Lipovo Polje, as in the autumn of 2010.

It is unknown exactly how the hydrological modifications described above affected Congeria populations because there was no research on Congeria at the time the changes were made, but we can deduce that they had devastating consequences. For example, Bole (1962) reported that Congeria was growing like clusters of grapes in the last chamber of Zira Cave. Today only individual bivalves can be seen on the walls in the deepest part of the cave. The water level in Jama u Predolcu dropped by 10 m, after Trebišnjica river dam was built. Since today the maximum depth of the lakes in the cave is 10 m, it is possible that more than half of the Congeria population was destroyed at that time. Further, thick layers of dead shells can be found throughout the passages of Markov Ponor, suggesting a catastrophic event, probably not too long ago. The large hydrological changes in the basin when the Lika hydroelectric power plant was built are the likely cause. In addition, bivalves vanished from two previously known localities: Crni Ponor and Izvor kod kapelu Sv. Mihovila. In the latter one we found a Congeria population in the mid 1990 but 20 years later there were no bivalves in the spring. The entrance to the spring was widened and today daylight penetrates into the entire cave including the subterranean pool where bivalves used to live, which may have caused their disappearance.

There are a number of other threats to Congeria populations: karst waters are in general very susceptible to contamination from domestic sources and agricultural runoff, and water extraction which can significantly reduce the water levels in the underground water bodies. In addition, tourism development in cave systems can also influence the subterranean habitats and fauna. Unfortunately, in the last few years Jama u Predolcu has also been used as a tourist site, and many changes were made without the proper assessment of the effects on Congeria and other fauna, and without establishing a baseline for subsequent monitoring of the habitat quality and Congeria population health.

Although population from Jama u Predolcu seems to be large and vigorous, it is important to stress that all three species of genus Congeria have been found in only 15 caves. Since Congeria is a long lived and slowly reproducing animal, it is highly sensitive to habitat changes. Data on ecology and population sizes are urgently needed to establish management plan for all three species, and for the whole distribution area, similarly to the plan already developed for Croatia (Bilandžija et al., 2014). Conservation of subterranean fauna and habitats should be of the highest priority since karst system in the Dinaric mountains is considered to be one of the most unique underground systems in the world (Culver & Sket, 2000; Deharveng et al., 2012).

**CONCLUDING REMARKS**

Bivalve species from genus Congeria are sedentary filter feeders. Unlike majority of cave species, they form populations with high densities. We estimated
the population size of Congeria kusceri in Jama
u Predolucu and it is between 72,454 and 72,906
individuals. The highest density, reaching maximum of
1,625 individuals per square meter, similar to surface
species from the family Dreissenidae, was observed between 1 and 3 m of depth.

Dinaric karst region, as one of the globally important subterranean hotspots, is under strong anthropogenic pressure. Our data show that hydrotechnological interventions have impacted all Congeria sites we monitored and due to the interconnectivity of the whole karst system they have likely influenced all C. kusceri and C. jalzici localities. This is clearly visible in the water level data that do not follow the expected natural cycle. Our results show that C. jalzici lives in colder caves and is exposed to much higher water level oscillations than C. kusceri. During periods of low water some of the bivalves become exposed to air, a condition that lasts for more than two months. Uniquely among bivalves, we found individuals exposed to the air to be active during that period.

ACKNOWLEDGMENTS

We are very grateful to cave divers that helped us count the bivalves: Robert Baković, Jana Bedek, Vedran Jalžić and Alen Kirin. In addition, a number of other cave researchers and friends helped during the field work: Ana Komerički, Kazimir Miculinčić, Domagoj Tomašković, Tatjana Vujnović, Andrej Turina, and Markica Vujica. The financial support for the research was provided by The State Institute for Nature Protection, Zagreb, Croatia and The Environmental Protection and Energy Efficiency Fund, Zagreb, Croatia. We are grateful to the Ministry of Culture, Directorate for Nature Protection for issuing the research permits. We are also very grateful to prof. W.R. Jeffery for his useful comments on the manuscript.

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http://dx.doi.org/10.1006/brht.2011.02.007


Usery T.A. & McMahon R.F., 1995 – Comparative study of the desiccation resistance of zebra mussels (Dreissena polymorpha) and quagga mussels (Dreissena bugensis). Technical Report EL-95-6, U.S. Army Corps of Engineers, Waterways Experiment Station: Vicksburg, MS.
Tracer tests are frequently used to delineate catchment area of water supply springs in karstic zones. In the karstic chalk of Normandy, the main tracers used are fluorescent: uranine, sulforhodamine B, naphthionate, and Tinopal®. In this area, a statistical analysis shows that less than half of the injected tracers joins the monitored restitution points and enters the drinking water system where they undergo chlorination. Most of the injected tracers is absorbed in the rock matrix or is thrown out of the aquifer via karstic springs: then it can join superficial waters where it is degraded due to the sun and air action. The paper presents firstly the laboratory degradation of a first batch of fluorescent tracers in contact with chlorine, in order to simulate their passage through a water treatment system for human consumption. A second batch of the same tracers is subjected to agents of natural degradation: ultraviolet illumination, sunlight and air sparging. Most tracers is degraded, and toxicity and ecotoxicity tests (on rats, daphnies and algae) are performed on degradation byproducts. These tests do not show any acute toxicity but a low to moderate ecotoxicity. In conclusion, the most used fluorescent tracers of the Normandy karstic chalk and their artificial and natural degradation byproducts do not exhibit significant toxicity to humans and the aquatic environment, at the concentrations generally noted at the restitution points.
called Normandy (Gombert, 2007, 2008). This is a 10,000 km² chalky area that contains nearly 500 groundwater wells and karstic springs used for water supply (Fig. 1). The previous studies showed that 47% of the injected tracers reach a drinking water extraction point, and 53% of them are lost.

Fig. 1. Location of Normandy in the Paris basin (according to Crampon et al., 1993).

**Nature of tracers**

In the context of limestone (Käss, 1998) as well as in the context of Normandy chalk (Gombert, 2007), fluorescent and ionic tracers are the most common tracers. The fluorescent tracers are mainly used because of their moderate cost, their low detection level and their easy detection method (Goldscheider et al., 2008). The main fluorescent tracers are uranine, sodium naphthionate, sulforhodamines, stilbenes such as Tinopal®, eosin, rhodamines, etc. They are molecules with long carbon chains or aromatic cycles with 10 to 30 carbon atoms. This molecular structure produces the fluorescent phenomena but also some predisposition to adsorption and the degradation process.

We selected the four main fluorescent tracers used in the karstic chalk of Normandy (Gombert, 2008): uranine (C20H10O5Na2), sulforhodamine B (C27H30N2O7S2Na), sodium napthionate (C10H8NSO3Na), and Tinopal® CBS-X (C28H22S6O2Na2). The first two have an intense, distinctive color (green and red, respectively), but the last two are colorless tracers, detectable only by ultraviolet excitation.

**Dose of tracers**

Most tracers are available in powder form and must be diluted in water prior to injection. The required dose is generally calculated by empirical formulas (Field, 2003) mainly dependent on the nature of the tracer, the distance to trace, the flow of the breakthrough point and/or the concentration expected at this point. The most commonly used dose is approximately 1 kg of tracer per 1 km of distance to trace (Gombert, 2007, 2008). This dose is mainly valid for uranine, and several authors have proposed a correction factor for other tracers: 1 (Gombert, 2008) to 4 (Käss, 1998; Benischke et al., 2007) for sulforhodamine B, 2 for Tinopal® (Gombert, 2008), 2.4 (Gombert, 2008) to 15 (Käss, 1998; Benischke et al., 2007) for naphthionate. However, in the context of the karstic chalk of Normandy, the analysis of 87 tracer injection data provides a 1.4 kg·km⁻¹ average uranine dose: this corresponds to an average 1.4 correction factor, close to the most commonly used empirical dose. This also indicates that the injection solution must be highly concentrated, usually several hundred g·L⁻¹.

At the other end of the tracing system, Gombert & Carré (2011) have examined 210 recoveries of fluorescent tracer tests in the same karstic context: the average concentration calculated at the breakthrough peak is 19 µg·L⁻¹ and the maximum concentration exceeds 500 µg·L⁻¹ in 3% of cases with a maximum of 800 µg·L⁻¹. As a result of these findings, the tracer solutions tested in our experiments were prepared at an intermediate value between the concentrations of the injection point (> 100 g·L⁻¹) and the breakthrough point (< 1 mg·L⁻¹). We chose a representative - and reasonable majorant - value of 1 g·L⁻¹ to provide an idea of the average tracer concentration that must circulate in this chalky karstic aquifer.

**STATE OF THE ART OF THE (ECO)TOXICITY OF FLUORESCENT TRACERS**

**Toxicity studies**

The effects of fluorescent tracers on human health have received little attention (Carré et al., 2007) and no acute toxicity problems related to the use of these products have been reported in any studies. International toxicological databases contain no information on these molecules, and the International Agency for Research on Cancer (IARC) website contains information only for rhodamine B, rhodamine 6G.
and eosin. Similarly, there is no literature on dose-response relationships, at least for oral exposure. In the absence of toxicological reference values and therefore the possibility to quantify the risk, two approaches were developed to decide on the latter and to define the conditions for utilization of the products.

The first approach concerns the toxicity of 12 dyes evaluated by the EPA under a method developed to assess the toxicity of industrial products (Smart, 1984). The risk level is low for uranine, low to medium for sulforhodamine B (and rhodamines B, Wt and G) and for the bleaching agent 28 (Tinopal®, family), and average for the bleaching agents 22 and 351 (Tinopal® family). Moreover, several authors, including Field et al. (1995) and Carré et al. (2007), demonstrated that none of the fluorescent tracers may induce significant health effects if their concentration is maintained below 1–2 mg·L⁻¹ during 24 h. The second approach was applied by a working group, initiated by the German Federal Environment Agency, which focused on the genotoxicity of 11 tracer dyes (Behrens et al., 2001). A genotoxic effect was observed only for rhodamine B, rhodamine 6G and naphthionate. No information is available for sulforhodamine B. The group recommended using uranine, naphthionate and Tinopal® (CBS-X and ABP) without limitations, but the use of rhodamines Wt, B, and 6G was not advised.

Ecotoxicity studies

Data regarding the ecotoxicological properties of fluorescent tracers are scarce. Molinari & Rochat (1978) have described a relatively low ecotoxicological risk for Rhodamine Wt, uranine and Phosphate, and a more important one - but not quantified - for Rhodamine B and Sulforhodamine B. In Behrens et al. (2001), ecotoxicological assessments were based on the determination of acute toxicity to daphnids and to zebrafish. Uranine, eosin yellow, amidorhodamine G, sodium naphthionate, pyranine and Tinopal® (CBS-X and ABP liquid) showed no effects. Conversely, significant inhibitory effects were observed for sulforhodamine B on the mobility of Daphnia magna: the 0% Effect Concentration (EC₀) for 48 h is 0.7 mg·L⁻¹ and the 50% Effect Concentration (EC₅₀) for 48 h is 0.16 mg·L⁻¹. Regarding primary producers, due to interferences caused by the fluorescence of some of the tracers, the authors concluded that the algae test provided no conclusive results and therefore could not be used for ecotoxicological assessments.

Rowinski & Chrzanowski (2011) studied the acute toxicity of rhodamine B and rhodamine WT using larvae of the microcrustacean Thamnocephalus platyurus. Their results showed a higher toxicity of rhodamine B compared with rhodamine WT (EC₅₀ 24 h: 8.1 mg·L⁻¹; EC₅₀ 24 h: 1698 mg·L⁻¹, respectively).

In addition to the data presented above, the environmental database (European Chemical Agency (ECHA)/Registered Substance Database, U.S. Environmental Protection Agency (USEPA)/Ecotox Database) and material safety datasheets (MSDS) submitted by the suppliers of fluorescent tracers gather some EC50 and No Observed Effect Concentration (NOEC) values regarding microcrustaceans and algae (Table 1). Generally speaking, these results showed a low toxicity of the selected tracers towards daphnids compared with algae. According to these data, the algal growth inhibition test seems a more appropriate tool to assess ecotoxicity of these compounds.

Table 1. Ecotoxicity data available in Ecotoxicity database and MSDS. D. pulex: Daphnia pulex; D. magna: Daphnia magna; C. dubia: Ceriodaphnia dubia; D. subspicatus: Desmodesmus subspicatus; LCX: Lethal Concentration for X% of affected individuals; n.a.d.: no available data; * data from Material Safety Data Sheets (MSDS).

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Microcrustacean immobilization test</th>
<th>Algal growth inhibition test</th>
<th>Toxicity on fish</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uranine</strong></td>
<td>EC₅₀ 48 h: 337 mg·L⁻¹ (D. pulex) (USEPA, 2015; Walthall &amp; Stark, 1999)</td>
<td>n.a.d. due to interferences caused by the fluorescence of tracers</td>
<td>LC₅₀ 48 h*: 10 to 100 mg·L⁻¹ (Rainbow trout)</td>
</tr>
<tr>
<td><strong>Sulforhodamine B</strong></td>
<td>EC₅₀ 48 h: 0.16 mg·L⁻¹ (Behrens et al., 2001)</td>
<td>n.a.d. due to interferences caused by the fluorescence of tracers</td>
<td>LC₅₀ 48 h*: 100 to 500 mg·L⁻¹ (Rainbow trout)</td>
</tr>
<tr>
<td><strong>Sodium naphthionate</strong></td>
<td>EC₅₀ 48 h: 2791 mg·L⁻¹ (D. magna) Predicted data QSAR toolbox (ECHA, 2015)</td>
<td>EC₅₀ 72 h: 63.2 mg·L⁻¹ (P. subcapitata) Predicted data QSAR toolbox (ECHA, 2015)</td>
<td>n.a.d.</td>
</tr>
<tr>
<td><strong>Tinopal®</strong></td>
<td>EC₅₀ 24 h*: &gt;1000 mg·L⁻¹ (D. magna) (ECHA, 2015)</td>
<td>NOEC 72 h: 3.13 mg·L⁻¹ (D. subspicatus) (ECHA, 2015)</td>
<td>LC₅₀ 96 h*: 76 mg·L⁻¹ (Zebra fish)</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS OF NEW DEGRADATION EXPERIMENTS**

**Tracer degradation protocols**

Two different degradation protocols have been used to simulate the two main degradation conditions of fluorescent tracers: (i) a strong and rapid degradation due to contact with artificial oxidizing agents, such as chlorine, during transit through the water treatment system used for drinking water production, and (ii) a low degradation due to contact with the natural oxidizing agents sunlight and air, during the outflow of traced groundwater out of the karst.

To simulate rapid degradation due to strong oxidizing agents, a volume of 1 L of each tracer solution at a concentration of 1 g·L⁻¹ was submitted for 20 minutes to sparging with chlorine gas at a temperature of 20°C (Fig. 2A). This chlorine was produced by a chemical reaction due to the introduction of 100 mL of 50% sulfuric acid in 600 mL of bleach containing 2.6% active chlorine. At the end of the sparging, the mixture of tracer and chlorine solution was left in contact for half an hour to simulate contact between water and chlorine in drinking water treatment basins.

To simulate low degradation due to the gentle natural agents sunlight and air, a volume of 2.5 L of
each tracer solution at a concentration of 1 g∙L⁻¹ was poured into a flat 50 x 35-cm box, forming a 3-cm thick film, and subjected for 24 hours to the following conditions (Fig. 2B): (i) aeration by continuous agitation using 4 magnets turning at low speed and (ii) a simulated 20,000 lux illumination with a 4,000-K white luminotherapy sunlight lamp and a 400-W UV lamp. Note that, for comparison, at its zenith the summer sun emits a 5,800-K light with an average intensity of 50,000 lux, and that we need to add a UV lamp because the luminotherapy lamp used here has a UV filter.

Analytical methods
The tracer solutions, before and after degradation, were analyzed by a high-performance liquid chromatography (HPLC) instrument, the DIONEX U3000. The injected sample volume was 20 µL. The separation of compounds was carried out with a C8 column, 250 mm in length and with a particle size of 5 µm, with an elution gradient and at a rate of 0.6 to 1 mL·min⁻¹. The eluent phase was composed of water and acetonitrile. Compounds were detected using two sensors connected in series: a fluorimeter and a diode-array.

The use of a diode-array detector helped to identify the wavelength of maximum absorption for the detection of compounds by plotting the UV spectrum of the molecule. Fluorescence detection, more sensitive than the UV method, was used at the following wavelengths (excitation/emission): 455/510 nm for uranine, 561/581 nm for sulforhodamine B, 420/454 nm for naphthionate and 340/429 nm for Tinopal®. Thus, only compounds with a chemical formula or with excitation and emission properties similar to those of the tracers could be detected at these wavelengths.

The degradation products that appeared were then isolated in the eluate from the liquid chromatography column and analyzed by mass spectrometry (LC-MS) according to both ionization modes (positive Electro-Spray Ionization ESI⁺ or negative Electro-Spray Ionization ESI⁻), to isolate their mass spectrum in order to identify the molecules.

Toxicity tests
The toxicity of tracers without degradation is known from the literature and has been compiled by Gombert & Carré (2011). We then studied the acute toxicity of the degradation products of tracers after chlorination by oral administration to rats. The tracer solutions have been tested according to a protocol derived from the OECD guideline for testing chemicals no. 423 “Acute oral toxicity – Acute toxic class method”.

Batches of 3 female rats were formed and the animals received the test solution administered orally at a concentration of 10 mg·kg⁻¹ in a single dose on the first day. The animals had fasted overnight and access to food was restored 4 hours after treatment. The dose tested belongs to group II of the packing groups described in “abstract ADR 2005-toxic substances,” corresponding to moderately toxic substances (LD50 > 5–50 mg·kg⁻¹). A control batch received the medium alone under the same conditions as the animals tested. All animals were observed for 14 days during which their weight, clinical signs and mortality were recorded daily. They were then sacrificed and a complete autopsy was carried out by macroscopic examination.

Ecotoxicity tests
The two standardized tests, described in Table 2, were selected to assess the ecotoxicity of the tracers’ solutions after the degradation steps, i.e., the Daphnia magna immobilization test and the algal growth inhibition test. These two organisms are conventionally used to assess the effects of chemicals and effluents on the aquatic environment.

Both tests were carried out according to their respective standardized protocol. The organisms were exposed to a concentration range of the tracers’ solution after the degradation steps and diluted with the respective test medium in order to determine ECx values. These values were derived from the concentration-response curves using a logistic Hill
model. The 95% confidence intervals were estimated using a “bootstrap” simulation method. Calculations were performed with REGTOX software v.7.0.5.

The tracer solutions were filtered on 0.45-µm filters prior to the preparation of the different concentrations for the algal growth inhibition test.

### Table 2. Test conditions of the selected bioassays.

<table>
<thead>
<tr>
<th>Test</th>
<th>Daphnia magna immobilization test</th>
<th>Algal growth inhibition test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organism</td>
<td>Daphnia magna Straus 1820</td>
<td>Pseudokirchneriella subcapitata</td>
</tr>
<tr>
<td>Test method</td>
<td>NF EN ISO 6341</td>
<td>NF EN ISO 8692</td>
</tr>
<tr>
<td>Endpoint</td>
<td>Mobility</td>
<td>Population growth</td>
</tr>
<tr>
<td>Type of effect</td>
<td>Acute</td>
<td>Chronic</td>
</tr>
<tr>
<td>Temperature</td>
<td>20 ± 2°C</td>
<td>22 ± 1°C</td>
</tr>
<tr>
<td>Lighting</td>
<td>Darkness</td>
<td>Continuous lighting (cool white light, within the range 5,760–7,010 lux)</td>
</tr>
<tr>
<td>Test duration</td>
<td>48 hours</td>
<td>72 hours</td>
</tr>
<tr>
<td>Measurement</td>
<td>24 and 48 hours</td>
<td>Cell counting at 24, 48 and 72 hours</td>
</tr>
<tr>
<td>Control and dilution medium</td>
<td>Synthetic medium (NF EN ISO 6341)</td>
<td>Synthetic medium (NF EN ISO 8692)</td>
</tr>
<tr>
<td>Agitation</td>
<td>None</td>
<td>Continuous (125 rpm, orbital shaker)</td>
</tr>
<tr>
<td>Test design</td>
<td>4 replicates per test condition, 4 controls</td>
<td>3 replicates per test condition, 6 controls</td>
</tr>
<tr>
<td>Test vessel</td>
<td>Glass tubes filled with 10 mL of test solution</td>
<td>Erlenmeyer flasks filled with 100 mL of test solution</td>
</tr>
<tr>
<td>Number of organisms</td>
<td>5 per replicate</td>
<td>Initial density: 10000 cells∙mL^{-1}</td>
</tr>
<tr>
<td>Expression of results</td>
<td>EC50 48 hours</td>
<td>EC10, EC50 72 hours</td>
</tr>
</tbody>
</table>

**TOXICITY AND ECOTOXICITY OF DEGRADED TRACERS**

### Nature of degradation byproducts

The analytical results obtained by HPLC are presented in Fig. 3.

![Fig. 3. Chromatograms of fluorescent tracers in fluorescence detection. A) Uranine; B) Sulforhodamine B; C) Naphtionate; D) Tinopal®.](image)

International Journal of Speleology, 46 (1), 23-31. Tampa, FL (USA) January 2017
Uranine

After chlorination, we observed the complete disappearance of uranine, for which the retention time is 13.5 min. (Fig. 3A). In addition, we noted the appearance, in fluorescence detection, of several fluorescent compounds, the most significant of these having a retention time of about 7 min. Unlike other minor compounds observed, however, this fluorescent compound was not detected by UV.

Four fractions of the eluent containing four major peaks detected by fluorescence were collected at around 5, 7, 15, and 16 min. These fractions were analyzed by LC-MS in an attempt to identify the generated degradation compounds. However, regardless of the ionization mode, it was not possible to isolate their mass spectrum, probably because their concentrations were too low.

Another breakdown product, not fluorescent but detectable by UV at 210 nm and 220 nm, was generated. Its HPLC retention time is 3.5 minutes. However, this compound is not present in sufficient quantities to allow for a possible identification by LC-MS.

Regarding light and air degradation (see also Fig. 3A), we observe a 10% decrease of the initial concentration, but no byproducts were noted either with UV or with fluorescence. That means the byproducts induced by the partial degradation of uranine are not fluorescent, and/or their concentration is too low to be detectable with UV.

Sulforhodamine B

After chlorination, we observed the complete disappearance of sulforhodamine B, for which the retention time is 1.30 min, (Fig. 3B). In addition, we noted the appearance in fluorescence detection of a single fluorescent compound at a retention time of 1.48 min. Due to the significant dilution factor, the signal intensity of this new peak is about 500 times smaller than that of the initial sulforhodamine B peak.

The fraction corresponding to this peak was not collected for LC-MS analysis but a chromatogram in UV detection was performed. At a 210-nm wavelength, a main peak appears at 1.36 min., for which a UV spectrum was plotted. The latter showed that the newly formed product has, instead of the 559-nm initial peak of sulforhodamine B, a main absorption peak at 196 nm and a secondary peak at 297 nm.

Note: for technical reasons, the degradation of this tracer by light and air action could not be carried out.

Naphtionate

During the chlorination of this tracer, with a retention time of 5 min., there is a decrease of about 20% in its concentration. In addition, a degradation compound appeared at a retention time of 13 min. This new compound is detectable by fluorescence (Fig. 3C) but hardly detectable in UV.

The fraction corresponding to this peak has been collected and analyzed by LC-MS in an attempt to identify the generated degradation compound. However, it was not possible to isolate the mass spectrum of this compound regardless of the ionization mode.

Regarding light and air degradation (see also Fig. 3C), we observe a 15% decrease of the initial concentration. A byproduct also appears that is easily detectable by fluorescence but very weakly by UV. As its retention time is similar to those induced by chlorination (13 min.), this byproduct seems to be the same.

Tinopal®

During the chlorination of this tracer, for which the retention time is 7.8 min., we observed the appearance of a degradation compound at a slightly lower retention time (7.6 min.) accompanied by other products between 6.5 and 10 minutes (Fig. 3D). These new compounds were also detected in UV.

The fraction of eluent containing the predominant peaks detected by UV (between 6.5 and 7.6 min., i.e., just before the Tinopal®) was collected. This fraction was analyzed by LC-MS in an attempt to identify the degradation compounds generated. No degradation products could be highlighted in ionization ESI+, but in ionization ESI-mode, an ion of molar mass 185 was detected (Fig. 4A). This molar mass corresponds to that of the benzaldehyde-2-sulpho-acid salt mentioned in previous studies as a degradation product of Tinopal® (Richner & Kaschig, 1999). The compound causing this mass peak was isolated and fragmented in MS/MS (Fig. 4B). This operation consists in subjecting the product to a bombardment of argon under pressure to break the molecules into sub-constituents of which the molar mass can then be calculated. The spectrum obtained confirmed this assumption: the presence of an ion of molar mass 80 (likely the ion SO₃⁻), of an ion of molar mass 157 (likely the benzene sulfonic acid C₆H₅SO₃⁻).

Regarding light and air degradation (see also Fig. 3D), we observed a 30% decrease but no byproduct was detected by fluorescence or UV. That means the byproducts induced by partial degradation of Tinopal® are not fluorescent, and/or their concentration is too low to be detectable by UV.

Toxicity of degradation byproducts

The pH of the solutions of degraded tracers was in the appropriate range to expose rats to the Tinopal® and the naphtionate solutions but too low for the uranine solution (approximately 2.2). This solution has therefore been diluted to 1/3 prior to administration; the final concentration of the tested degraded solutions is therefore 1 g·L⁻¹ for Tinopal® and naphtionate and 0.33 g·L⁻¹ for uranine. Given this low pH, sustained monitoring has been performed, but the animals showed no clinical signs. This suggests either that the product was injected directly into the stomach (the low pH of which is compatible with that of the injected product), or that it was not overly harmful to the esophagus. In the absence of mortality and taking into account the absence of an inflammatory reaction observed during the autopsy on the esophagus, OECD Test Guideline 423 advocates in a 2nd phase exposing a new batch of three rats to the same concentration in order to achieve a satisfactory statistical ability to determine whether or not the product is toxic at the concentration used.
The pH measured in the degraded solutions of phase 2A was found to be identical to that of phase 1.

None of the tracer solutions degraded by chlorine demonstrated toxicity in rats. No deaths or clinical effects were observed from a solution of 0.33 to 1 g·L⁻¹, administered at a dose of 10 mg·kg⁻¹. During the autopsy, direct examination showed no macroscopic anomaly visible on the digestive tract. The average weight curves of animals in the various batches remained perfectly parallel to those of the control group for the two phases, leaving no evidence of the harmful effects of tracer ingestion (Fig. 5). This study thus confirms that the products tested are not of Group I and II and that at concentrations by far exceeding environmental concentrations, they present no toxic risk to rats, and very likely neither to humans.

Ecotoxicity of degradation products

The results obtained for the three selected tracers are reported in Table 3.

Due to the lack of baseline data, as shown in Table 1, this study did not allow determination of the ecotoxicity of the degradation products alone. It therefore focused on the assessment of the ecotoxicity of the solutions of degraded tracers.
These results showed a residual toxicity of the three tracers’ solutions after the degradation steps, allowing classification: sodium naphtionate >> uranine > Tinopal® and a higher sensitivity of P. subcapitata compared with D. magna. For the most toxic one (i.e., sodium naphtionate), the EC50 72 h, expressed as initial nominal concentration of the tracer, was equal to 40 mg L⁻¹. For the other two, the EC50s of the most sensitive species were higher than 750 mg L⁻¹, reflecting the lack of short-term effects of the tested solutions for aquatic organisms.

**CONCLUSION**

In some specific contexts, as in chalky karst of Normandy, only a minority of tracer tests are positive. The injected tracers reach the water point and are introduced into the drinking water supply network. Under the impact of the water purification treatment (most often, disinfection by chlorination), they may degrade into byproducts. If swallowed, tracers and their degradation products could possibly be toxic to humans consuming the water.

A similar problem occurs for tracers that do not reach the targeted water point. They can be partially adsorbed in the aquifer but a significant part can be eliminated via karstic springs. In this second case, they join the surface waters where they may degrade due to sunlight and air interactions. These tracers can also degrade into byproducts, the composition and ecotoxicity of which are unknown for aquatic organisms.

Four of the most frequently used fluorescent tracers were studied here: uranine, sulforhodamine B, naphtionate and Tinopal®. Previous studies have shown their toxicity and ecotoxicity to be globally negligible at the concentrations typically used in tracer tests, but the nature and toxicity of the degradation product(s) of these tracers are not mentioned.

We therefore reproduced in the laboratory an artificial degradation of these tracers by chlorination or by air and sunlight action. Several degradation byproducts identifiable by HPLC appeared in most cases. Because of their low concentration and the strong dilution inherent to the detection technique, it was unfortunately not possible to characterize these degradation byproducts, except for Tinopal®. In this case, the product obtained by chlorination is identical to the one mentioned in the previous studies for degradation of this tracer in natural conditions, i.e., under the influence of sunlight and air. This could mean that, for the large organic molecules that fluorescent tracers are, the weakest chemical bonds are the same regardless of the intensity of the degradation.

For each of the above-mentioned degradation pathways, the toxicity of the byproducts has been tested, except for sulforhodamine B. The tested concentrations are on the order of g L⁻¹, that is to say medial between those used during injection and those observed in recovery points. No signs of acute toxicity have been demonstrated in rats after injection of solutions of degraded tracers. Regarding aquatic organisms, a residual ecotoxicity of these highly concentrated tracer solutions has been observed, mainly for sodium naphtionate.

In conclusion, these four fluorescent tracers have been tested at concentrations in excess of 50 times the maximum observed in recovery at drinking water capturing sites in the chalk karstic aquifer of Normandy. The tracers and their degradation byproducts appear to have no significant toxicological and ecotoxicological effects at the concentration commonly found in such context.

To obtain a complete view of the toxicity of the main hydrogeological tracers, work remains to be done for other fluorescent (amino G acid, eosin, etc.) or ionic (iodide, bromide, lithium, etc.) tracers.

**ACKNOWLEDGEMENTS**

We thank the DREAL (Regional Directorate of Environment, Planning and Housing) of Normandy (France) for financing this study on the degradation of four of the main fluorescent tracers used in the karstic chalk of Normandy. We are also grateful to our three anonymous reviewers for their constructive comments.

**REFERENCES**


Petrographical and geochemical changes in Bosnian stalagmites and their palaeo-environmental significance

Veronica Chiarini¹,²*, Isabelle Couchoud²,³, Russell Drysdale²,³, Petra Bajo³, Simone Milanolo⁴,⁵, Silvia Frisia⁶, Alan Greig⁷, John Hellstrom⁷, and Jo De Waele¹

¹Department of Biological, Geological and Environmental Sciences (BIGEA), University of Bologna Alma Mater, Italy
²Laboratoire EDYTEM, UMR CNRS 5204, Université Savoie Mont Blanc, Le Bourget du Lac, France
³School of Geography, University of Melbourne, Australia
⁴Hydro Engineering Institute Sarajevo – HEIS, Sarajevo, Bosnia and Herzegovina
⁵Center for Karst and Speleology – CKS, Sarajevo, Bosnia and Herzegovina
⁶Earth Sciences, SELS, The University of Newcastle, NSW Australia
⁷School of Earth Science, University of Melbourne, Australia

Abstract: Detailed petrographic observations have been coupled with trace element and δ¹³C - δ¹⁸O analyses in order to investigate their dynamics in two Holocene Bosnian speleothems. The potential of this multiproxy approach in providing a means to extract palaeo-environmental information from stalagmites whose stable isotope signals are noisy and without obvious trends has been tested. The studied stalagmites are mostly characterized by columnar microcrystalline fabric. At the sub-millimetre scale of lamination, different microcrystalline columnar sub-types (open and closed) have been detected and classified on the basis of the observed porosity and the crystallite size. The presence of variations in crystallite arrangement at the lamina scale suggests the occurrence of small-scale environmental changes recorded in the studied samples. A positive correlation was found between Mg concentration, δ¹³C and fabric variations, while a negative correlation relates those parameters with Sr concentration. Both δ¹³C and fabric changes appear to be directly related to changes in hydrology. The detailed observation of calcite fabrics combined with stable isotope and trace element profiles allowed for the interpretation of the conditions under which the speleothems were deposited.

Keywords: speleothems, stable isotopes, trace elements, calcite fabrics, microstratigraphy

Citation: Chiarini V., Couchoud I., Drysdale R., Bajo P., Milanolo S., Frisia S., Greig A., Hellstrom J. and De Waele J., 2017. Petrographical and geochemical changes in Bosnian stalagmites and their palaeo-environmental significance. International Journal of Speleology, 46 (1), 33-49. Tampa, FL (USA) ISSN 0392-6672 https://doi.org/10.5038/1827-806X.46.1.2057

INTRODUCTION

Stalagmites are considered excellent continental materials for the study of past climate changes thanks to their formation in a relatively stable environment, protected from erosion and weathering processes, and to the possibility of obtaining precise ages with the U-Th disequilibrium technique. Oxygen and carbon stable isotope ratios are the most commonly used climate and environmental proxies extracted from stalagmites (e.g., McDermott, 2004; Fairchild & Baker, 2012). The interpretation of the climate signal relies on the assumption that carbonate precipitation and stable isotope fractionation occurred under equilibrium conditions. This condition has been routinely validated with the Hendy test (1971). However, this test presents some limitations (e.g., Dorale et al., 2002; Spötl & Mangini, 2002; Couchoud, 2008; Dorale & Liu, 2009; Lachniet, 2009). In fact, there are cases in which stalagmites apparently affected by disequilibrium fractionation still preserve climate signals (e.g., Dorale & Liu, 2009; Lachniet, 2009; Kennett et al., 2012; Ridley et al., 2015). In addition, even stalagmites influenced by disequilibrium isotope fractionation, which occurs more frequently than previously thought, can indirectly provide information about climate and environmental changes, as disequilibrium conditions can be triggered by factors related to climate (e.g., Hellstrom et al., 1998; Plagnes et al., 2002; Genty et al., 2006; Lachniet, 2009).

The fabric of stalagmites (i.e., the shape and arrangement of the calcium carbonate crystals) changes under different physical and hydrochemical conditions, and provides additional information on the environment of formation. This can facilitate the
interpretation of the stable isotope signal (e.g. Frisia et al., 2000; Couchoud, 2006; Frisia & Borsato, 2010; Mattey et al., 2010; Belli et al., 2013; Riechelmann et al., 2014; Frisia, 2015). According to Frisia (2015), the speleothem fabric is a useful, supplementary tool to recognize the presence, and evaluate the intensity, of disequilibrium isotopic fractionation. For example, calcite fabric and the $\delta^{13}C$ signal can constrain interpretations because this isotopic ratio is more responsive to conditions associated with local soil CO$_2$ production and hydrology. In particular, drip-rate variations influence the duration of degassing, which can result in enrichment in $^{13}C$ in the solution covering the stalagmite (Dreybrodt, 1988; Usdowski & Hoefs, 1990).

The most recent and comprehensive speleothem calcite fabric classification has been proposed by Frisia (2015). It reports a methodology for fabric identification and for the sequential coding of fabrics, reflecting, in a hierarchic system, the conditions of precipitation: drip rate, supersaturation, Mg/Ca ratio, and the presence of impurities in the feeding waters. The coded fabrics can then be plotted along with the other geochemical parameters on a time- or depth scale of the speleothem.

In this study, we examined two stalagmites found already broken in Mračna Pećina Cave (Bosnia and Herzegovina). We carried out a detailed petrographic study and combined these with trace element and stable isotope measurements to explore the possibility of extracting palaeo-environmental signals from samples that present high-variability (i.e., noisy) stable isotope profiles that apparently lack trends that might be interpretable in terms of past climate changes.

**STUDY SITE**

Mračna Pećina (also known as Banja Stijena) is a relatively small cave located on the left flank of the narrow valley cut by the Praća River (ca. 45 km East of Sarajevo, Republika Srpska, Bosnia and Herzegovina, Fig. 1). The cave was discovered at the beginning of the 20th century; an artificial entrance and concrete stairs were built between the First and Second World War to allow tourists to access the first chamber (Daneš, 1921). The artificial entrance is located at 597 m a.s.l., about 20 m above the Praća River, at the foot of a limestone cliff. The natural entrance, now occluded by a rockfall, is located on the north-eastern upper side of the first chamber, not far from the artificial entrance (43°46'20.534” N; 18°53'14.049” E).

The cave has developed at the foot of the Romanija Plateau, which reaches the highest elevation of 1500 m a.s.l. in its western part, while the central and southern parts overlying the cave reach elevations between 800 and 1,000 m a.s.l. The Romanija massif consists of a Triassic succession of basal sandstone followed by massive limestone with ammonites, crinoids, and lenses of dolomite, reef limestone and light grey limestone with megalodonts and dolostone. The Mračna Pećina Cave network develops in the Triassic massive limestone and is characterised by a W-E orientation, coherent with a major thrust line that separates the massive reef limestone from the massive limestone containing ammonites and dolomites. The cave comprises a chamber 10 m wide and up to 6 m high, decorated with stalactites and flowstones, and located close to the entrance. A series of galleries totalling 1,148 m in length occurs on the same principal level, with a total depth with respect to the entrance of 37 m. In the entrance area, a dark coating is ubiquitous on the cave walls, and is probably related to the use of torches during tourist visits at the beginning of the last century.

Nowadays, the surface above the cave is modified due to grazing and timber cutting (Milanolo et al., 2013). Evidence of human occupation and activities in the area dates back to the Neolithic period (Srejović, 1994). The plateau is characterised by conifer-dominated woods and pastures, while the valley where the cave entrance is located hosts deciduous vegetation. The present-day climate is temperate, with warm summers and cold and snowy winters. Rainfall is distributed all year round with the absence of a distinct dry season. Annual precipitation in the Sarajevo region reaches 946 ± 160 mm, while mean annual temperature is 11.0 ± 0.7°C (for the time interval 1992-2015; Sarajevo meteorological station, Federal Institute of Hydrometeorology BIH; Fig. 2).
MATERIALS AND METHODS

The sampled stalagmites

The two stalagmites examined in this study (BS14 and BS15) were found already broken near the main chamber (between 50 and 100 m from the artificial entrance). Although it has not been possible to reconstruct their original position, the presence of a dark coating on their surfaces, similar to the one deposited on the cave walls and other stalagmites in this area, suggests the broken specimens were lying close to their original positions in the cave. They are both characterised by a flat and regular lamination and by a squat shape (BS14: 9 cm tall and average diameter of 7.5 cm; BS15: 10 cm tall and average diameter of 6.5 cm) (Fig. 3). Preliminary dating was performed with the U/Th method at the University of Melbourne (School of Earth Science). Uranium and thorium were separated from the bulk of the carbonate matrix through a procedure involving the dissolution of the sample and addition of a mixed spike that enables to constrain the yield of the procedure. The uranium and thorium of the spiked samples were extracted by elution through TRU-Spec columns. Ages have been corrected using a \(^{230}\text{Th}/^{232}\text{Th}\) of 0.30 ± 0.20 for BS14 and 0.65 ± 0.19 for BS15, which have been calculated according to the stratigraphic approach described in Hellstrom (2006). The results show that these stalagmites grew during the later half of the Holocene (from ~ 6 to ~2 ka for BS14 and from 4 to 1 ka for BS15; see Table 1).

Stable isotope analyses

Samples for \(\delta^{13}\text{C}\) and \(\delta^{18}\text{O}\) analyses were drilled along the vertical growth axis at a 1 mm increment. About 2 mg of powder were collected for each sample using a 1 mm diameter drill bit mounted on a MicroProto systems MicroMill 2000. Samples designed for the Hendy test (Hendy, 1971) were drilled every 3 to 5 mm along each of three distinct laminae on each stalagmite using a 0.6 mm diameter drill bit (see sampling tracks in Fig. 3).

All carbonate isotope samples were prepared and analysed at the School of Geography, University of Melbourne. For the growth-axis samples, about 0.7 to 0.8 mg of powder were weighed into glass vials, which were purged with helium before sample acidification using 105% H\(_3\)PO\(_4\) at 70°C. The sample CO\(_2\) gas was then carried into an Analytical Precision AP2003 continuous-flow isotope-ratio mass spectrometer using an ultra-high-purity (99.9995%) helium carrier gas. External reproducibility was better than 0.05 and 0.10‰ for C and O, respectively. For the Hendy test samples, about 0.05 to 0.10 mg of calcite powder were weighed and placed in glass vials and analysed on a Nu Instruments Perspective dual-inlet isotope-ratio mass spectrometer. The samples were acidified with 105% H\(_3\)PO\(_4\) at 70°C in a Nu Instruments NuCarb sample preparation unit and the sample gas CO\(_2\) admitted into the ion source under vacuum.
Table 1. U-Th ages of stalagmites BS14 and BS15 performed in the laboratory of the School of Earth Sciences at the University of Melbourne (April 2015) according to methods exposed in Hellstrom (2006) and Drysdale et al. (2012).

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>$^{238}$U (ng/g)</th>
<th>2σ</th>
<th>Depth (mm from top)</th>
<th>$^{230}$Th/$^{238}$U</th>
<th>2σ</th>
<th>$^{234}$U/$^{238}$U</th>
<th>2σ</th>
<th>Uncorrected Age (ka)</th>
<th>2σ</th>
<th>$^{232}$Th/$^{238}$U</th>
<th>2σ</th>
<th>$^{230}$Th/$^{232}$Th</th>
<th>2σ</th>
<th>Corrected age (ka)</th>
<th>2σ</th>
<th>2σ</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS14-2e</td>
<td>21.36</td>
<td>1.60</td>
<td>2 ± 0.5</td>
<td>0.0395</td>
<td>3.56E-3</td>
<td>1.4754</td>
<td>0.0058</td>
<td>2.956</td>
<td>0.274</td>
<td>0.04263</td>
<td>1.3E-3</td>
<td>0.30 ± 0.20</td>
<td>0.92567</td>
<td>2.011</td>
<td>0.690</td>
<td></td>
</tr>
<tr>
<td>BS14-3a</td>
<td>38.66</td>
<td>2.94</td>
<td>45.5 ± 1</td>
<td>0.0681</td>
<td>3.72E-3</td>
<td>1.3737</td>
<td>0.0189</td>
<td>5.534</td>
<td>0.318</td>
<td>0.00289</td>
<td>0.5E-4</td>
<td>0.30 ± 0.20</td>
<td>23.6022</td>
<td>5.462</td>
<td>0.322</td>
<td></td>
</tr>
<tr>
<td>BS14-1a</td>
<td>34.47</td>
<td>2.59</td>
<td>80.5 ± 1</td>
<td>0.0814</td>
<td>2.22E-3</td>
<td>1.3174</td>
<td>0.0064</td>
<td>6.939</td>
<td>0.196</td>
<td>0.03285</td>
<td>1.9E-4</td>
<td>0.30 ± 0.20</td>
<td>2.47759</td>
<td>6.121</td>
<td>0.578</td>
<td></td>
</tr>
<tr>
<td>BS15-2d</td>
<td>29.67</td>
<td>2.23</td>
<td>3 ± 0.5</td>
<td>0.0143</td>
<td>1.36E-3</td>
<td>1.5043</td>
<td>0.0064</td>
<td>1.041</td>
<td>0.103</td>
<td>0.00257</td>
<td>0.7E-4</td>
<td>0.65 ± 0.19</td>
<td>5.57631</td>
<td>0.920</td>
<td>0.109</td>
<td></td>
</tr>
<tr>
<td>BS15-1c</td>
<td>38.03</td>
<td>2.86</td>
<td>51 ± 1</td>
<td>0.0276</td>
<td>1.86E-3</td>
<td>1.3770</td>
<td>0.0057</td>
<td>2.206</td>
<td>0.154</td>
<td>0.00149</td>
<td>0.2E-4</td>
<td>0.65 ± 0.19</td>
<td>18.4251</td>
<td>2.130</td>
<td>0.156</td>
<td></td>
</tr>
<tr>
<td>BS15-2a</td>
<td>26.87</td>
<td>2.02</td>
<td>98 ± 1</td>
<td>0.0576</td>
<td>4.54E-3</td>
<td>1.4226</td>
<td>0.0054</td>
<td>4.498</td>
<td>0.357</td>
<td>0.00409</td>
<td>1.1E-4</td>
<td>0.65 ± 0.19</td>
<td>14.1026</td>
<td>4.295</td>
<td>0.362</td>
<td></td>
</tr>
</tbody>
</table>
External reproducibility was 0.03 and 0.06‰ for C and O, respectively. Sample data from both instruments were normalised to the V-PDB scale using two in-house calcite standards (a Carrara Marble standard, ‘NEW1’, and a powdered homogeneous calcite prism of unknown origin, ‘NEW12’), both previously calibrated against the international reference standards NBS-18 and NBS-19.

**Trace element analyses**

Trace element concentrations were analysed continuously along the vertical growth axis of stalagmite BS15 at the School of Earth Sciences (University of Melbourne) by a Helex laser-ablation system, equipped with a customised Resonetics Resolution 193 nm ArF laser system, in conjunction with an Agilent 7700x quadrupole ICP-MS instrument, as described in Woodhead et al. (2007) and Drysdale et al. (2012). The polished speleothem surface was pre-ablated with a 188-µm-diameter laser spot at an intensity of 10 Hz in order to eliminate surface contamination. A 8 x 140 µm laser slit and a pulse rate of 10 Hz was used for the analysis, which was carried out in a high purity helium atmosphere. One continuous track has been performed at the velocity of 30 µm/s, covering the entire length of stalagmite BS15. In order to compare the data with the isotopic values and conform the statistical analyses, an average value for each element has been calculated at a millimetre interval corresponding to the stable isotope profiles. Correlation coefficients have been calculated between individual trace elements and between the trace element and stable isotope values.

**Petrography**

Both stalagmites were cut along their vertical growth axis. The two halves were polished and subsequently scanned at high resolution for stratigraphic observation. Two thin sections containing the full length of both stalagmites were made. Petrography was studied on an Olympus BH-2 BHS polarising microscope. Pictures of magnified thin sections were taken with a Leica MC 120 HD camera mounted on the microscope.

Growth discontinuities were identified and calcite textures were recognised and classified following the criteria proposed in Frisia (2015). At the scale of lamination, calcite crystallite size was inferred on the basis of: 1) the composite crystallite boundary geometries, from which intracrystalline microporosity has been deduced; and 2) intercrystalline porosity features (i.e., pore orientation and geometry and their relative distribution). From this, a sub-classification scheme for the columnar microcrystalline texture was constructed for this case study. Following the hierarchical classification of fabrics proposed by Frisia (2015), a ranking has been associated with each fabric subtype that expresses a progressive increase of inter- and intra-crystalline porosity within the microcrystalline subtypes, which is related to the presence of impurities directly affecting the crystallite lattice development. In addition, by analogy with the calcite classification proposed in Frisia (2015), which was considered as a starting point for this specific subdivision, the deposition of the classified textures under increased hydrological stress (i.e., less regular dripping) is inferred, which may also have influenced the stable isotope fractionation of the precipitated calcite.

**Statistical analyses used for comparing petrography and geochemical signals**

Each petrography class was examined on the basis of calcite stable isotope and trace element composition to investigate the possible presence of geochemical signatures. In order to test whether isotopic variations are associated with petrographic changes, a Kruskall-Wallis test was performed. An iso-cope-fabric plot (IsoFab) was built according to the method described by Frisia (2015), calculating the average δ13C and δ18O values for each fabric and the associated standard deviations. In order to investigate the statistical significance of isotopic differences/similarities between the petrography groups, multiple comparisons using the Bonferroni correction have been performed (e.g., Dunn, 1961). Finally, to further investigate the presence of geochemical signatures in the classified petrography groups, discriminant analysis was performed using SPSS IBM software®, considering first the δ13C and δ18O composition of petrography classes in stalagmites BS14 and BS15, then δ13C, δ18O, Sr, and Mg composition (e.g., Green et al., 2008). Indeed, this particular multivariate analysis allows for the attribution of single individuals to different groups according to chosen variables. These variables are used to construct canonical discriminant functions as a function of which individuals are plotted.

**RESULTS**

**Stable isotope profiles (δ13C and δ18O)**

The stable oxygen isotope profiles in both samples do not have any evident long-term trend and are dispersed in the same range of values (BS14 δ18O mean value: -8.0 ± 0.4‰; BS15 δ18O mean value: -8.1 ± 0.3‰; Fig. 4). Conversely, δ13C values are much more dispersed in the BS14 record than in BS15 (BS14 δ13C mean value: -8.4 ± 1.0‰; BS15 δ13C mean value: -9.4 ± 0.4‰). While BS14 shows no evident trend, a long-term trend towards higher δ13C values is evident in the upper half of BS15, starting at 40 mm from the top (Fig. 4).

Stable oxygen and carbon isotope fluctuations show a statistically significant correlation in stalagmite BS14 (r2 = 0.466), suggesting that calcite precipitation likely occurred under disequilibrium conditions (Hendy, 1971). In stalagmite BS15, stable oxygen and carbon isotope values show no significant correlation (r2 = 0.095), suggesting that precipitation possibly occurred closer to isotopic equilibrium (although equilibrium conditions are unlikely in most cave environments; Mickler et al., 2006 and references therein). To support this first inference, we performed the Hendy test on a few laminae:
• BS14 stalagmite:
The samples along the lamina at 59 mm from the top (H3 in Fig. 5) show a lateral enrichment of 0.86‰ for \( \delta^{13}C \) and of 0.51‰ for \( \delta^{18}O \), suggesting significant disequilibrium fractionation. The two laminae sampled at 31.5 and 16.5 mm from the top (H2 and H1 in Fig. 5) show a smaller lateral variation in stable isotope values (0.32‰ and 0.13‰ for \( \delta^{13}C \) and 0.17‰ and 0.26‰ for \( \delta^{18}O \), respectively) pointing to a lower degree of disequilibrium fractionation in this portion.

• BS15 stalagmite:
Variations along the laminae at 79 mm and 32.5 mm from the top are comparable (0.20‰ and 0.16‰ for \( \delta^{13}C \) and 0.17‰ and 0.26‰ for \( \delta^{18}O \), respectively; H3 and H2 in Fig. 5). Samples from the lamina at 8 mm from the top show stronger fluctuations for \( \delta^{13}C \) values (range of 0.62‰; H1 in Fig. 5) while \( \delta^{18}O \) values are more stable (range of 0.18‰), suggesting the possible occurrence of prolonged CO\(_2\) exchange with the cave air related to long residence time of a thin film of fluid at the growing stalagmite surface in this portion of the stalagmite (Dreybrodt & Scholz, 2011; Hansen et al., 2013).

Trace element concentration in stalagmite BS15
Trace elements were analysed by laser ablation only in stalagmite BS15. However, only the calculated average values at the millimetre scale have been considered for this study (Fig. 6). This choice was made to enable consistent comparisons amongst the proxies (i.e., trace elements, petrography and stable isotope profiles).

Correlation coefficients were calculated between each analysed element in order to support graphic observations (Table 2). Among divalent cations (Sr, Ba, and Mg), the results show a strong covariation...
between Sr and Ba, and an antiphase trend between Mg and both Sr and Ba. The trivalent cation Y, the metals Cu and Zn, and the electronegative element P are linked by positive correlations, resulting in common trends. These elements are characterised by strong adsorption behaviour in soils, compared to the solution preference of the divalent cations like Sr and Mg (Borsato et al., 2007 and reference therein). No correlation is found between Mg and P. However, from the observation of their profiles, it is possible to distinguish a trend towards higher values from about 40 mm from the top (Fig. 6). The Mn signal shows little variability and has a long-term trend towards slightly higher values until 28 mm from the top, when it starts decreasing again. Considering Th, even if its concentration is just above the instrument detection limit, it shows a higher frequency of spikes in the upper part of the stalagmite starting ca. 48 mm from its top (Fig. 6).

**Petrography**

The polished sections of both stalagmites appear compact (especially stalagmite BS15) and finely laminated in the central portion, while the flanks are more porous and milky. On the polished sections the presence of composite columnar crystals (sensu Frisia et al., 2000) elongated along the growth direction can be distinguished. Two main “palaeo” surfaces marking the temporary interruption of the stalagmite growth have been identified on the polished surface of stalagmite BS14 (at 15 and 25 mm from the top; 1 & 3 in Fig. 3) and one in BS15 (at 18 mm from the top; surface 1 on Fig. 3). These surfaces have been recognised also in thin section as sharp interruptions of crystal growth.
Table 2. Pearson correlation coefficients (r) between trace element profiles measured on stalagmite BS15. LOD represents the instrument detection limit. Correlation coefficients (r) have been calculated among the data series using the average trace element content millimetre, and corresponds to the same depth scale as the stable isotope data. Statistically significant correlations are indicated in bold.

<table>
<thead>
<tr>
<th>Trace elements</th>
<th>Mg</th>
<th>P</th>
<th>Mn</th>
<th>Cu</th>
<th>Zn</th>
<th>Sr</th>
<th>Y</th>
<th>Ba</th>
<th>Th</th>
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</thead>
<tbody>
<tr>
<td>LOD</td>
<td>0.021</td>
<td>1.2</td>
<td>0.16</td>
<td>0.014</td>
<td>0.025</td>
<td>0.0021</td>
<td>-</td>
<td>0.00067</td>
<td></td>
</tr>
<tr>
<td><strong>CORRELATION COEFFICIENT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td>0.06</td>
<td>0.54</td>
<td>0.29</td>
<td>-0.01</td>
<td>0.02</td>
<td>-0.62</td>
<td>-0.25</td>
<td>-0.48</td>
<td>0.13</td>
</tr>
<tr>
<td>P</td>
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</tr>
<tr>
<td>p value</td>
<td>0.54</td>
<td>1.3E-6</td>
<td>2.1E-5</td>
<td>1.5E-18</td>
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marked by visible crystal terminations associated with brown, dense surfaces coating crystal tips, especially in stalagmite BS14. Several other similar surfaces, presenting a sharp contact with the underlying laminae, are visible under the microscope only and possibly represent short-term hiatuses (e.g., a surface observed at 18 mm from the top in BS14 presents evidence of crystal-growth competition, demonstrating the presence of a growth interruption; surface 2 in Fig. 3).

In thin section, the textures observed on the polished stalagmite surfaces result mainly in large, columnar, microcrystalline, composite crystals, with the exception of dendritic calcite in a few areas. These composite crystals consist of aggregates stacked with their c-axes oriented parallel to the vertical growth axis of the whole stalagmite, which may show mismatched stacking relative to the vertical growth direction in the upper portion of the stalagmite. The crystallites forming these composite crystals show optical continuity under polarised light. Relatively irregular vertical limits, which alternate with interfingered portions, characterise the composite crystals. This feature suggests subtle transitions to zones of higher proportion of impurities favouring the formation of crystal defects, resulting in high, intracrystalline microporosity (Frisia, 2015; Fig. 7). Brownish lamination related to periodic input of detrital/colloidal particulates is visible in both stalagmites (cf. Frisia et al., 2000).

The following detailed classification of fabrics from our samples is a subdivision of the columnar microcrystalline fabric, based on the observed porosity and composite crystal boundaries, from which crystallite size at the laminae scale was inferred (Fig. 8):

1) Highly compact and translucent laminae consisting of welded crystallites, suggesting negligible presence of particulate and/or foreign

Fig. 7. Vertical log of a portion of the thin section of stalagmite BS15 showing the composite columnar crystals characterised by the presence of relatively straight and irregular/interfingered boundaries. Different calcite crystallites arrangements are visible at the scale of lamination inside the composite crystals.
ions that would create lattice deformation or occlude growth sites. This fabric subtype has here been classified as **compact columnar microcrystalline calcite** (Ccm, Fig. 8a). Laminae formed by this fabric are bound by “wavy” brownish surfaces, corresponding to flattened calcite crystal terminations and to cavities in the below compact carbonate lamina (Supplemental Fig. S1).

2) Some groups of laminae differentiate from the previous fabric (Ccm) by the occurrence of linear intercrystalline porosity. The boundaries between the composite crystals are irregular, suggesting that foreign ions or particulates occluded growth sites. Overall, the laminae appear milkier than in Ccm, and, under the optical microscope, show the presence of colloid-rich brown laminae with similar features as in Ccm. This fabric subtype has been classified as **open columnar microcrystalline calcite** (Ocm, Fig. 8b).

3) Another fabric, observed only in the younger portion of stalagmite BS14, is characterised by elongated aggregates of crystallites (Fig. 8c). The composite crystal boundaries range from straight to interfingered. This is possibly related to a different content in particulate. This fabric is very similar to the elongated columnar calcite (sensu Frisia, 2015), which occurs under relatively high (> 0.3) Mg/Ca ratio of parent drip water associated with relatively fast dripping; when this fabric is associated with micrite and lateral overgrowth, diagenesis may have occurred (Frisia, 2015). However, the elongated appearance in this case is given by the succession of vertically oriented

<table>
<thead>
<tr>
<th>Thin section – Plane polarised light</th>
<th>Thin section – Cross polarised light</th>
<th>Scheme of crystallite arrangements (left)</th>
<th>Sub-patterns of columnar microcrystalline calcite (cm)</th>
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</thead>
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<td><img src="image2.png" alt="Image" /></td>
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<td><img src="diagram4.png" alt="Diagram" /></td>
<td><strong>4) Pcm</strong> “Porous”</td>
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<td><img src="image10.png" alt="Image" /></td>
<td><img src="diagram5.png" alt="Diagram" /></td>
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<td><img src="diagram6.png" alt="Diagram" /></td>
<td><strong>6) D Dendritic</strong></td>
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Fig. 8. Fabric subdivision and classification (plane and cross polarised light) modified from Frisia (2015). The white scale on the picture represents 1 mm. The red frames surround the targeted fabric for each sub-type, as described in the columns on the right.
pores, which, in turn, are characterised by irregular boundaries preventing the identification of size and geometry of single crystallites. This suggests the presence of foreign particles favouring the formation of crystal defects, which are reflected in the observed irregular shapes (Frisia, 2015). Thus, this fabric has been referred to as elongated microcrystalline calcite (Ecm; Fig. 8c).

4) Some sets of laminae consist of microcrystalline fabric, crossed by narrow, vertically oriented linear porosities and composite crystals with irregular boundaries similar to those illustrated by Frisia et al. (2000). Thus, the fabric has been classified as porous microcrystalline calcite (Pcm; Fig. 8d). It differentiates from Ocm by the presence of a less regular intercrystalline linear porosity and a higher intracrystalline porosity inferred from the more irregular boundaries of the composite crystals. As for Ocm, some intervals attributed to the porous microcrystalline subtype contain brownish laminae, testifying the input of colloidal particulates, which, in this specific fabric, must have efficiently occluded growth sites.

5) The last columnar microcrystalline subtype identified in the Bosnian stalagmites is most common in the younger portion of BS15, and rare in BS14. The composite crystals show irregular boundaries more pronounced than in Pcm, but absent, or rare, elongated porosities. This fabric has thus been classified as stricto sensu microcrystalline calcite (SScm; Fig. 8e).

6) The only non-columnar fabric identified in the stalagmites is dendritic (D), as described in Frisia et al. (2000), Couchoud (2006), Banks et al. (2010) and Frisia (2015). It can be observed on the flanks of both stalagmites and in the upper part of BS14 (Fig. 8f). The dendritic fabric is characterised by crystals that are not elongated along the growth axis of the stalagmites, and the composite crystals form branching assemblages, with a 60° angle.

The brownish lamination cited above, which is believed to be related to periodic input of colloidal/detrital particulate, has not been considered for this classification, but has been treated separately, assigning the value 0 to the pattern lacking the brownish lamination and the value 1 to the laminated one. This feature and the fabric subtypes are then represented as a function of distance from the stalagmite top, in profiles that can be compared with the geochemical data to discuss hydrological changes (Fig. 4).

**Statistical approach used to couple petrography and geochemical signals**

According to the statistical approach described in the methods section, the classified petrography groups present a different and specific δ13C and δ18O composition (Kruskal-Wallis test; Table 3). In particular, the IsoFab plot highlighted progressively higher isotopic values from Ccm to D (Fig. 9). The multiple tests between the groups display a statistically different δ13C composition of petrography classes and a less marked δ18O signature (Table 4 and Fig. 9). According to discriminant analyses, 47% (stalagmite BS14) and 55% (stalagmite BS15) can be well classified into their petrography group based on their stable isotope composition: the graphic representation displays the presence of distinct isotopic composition for each petrography group (Fig. 10). The discriminant analyses performed considering 4 variables (δ13C, δ18O, Mg, and Sr) on stalagmite BS15 petrography, results in a good distinction of the different petrography groups (67.7% of samples well classified; Fig. 10).

**DISCUSSION**

**Trace element variations in stalagmite BS15**

The trace element composition of speleothems is related largely to hydrochemical processes in the unsaturated zone overlying the cave (i.e., Roberts et al., 1998; Huang & Fairchild, 2001; Fairchild et al., 2006; Borsato et al., 2007; Fairchild & Treble, 2009). Variations in calcite trace element concentration thus provide information on water-rock interaction, which relates to climate (Fairchild & Treble, 2009). In addition, the type and density of vegetation cover, which can be either related to climate or human activities, can influence trace element variation (Borsato et al., 2007). The understanding of the mechanisms responsible for their fluctuations in a given cave site is crucial for a correct interpretation of the trace element environmental significance.

In the studied stalagmite, a negative correlation appears between Sr and Mg at a millimetre scale (Fig. 11; Table 2). This opposed behaviour, also observed by Roberts et al. (1998) and Treble et al. (2013) would exclude the occurrence of prolonged and intense dry periods, causing relevant phases of prior calcite precipitation (PCP), at least at a decadal time scale. This phenomenon, in fact, would be reflected in a positive correlation of both trace elements (Fairchild & Treble, 2009). Mucci & Morse (1990) demonstrated that temperature influences variations in Mg concentration. However, Mg concentration in parent drip water is also closely related to water residence
time: drier conditions induce longer residence times and allow for selective leaching of Mg from soil or host rock, when the host rock is dolomite or Mg-bearing carbonate/metamorphic/igneous rock (Plummer, 1977; Fairchild et al., 2000). Sr can be related to calcite precipitation rate, where higher concentrations would indicate faster speleothem growth (Huang & Fairchild, 2001). Thus, the antiphased variations of both elements in the studied stalagmite may be related to differential weathering of limestone and dolomite/Mg-bearing carbonate bedrock as a result of groundwater residence time (e.g., Huang et al., 2001). Their trends could thus be explained as the result of hydrological processes: the alternation of relatively dry periods characterised by longer groundwater residence time and wetter periods, with a faster aquifer transmission and, possibly, higher growth rates (cf. Treble et al., 2013).

P concentration in speleothems is commonly interpreted as being related to vegetation die-back or microbial mats (Huang et al., 2001; Treble et al., 2005; Borsato et al., 2007; Fairchild and Treble, 2009). The mobility of P in soils is higher at a pH between 4 and 6 (Giesler et al., 2005), conditions that usually occur in summer (Sartori et al., 2005). However, P is commonly re-adsorbed by the lower-alkaline soil layer, maintaining its mobility only in presence of extremely rapid infiltration, as witnessed by its positive correlation with Y, Zn and Cu (e.g., Borsato et al., 2007). Given that plants consume P during their growing period, and that autumn is one of the wettest periods, its mobilisation should mainly occur during this season, when vegetation becomes dormant in temperate climates (Frisia, 2015 and reference therein). Autumnal organic matter flushing

Table 4. Bonferroni test results between petrography groups in stalagmites BS14 and BS15 for $\delta^{18}O$ and $\delta^{13}C$ ratios. Probability value is indicated for both isotopic ratios. Bold italic values indicate significantly different groups in terms of $\delta^{18}O$ and/or $\delta^{13}C$ composition (0.05 significance level).

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<th>4) Pcm</th>
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International Journal of Speleology, 46 (1), 33-49. Tampa, FL (USA) January 2017
often results in a brownish coating at stalagmite tips that can act as a growth inhibitor. The hypothesis of autumnal flushing of P is further supported by its negative correlation with Sr \( (r = -0.58; p < 0.001) \), since Sr is usually incorporated into stalagmite calcite lattice during period of high growth rate (Huang & Fairchild, 2001).

More frequent Th peaks are finally observed in the upper 40 mm of stalagmite BS15. Considering that Th has an extremely low solubility in water, its increase
in this portion of the stalagmite is likely related to a higher detritus load in the parent drip water.

**Stable isotope variability in stalagmites BS14 and BS15 and comparison with trace element profiles in stalagmite BS15**

In stalagmite BS15, the $\delta^{18}$O and $\delta^{13}$C profiles present similar trends at the centennial scale more visible in its upper half (Fig. 4). Considering the Mg-Sr negative correlation described earlier, the occurrence of prior calcite precipitation (PCP) is excluded as a possible factor for such a long $\delta^{13}$C and $\delta^{18}$O covariation. In stalagmite BS14, covariation of the two stable isotope ratios is observed (Fig. 4). According to Hendy (1971), covariation along the growth axis is one of the indicators of calcite deposition under disequilibrium conditions. However, several other authors recognised the role played by climate fluctuations in causing covariation of $\delta^{13}$C and $\delta^{18}$O (i.e., vegetation changes associated with climate changes; Cerling, 1984; Dorale et al., 1992; 1998). Considering the temperature-dependent fractionation factor (between ~0.37 and 0.17‰/°C) of the stable isotope composition of precipitation in the Balkans (Vreča et al., 2006), the $\delta^{18}$O variation observed in BS15 and BS14 would indicate an average temperature fluctuation of at least $\pm$10°C during the last 5 ka, if calcite precipitated under equilibrium conditions. This is unrealistic for the Holocene. Moreover, the Hendy test was performed on three laminae in both stalagmites to test the conditions of precipitation. It indicates the presence of clear isotope fractionation on lamina H3 of stalagmite BS14, while the remaining laminae tested in BS14 and BS15 display lower stable isotope variations (Fig. 6). However, the asymmetric lateral enrichment of isotope composition observed in these laminae is also inconsistent with calcite precipitated under equilibrium conditions along a single lamina. Thus, it is likely that the thinness of single laminae did not allow a sufficient sampling resolution resulting in isotopic values biased by cross-contamination. Thus, disequilibrium conditions have been clearly identified only in lamina H3 of stalagmite BS14. Although isotopic fractionation cannot be evaluated with reliability by the Hendy test, a palaeo-environmental signal may still be recorded if disequilibrium fractionation (which results of environmental forcing) and environmental changes act in the same direction on the isotopic ratios.

The relation between CO$_2$ dynamics and speleothem calcite stable isotope ratios has been widely investigated. CO$_2$ loss to the cave atmosphere is a fast process that is already well underway by the time the water drop hits the stalagmite surface (Dreybrodt, 2011). However, when calcite starts precipitating, CO$_2$ is produced and released to the cave atmosphere. When this process occurs from a thin film of fluid, it causes $\delta^{13}$C and $\delta^{18}$O enrichment of the DIC (dissolved inorganic carbon) in the solution. While the increase of $\delta^{13}$C is an irreversible process, $\delta^{18}$O ratios can return to equilibrium values through slow isotopic exchange with water in the hydration/dehydration processes of CO$_2$ (Affek & Zaaour, 2014). However, this re-equilibration is an extremely slow process. Dreybrodt & Scholz (2011) and Hansen et al. (2013) demonstrated how the rate of outgassing is related to the thickness of the water film on speleothems, preventing complete $\delta^{18}$O DIC re-equilibration in thin water films. Considering that the occurrence of disequilibrium conditions in DIC isotope ratios is recorded in the precipitating calcite, speleothems forming from thin films of fluid, which are the result of long drip intervals, may experience enriched $\delta^{13}$C and $\delta^{18}$O values (Mühlinghaus et al., 2007; Riechelmann et al., 2013; Caddeo et al., 2015). Thus, the large fluctuations of $\delta^{13}$C and $\delta^{18}$O observed in both stalagmites (especially BS14) suggest a probable influence of variations in water-film thickness on both isotope ratios, with $\delta^{13}$C and $\delta^{18}$O enrichments during periods of low drip rates, reflecting more arid conditions.

To better interpret the dynamics behind stable isotope variations, trace elements and $\delta^{13}$C profiles in stalagmite BS15 were compared. There is a strong ($r = 0.72, p < 0.001$) positive correlation between $\delta^{13}$C and Mg profiles in BS15 (Fig. 11; Table 2). Considering the Mg interpretation, as related to precipitation patterns, its covariation with $\delta^{13}$C suggests that the latter is a hydrological proxy in this case, where higher values are indicative of drier periods.

**Petrography variations and their relation to environmental changes**

**Chemical and physical parameters influencing fabric variation**

The dimension of the crystallites forming the identified composite crystals has not been disentangled at the optical microscope level, suggesting the presence of extremely small calcite crystallites arranged in optical continuity within the crystal aggregates to which they belong. This general structure can be referred to as columnar microcrystalline (Frisia et al., 2000; Frisia & Borsato, 2010; Frisia 2015). According to Frisia (2015), this fabric can be found in temperate regions characterised by seasonal contrast in temperature, precipitation and vegetation activity. The presence of brown laminae, which is believed to be related to organic matter flushing following vegetation die-back (mainly during autumn at mid-latitudes, Frisia et al., 2000), suggests seasonal changes of water discharge. Although the Sarajevo area is not currently affected by a strong seasonality of precipitation, the significant seasonal temperature contrast must play an important role on evapotranspiration, and thus water balance and year-round variations in drip rate, especially when summer periods are synchronous with relatively low precipitation (Fig. 2). Present-day monitoring of drip sites in the area where the stalagmites were found shows a strong connection with the external environment (Chiarini et al., unpublished data). In particular, higher discharge is found during late autumn to early spring precipitation, while low-to-absent dripping occurs in summer. Therefore, calcite fabric alternation is likely reflecting changes in drip water saturation.
and drip rate, which are related to environmental changes during the second half of the Holocene in the study area.

Stable isotope profiles and trace elements were compared with petrographic changes in order to better understand the factors involved in crystallite changes. Similar trends between fabric-class variations and δ¹³C are visible in both profiles, suggesting the presence of a common factor driving changes in both parameters (i.e., increased calcite porosity and calcite δ¹³C; Fig. 4 and 9). Kruskal-Wallis and multiple tests associated with the Bonferroni correction confirmed the presence of a strong link between δ¹³C and fabric sub-types which associates progressively higher isotope ratios with the more porous and impurity-rich fabrics; a similar but markedly weaker trend affects the petrography classes δ¹³C composition with progressively higher values from Ccm to Scsm and D (Fig. 9).

Considering the interpretation of δ¹³C as a hydrologic proxy, the formation of a particular calcite texture under progressively increased hydrological stress is clear. Petrographic changes induced by variations in drip rate were first reported by Kendall & Broughton (1978), then subsequently by Frisia et al. (2000) and Frisia & Borsato (2010); Mühlinghaus et al. (2007) also found that δ¹³C values in speleothem calcite vary with the drip interval. However, this cannot be considered the sole factor triggering changes in calcite stable isotope composition.

The positive correlation between Mg and δ¹³C, and their negative correlation with Sr, in stalagmite BS15 suggests a possible trace element signature for each petrography class, which was highlighted by the discriminant analyses performed using δ¹³C, δ¹⁸O, Mg, and Sr as variables (Fig. 10 and 11). Riechelmann et al. (2014) described the role of Mg in triggering petrographic changes: the Mg content, which is related to hydrology, likely contributed to the fabric variations in the studied stalagmites contributing in the formation of crystallites presenting a higher density of defect sites.

Another factor that could influence calcite fabric and δ¹³C changes is the mean annual temperature, which causes changes in the vegetation cover and, consequently, in soil CO₂ production. However, the studied stalagmites did not grow over periods long enough to experience significant temperature or climate-driven vegetation changes. Thus, it is more plausible to consider hydrological variations as the major factor triggering both δ¹³C and fabric changes, while the impact on seasonality changes in temperature could have played an indirect role in aquifer recharge via evapotranspiration. Over the long term, a marginal role of vegetation cover in influencing δ¹³C ratios cannot be excluded, as these stable isotope variations are not fully explained by hydrological changes in the studied stalagmite. In particular, in BS15 stalagmite, the uppermost 40 mm show more numerous Th peaks, suggesting increased soil erosion possibly related to anthropic activities on the overlying plateau. The same portion does not display brown lamination, indicating the absence of flushing events and possibly a relatively regular input of detritus load (Fig. 4).

**Palaeo-environmental information recorded by calcite fabrics**

It was demonstrated that drip interval played a fundamental role in the type of calcite fabric of the studied stalagmites, and that the Mg concentration in drip water possibly influenced fabric development: a longer drip interval caused the calcite δ¹³C ratios to increase, which corresponds to lower Sr and higher Mg concentration in stalagmite calcite, also resulting in higher defects and smaller-dimension of the calcite crystallites.

At the more detailed level, groups of laminae formed by **compact columnar microcrystalline (Ccm)** calcite are those characterised by the highest Sr and lowest Mg content. These laminae are separated by wavy brown surfaces, which show evidence of local dissolution of the underlying crystal tips and joints, suggesting flushing events carrying colloids (Supplemental Fig. S1). The presence of flattened calcite crystal terminations and cavities within the compact carbonate lamina below the brownish lamination suggest local (at stalagmite tip), organic-matter oxidation following flushing events (organic colloids from the soil zone), which created an acidic environment leading to very localized dissolution (Frisia, 1996). This pattern can be related to a wet season followed by a relatively dry period, before a strong infiltration event (in autumn) causing a massive flushing of organic matter. These conditions are found in Mediterranean-like climate, which is now present in the coastal area of the Balkans (Vreča et al., 2006).

The **open columnar microcrystalline fabric (Ocm)** is instead characterised by increased porosity, which suggests a less-regular crystallite stacking related to an increased amount of foreign particles/colloids. These particles may have caused higher crystal defects, possibly inducing a smaller crystallite size. Brown surfaces, where present, are less sharp than in the previous sub-pattern and contain less evidence of dissolution features, suggesting the occurrence of weaker infiltration events. If compared to the **Ccm** fabric, a slower growth rate can be inferred from the lower Sr content. The slightly higher Mg content suggests instead the presence of marginally drier conditions during its formation. The δ¹⁸O values detected in the IsoFab plot between **Ocm** and **Ccm** are comparable and indicate the absence of strong differences in the water film on the tip of the stalagmites. However, slightly drier conditions may have translated into more irregular dripping, triggering the precipitation of this fabric.

The **elongated columnar microcrystalline (Ecm)** fabric has only been found in the younger portion of stalagmite BS14, followed by dendritic fabric at the very top of the stalagmite. Its formation possibly occurred under conditions similar to the ones that prevailed during deposition of elongated columnar calcite associated with the influence of impurities (Frisia, 2015). An increasing supply of foreign particles...
is thought to have caused the higher density of crystal defects, and thus the increased porosity. Its $\delta^{18}$O and $\delta^{13}$C values are higher when compared to $\text{Ocm}$ and $\text{Ccm}$, suggesting its formation under thin films of fluid related to a generally drier climate.

The **porous columnar microcrystalline (Pcm)** calcite, observed in both stalagmites, coincides with higher $\delta^{13}$C and $\delta^{18}$O values when compared to the $\text{Ocm}$, but with similar values when compared to the $\text{Ccm}$. This fabric is also characterised by lower Sr and higher Mg content, indicating, respectively, slower growth rate and drier conditions. Increased summer temperature may have played a role together with increased aridity causing further irregularities in drip rate.

Finally, the **stricto sensu microcrystalline (SScm)** fabric and the **dendritic** fabric mainly occur, respectively, in the upper 18 mm of stalagmite BS15 and in the upper 3 mm of stalagmite BS14. The first is associated with higher $\delta^{13}$C values but $\delta^{18}$O values are comparable to those of Pcm. Brown laminae are faint or absent, suggesting the lack of major flushing events and/or lower soil and vegetation activity. The presence of dendritic calcite at the top of BS14, associated with isotopic values comparable to the SScm of the same stalagmite, would also indicate the presence of generally drier conditions (McDermott et al., 1999; Frisia, 2000). The presence of SScm in BS15 would also be in agreement with a less dense vegetation cover on the overlying plateau. In addition, a black layer preserved in both stalagmites close to their top is likely due to a soot coating. It indicates a likely human frequentation of the cave at this time, and thus settlements in the area.

**CONCLUSIONS**

In this study, we analysed two Bosnian speleothems using a multiproxy approach and applying the petrography micrologging method proposed in Frisia (2015) to extract robust environmental interpretations. Comparison of stable isotopes, micro-petrography and trace elements has allowed us to identify hydrological changes as the main forcing parameter.

Petrography micrologging of these stalagmites was found to be a useful tool. Its employment is strongly encouraged when calcite precipitation under disequilibrium conditions is suspected. Indeed, this method can represent a powerful instrument for the detection and interpretation of palaeo-environmental signals in speleothems where stable isotope fractionation under disequilibrium conditions cannot be ruled out. Petrography also helps to better understand the conditions of precipitation. However, calcite pattern variations can be subtle, and are not always readily resolved; sub-type classifications may have to be defined for specific samples. Comparison with other speleothem proxies is always necessary in order to refine the interpretations.

Finally, as petrography becomes more widely used in speleothem research, it will be necessary to ensure consistency in nomenclature, and to avoid the multiplication and redundancies of sub-classification and terminologies. Accordingly, the development of a working group would be highly desirable, facilitated via workshops and an online discussion platform, including a petrographic database.

**ACKNOWLEDGMENTS**

We are grateful to the “Gruppo Speleologico Bolognese/Unione Speleologica Bolognese” for the technical help during sampling campaigns in Bosnia, to the “Federazione Speleologica dell’Emilia Romagna” and the “Gruppo Speleologico Faentino” for funding provided and to the Federal Hydrometeorological Institute of Sarajevo for providing historical meteorological data. A special thanks to Emilie Chalmin (Université Savoie Mont-Blanc, France) and Diego Ercole Angelucci (University of Trento, Italy) for the support provided during microscopic observations; Roland Maas (University of Melbourne, Australia) is thanked for the support provided for U-Th dating; Fausto Desalvo (Department of Mathematics, University of Bologna, Italy) for the help provided in the statistical analyses. Finally, we want to thank the reviewers for their constructive comments.

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Chiarini et al.


Secondary minerals from salt caves in the Atacama Desert (Chile): a hyperarid and hypersaline environment with potential analogies to the Martian subsurface

Jo De Waele1*, Cristina Carbone2, Laura Sanna3, Marco Vattano4, Ermanno Galli5, Francesco Sauro1, and Paolo Forti1

Abstract: Over the past 15 years several expeditions by French, American and especially Italian cavers have unveiled over 50 caves in the Cordillera de la Sal (Atacama Desert, Northern Chile). Many of these caves contain a variety of speleothems and minerals, some of which have rarely been observed within karst systems. Most of the secondary deposits in these caves are composed of halite, but also other halide, carbonate, sulphate, nitrate, phosphate, and silicate minerals have been found. Among the sixteen cave mineral species recognized, atacamite, darapskite, blödite, leonite, anhydrite, and especially antarcticite are worth mentioning. In one of the samples an unknown Ca-Sr-bearing chloride mineral has also been discovered, but it has not been possible to carry out detailed mineralogical analyses. These often-rare minerals have formed in this region due to the very extreme hyperarid and salt-rich environment. This research reports the mineralogical results and proposes the geogenetical mechanisms leading to the formation of antarcticite, powdery anhydrite, and the paragenesis of the halite-darapskite-blodite. This study also shows that Atacama caves may be excellent analogues to study weathering processes and subsurface secondary minerals in hyperarid and hypersaline environments on Mars.

Keywords: cave minerals, salt caves, hyperaridity, minerogenesis, Mars analogues

Citation: De Waele J., Carbone C., Sanna L., Vattano M., Galli E., Sauro F. and Forti P., 2017. Secondary minerals from salt caves in the Atacama Desert (Chile): a hyperarid and hypersaline environment with potential analogies to the Martian subsurface. International Journal of Speleology, 46 (1), 51-66. Tampa, FL (USA) ISSN 0392-6672 https://doi.org/10.5038/1827-806X.46.1.2094

INTRODUCTION

Salt caves have been described in only a few regions worldwide, including the Dead Sea (Frumkin et al., 1991; Frumkin, 1994, 1998, Frumkin & Ford, 1995), the Zagros Mountains in Iran (Bosák et al., 1999; Bruthans et al., 2008, 2009, 2010; Filippi et al., 2011), and the Atacama Desert (Salomon, 1995; Sesiano, 2009). These desert areas are typically characterized by an extremely arid climate, with rainfall usually well below 200 mm a⁻¹ and often concentrated in short periods of time. Among hypersaline desert areas the central Atacama is the driest one, with mean annual rainfall below 25 mm a⁻¹, and often with several consecutive years without any rainfall (Houston & Hartley, 2003; Sesiano, 2006). The geological history and soil mineralogy data of the Atacama region suggest that extremely arid conditions have persisted for 10–15 million years (Erickson, 1983; Alpers & Brimhall, 1988; Sillitoe & McKee, 1996; Hartley et al., 2005; Clarke, 2006), which makes it possibly the oldest desert on Earth.

Despite this extreme hyperarid climate, there are several solution caves in the Oligocene-Miocene evaporites of the Cordillera de la Sal. Cave exploration in this area started only in the early 1990s (Salomon, 1995) and up to now almost 50 caves have been discovered, explored and surveyed for a total development of over 15 km (Fryer, 1995; Sesiano, 1998, 2006, 2007, 2009; Padovan, 2015). These caves and the evaporite karst area have been the subjects of scientific research only recently (De Waele et al., 2009a, 2009b, 2009c, 2009d; De Waele & Forti, 2010). For these peculiar environmental conditions, the Atacama Desert has been proposed as one of the most promising analogues for the chloride-bearing
evaporative deposits that have been found on the planet Mars (Bobst et al., 2001; Lowenstein et al., 2003). Peculiar hygroscopic minerals and perchlorates have long been postulated and finally found by Phoenix lander on Mars (Clark, 1978; Hecht et al., 2009). Several studies have demonstrated the importance of hygroscopic salts for the potential search of life on the red planet (Davila et al., 2016), also using analogue geomicrobiological studies from the Atacama Desert (Wierzchos et al., 2006; Davila et al., 2013). Solutional caves have also been postulated on Mars in several evaporitic environments (Baioni & Tramontana, 2015, 2016), thus the study of cave minerals in the Atacama desert could open new research fields on the expected secondary minerals present in the planet’s subsurface and their potential as astrobiological targets.

Cave minerals are the results of complex interactions between bedrock, circulating water, and sediments of various sources. Before this study, salt caves on Earth were expected to be generally very poor in secondary minerals, with most speleothems and secondary deposits composed of halite. In the early studies in Mount Sedom salt karst area (Israel) only five minerals have been identified (besides halite, also carnallite, sylvite, gypsum, and anhydrite), together with some unidentified iron oxides and hydroxides (Forti & Buzio, 1985; Frumkin & Forti, 1997; Hill & Forti, 1997). Among them, carnallite would be related to a Dead Sea flood event, so cannot be regarded as a true cave mineral.

Eight cave minerals of Atacama were described earlier based on samples taken in some of the first explored caves (De Waele et al., 2009a), but a new sampling campaign in November 2015 allowed to investigate newly discovered caves on the higher part of the Cordillera (Padovan, 2015). In this paper we describe the sixteen known cave minerals from the Atacama region and the very special conditions that allowed for their formation in this environment. Finally the potential of these caves as analogues for hypersaline subsurface environments on Mars is discussed.

**STUDY AREA**

**Location and climate**

The Cordillera de la Sal is situated in the Pre-Andean depression near the San Pedro de Atacama village (2,446 m a.s.l.) at about 150 km northeast from the Pacific coast of South America (Fig. 1).

From a general climatic point of view evaporation greatly exceeds precipitation in the Atacama Desert, with temperature of 35°C in summer and 5°C in winter (mean 14 °C), an average relative humidity of 40.5% (minimum 16.7%, maximum 80.9%), rainfall of 25–50 mm a⁻¹, and evaporation of 1,800–3,200 mm a⁻¹ (Boschetti et al., 2007). This extreme aridity is produced by the constant regime of the Pacific Anticyclone, a cold current along the west coast of South America, and the rain shadow effect from the Andean Cordillera to the east that produces rare precipitation events, a lack of fog, and the absence of dewfall. Data obtained from the weather station in San Pedro de Atacama during 2011 confirms that moisture is only detected on rock surfaces during rainfalls and for a short time after a rain event (DiRuggiero et al., 2013). Historical rainfall records show that the most extreme localized rain events are generally related to El Niño years, while La Niña brings a greater regional annual precipitation (Ortlieb, 1994; Morales et al., 2012). Water recharge in the area is mainly guaranteed from the Altiplano, through the Rio San Pedro stream that cuts the Cordillera de la Sal anticline in its northern part. This valley allows for the observation of the internal structure of this Oligocene-Miocene anticline.

**Geology**

From a geological point of view the Cordillera is composed of an over 1,800-m thick sequence of Tertiary continental sediments of the Paciencia Group, a package of alluvial conglomerates that interfinger with the San Pedro Formation, this last being a series of fine-grained clastic sediments including some 20 to 60 m thick interbedded salt units (Wilkes & Görler, 1994). The chlorides derive from the recycling enrichment of marginal marine deposits such as paralic clastics, limestones, and abundant evaporites (gypsum and halite) deposited from Cretaceous to Paleogene in the Salar of Atacama, which was an interior seaway at that time (Mpodozis et al., 2005). This basin has been closed and filled with clastic sediments since the Eocene, when the mountain chain started its uplifting (Dunai et al., 2005). During the Early to Mid-Miocene, the Paciencia Group started to deform into a series of salt folds along the axis of the present day Cordillera de la Sal. The Artola Ignimbrite (Vilama Group) overlies the folded Paciencia Group, thus dating the regional unconformity to 9.40 Ma (Evenstar et al., 2016). The
Caves

The caves are all located in the Cordillera de la Sal, a NE-SW elongated fold- and thrust belt a couple of kilometres wide and over 100 km long. This ridge is bounded to the east by the Salar de Atacama, bordered by the Salar Fault system at the foot of the Western Cordillera de los Andes (Altiplano), and to the west by the narrow Llano de la Paciencia at the margin of the Domeyko Range in the Precordillera. More than 50 caves are known in the Cordillera de la Sal, several of which are over 2 km long. The most important ones are “through caves” accessible from the sinking stream down to the resurgence. They often start with collapses that allow access to the below lying dry-cave riverbed. Once this floor is reached the caves are often easily traversed downstream (and upstream) until they lead to an open sky canyon. These skylights can be short, thus representing cave entrances (shafts), but can also be relatively long, thus dividing different parts of a single cave system. The underground passages are typically characterized by meandering behaviour with a cave floor very close to the horizontal. The long profile in fact often shows a gradient of less than 3%. In cross section the cave passages can be described as composed of stacked conduits with different sizes, probably deriving from different climate regimes in the past. The walls of a single conduit also show horizontal notches, demonstrating these conduits to have grown in response to a series of flood pulses, each of them having dissolved a part of the salt floor. Some of these older cave conduits still contain diamictons (debris flow sediments), deposited by a last major flood pulse. Dating of bones contained within these terrigenous infillings indicates that these caves formed in the last 6 ka (De Waele et al., 2009d). Cave microclimate conditions are characterised by in-cave temperatures ranging between 15 and 18°C, depending on altitude, cave depth below the surface, and size/number of their openings. Cave relative humidity is always very low (around 15%) enforced by the constant airflow through the entire cave length. The long periods of extreme dryness favour evaporation on cave walls, except during rare rain events. Sometimes decades can pass between one rain event and another.

Secondary minerals and speleothems have been sampled in eight caves, four of them visited in 2009, and the remaining four during the 2015 expedition (Fig. 1). On the east side of the Cordillera de la Sal, Mina de Chulacao Cave is a 1 km long giant salt cave passage, among the biggest and probably the oldest in the area. It is also the closest to the San Pedro de Atacama village (around 4 km west of the village). The canyon-like passage reaches a height of over 20 m, and width of 25 m (Fryer, 2005). The downstream entrance area has been used for the exploitation of copper minerals, of which remains are still clearly visible. Lechuza de Campanario Cave is located 3 km southwest of Chulacao Cave, with the downstream entrance on the same flank of the Cordillera de la Sal. Its passages are much smaller than Chulacao’s (average width 4 m, highest ceiling 10 m), for a development of around 600 m (Fryer, 2005).

Paredes de Vidrios Cave is a frequently visited cave because it is located at the second entrance gate before ascending to the Valle de la Luna Natural Park, in vicinity of a parking lot and a wide canyon floor (1.2 km southwest from Lechuza Cave). It is a 2–3 m wide and up to 10 m high meandering canyon with several skylights. Zorro Andina Cave is located 4 km further to the southwest, and a long walk is needed to reach this beautiful 300-m long cave. It has a 5 m wide and up to 7 m high meandering cave passage, decorated with beautiful halite speleothems.

The biggest cave systems are located on the west side of the Cordillera de la Sal, 35 km southwest of San Pedro de Atacama. These have been explored in the last few years by Italian cavers (Padovan, 2015). Arco de la Paciencia Cave opens out into the Llano de la Paciencia, and is a >2 km long meandering cave passage with a clear upper level rich in halite speleothems. Vučuña Seca Cave, with characteristics similar to Arco de la Paciencia Cave, is located 4 km north and is around 2 km long, while Ventanas Cave is found 2 km south and is only half a km long. The Cressi cave system, is located on the summit plain of the central core of the Cordillera de la Sal and has several entrances that give access to a canyon with multiple skylights. The total development of the cave trunks exceeds 5 km, making this system the longest cave in Chile, and among the longest halite caves of the world.

Methods

Secondary minerals sampled for this study include stalactites, flowstones, precipitates that form crusts in the streambeds and at the groundwater seeps, coatings along the cave walls, earthy masses from the cave floors, and efflorescence salts on ceiling rock outcrops. Cave mineral and speleothem samples were collected during two expeditions (2009 and 2015) with a knife or a geological hammer using small plastic containers or sampling bags.

Mineral phases were determined by combining X-ray diffraction data with semi-quantitative chemical analyses. X-ray analyses were performed on a Philips PW1050/25 diffractometer (Department of Chemical and Geological Sciences, Modena and Reggio Emilia University), or on Gandolfi cameras (Ø 114.6 mm, 24/48 h standing time) when the material was scarce. In both cases experimental conditions were 40 kV, 20 mA, filtered CuKα Ni radiation, λ = 1.5418 Å. All the samples, and in particular the fragments used in Gandolfi cameras, were characterised by means of semi-quantitative chemical analyses and high resolution frames from a scanning electron microscope (ESEM Philips XL40) equipped with EDS – EDAX 9900 microprobe (Centro Interdipartimentale Grandi Strumenti - C.I.G.S., Modena and Reggio Emilia University).
Samples analysed at the DISTAV (Genoa University) used a Philips PW3710 diffractometer (current: 20 mA, voltage: 40 kV, range 2θ: 5–80°, step size: 0.02° 2θ, time per step: 2 sec) equipped with a Co-anode and interfaced with Philips High Score software package for data acquisition and processing. Scanning electron microprobe analyses were performed with a SEM VEGA3 TESCAN (DISTAV, Genoa University) operated at 20 kV and equipped with the EDAX-APOLLO_X DPP3 energy-dispersive (EDS) X-ray spectrometer equipped with an ultrathin polymer window and with resolutions for Manganese Kα = 126 eV and typically ranging detection for chemical elements of atomic number greater than 5 (Boron). Data acquisition and elaboration were performed with the TEAM Enhanced Version: V4.2.2 EDS software.

Cave minerals

Mineralogical analyses have revealed a great variety of secondary deposits for the Atacama salt caves. In all, 16 mineral species have been identified (Table 1), six of which are quite rare for cave environments. Most secondary mineral phases are composed of halite but other minerals have been observed occasionally such as other halides, sulphates, phosphates, nitrates, carbonates, and silicates.

### Table 1. Cave minerals identified in Atacama caves: Ch - Mina de Chulacao; Lec – Lechuza de Campanario; Pav – Parede de Vidrios; Zra – Zorro Andina; Arc – Arco de la Paciencia; Vs - Vícuña Seca; Vent – Ventanas; Cre – Cressi cave system.

<table>
<thead>
<tr>
<th>Cave</th>
<th>Mineral</th>
<th>Nominal chemical formula</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>Halite</td>
<td>NaCl</td>
<td>Ubiquitous as speleothem. This mineral is sometimes present as small euhedral ice-luster millimetric crystals</td>
</tr>
<tr>
<td>Arc</td>
<td>Antarcticite</td>
<td>CaCl₂·6H₂O</td>
<td>White, ephemeroous, millimetre-long curls on clay-marly substrate</td>
</tr>
<tr>
<td>Ch</td>
<td>Atacamite</td>
<td>Cu₄Cl(OH)₃</td>
<td>In globular aggregates of emerald green radial elongated crystals or, rarely, as millimetric veins of euhedral crystals</td>
</tr>
<tr>
<td>Ch</td>
<td>Aragonite</td>
<td>CaCO₃</td>
<td>Crusts up to 5 mm thick of vitreous luster transparent to pale-blue or light-green tabular prismatic crystals</td>
</tr>
<tr>
<td>Ch, Zra, Cre, Arc, Vent, Vs</td>
<td>Anhydrite</td>
<td>CaSO₄</td>
<td>Small lens-shaped aggregates of milky white fibers over euhedral partially corroded gypsum crystals, or white powders filling fractures or solution pockets.</td>
</tr>
<tr>
<td>Zra</td>
<td>Bassanite</td>
<td>2CaSO₄·H₂O</td>
<td>Rare very small fibres inside an earthy material in white corrosion pockets on the surface of gypsum crystals</td>
</tr>
<tr>
<td>All</td>
<td>Gypsum</td>
<td>CaSO₄·2H₂O</td>
<td>Transparent centimetre-sized, euhedral crystals, partially transformed into bassanite and anhydrite</td>
</tr>
<tr>
<td>Cre, Arc</td>
<td>Barite</td>
<td>BaSO₄</td>
<td>Small micrometric inclusions in halite, not visible with naked eye</td>
</tr>
<tr>
<td>Cre</td>
<td>Celestine</td>
<td>SrSO₄</td>
<td>Small micrometric prismatic crystals in halite, not visible with naked eye</td>
</tr>
<tr>
<td>Cre</td>
<td>Blödite</td>
<td>Na₂Mg(SO₄)₂·4H₂O</td>
<td>Granular material in the yellowish crusts on dried out cave pools</td>
</tr>
<tr>
<td>Arc, Cre</td>
<td>Leonite</td>
<td>K₂SO₄·MgSO₄·4H₂O</td>
<td>Small amounts together with antarcticite, not visible with naked eye.</td>
</tr>
<tr>
<td>Cre</td>
<td>Darapskite</td>
<td>Na₂(NO₃)·(SO₄)·H₂O</td>
<td>Small submillimetric laminar crystals in yellowish crusts on dried out cave pools</td>
</tr>
<tr>
<td>Arc</td>
<td>Cl-Apatite</td>
<td>Ca₅[PO₄]₂·Cl</td>
<td>Traces in some samples</td>
</tr>
<tr>
<td>Pav</td>
<td>Biphosphammite</td>
<td>(NH₄)₃K₃H₂PO₄</td>
<td>Thin small pale yellow layered fibres</td>
</tr>
<tr>
<td>Pav</td>
<td>Guanine</td>
<td>C₂H₇(NH₃)N₂O</td>
<td>Earthy, silky-luster milky white to pale pink crystals</td>
</tr>
<tr>
<td>Ch</td>
<td>Clinoptilolite</td>
<td>Na₄K₂·3Ca₂·5Al₂·Si₄O₂·7H₂O</td>
<td>Small whitish to pale-pink earthy grains with a few euhedral crystals always strongly associated with halite</td>
</tr>
</tbody>
</table>

**Halides**

In the Atacama salt caves the majority of speleothems are composed primarily of halite, which forms macrocrystalline deposits/speleothems (euhedral crystals and clusters, stalactites, etc.), microcrystalline (or fine-grained) deposits/speleothems (bent stalactites, flowstone, crusts, helictites, etc.), solid deposits/speleothems (glaze crusts, water table films, etc.) (Fig. 2). In general the halite forms of Atacama (De Waele et al., 2009a, 2009c) resemble those of other salt karst areas in the world (Hill & Forti, 1997; Filippi et al., 2011).

Halite is ubiquitous in all of the caves, but other minerals from the same group have been observed occasionally, such as the very rare cave minerals atacamite and antarcticite, whose occurrence is consistent with the low relative air humidity (< 25%) in this karst environment (Onac, 1996; Reich, 2008).

Atacamite has been found close to the southern entrance of Mina de Chulacao Cave as rounded aggregates of emerald to dark green crystals and crystalline crusts on the cave walls, associated with small, thin, white to pale-green aragonite crusts.

Antarcticite, a highly hygroscopic mineral, occurs as efflorescences of acicular crystal aggregates (Fig. 3C-D) protruding outward from a marly interbedded layer on the cave walls in the Arco de la Paciencia Cave. This sample also contains traces of barite, anhydrite, Cl-apatite, and the rare K-Mg sulphate leonite (Fig. 4). Antarcticite crystallizes close to anhydrite zones (Fig. 3B), as required to create a Ca-enriched saline brine completely depleted in sulphate. Halides precipitate in hot and arid zones when 90% of salt water is evaporated, so they often occur along with other minerals that are created by evaporation, such as sulphates.
Fig. 2. Typical halite speleothems from the Atacama caves: A) Bent stalactites; B) Bent stalactites forming columns and some small stalagmites; C) Macrocrystalline halite stalactites; D) Cottonballs; E) Salt crust covering the dry riverbed; F) Glaze (toothpaste-like) halite flowstone (A and E: Photos Marco Vattano, La Venta Esplorazioni Geografiche; B, C, D, and F: Photos Riccardo De Luca, La Venta Esplorazioni Geografiche).

Fig. 3. SEM images of the association antarcticite-anhydrite at Arco de la Paciencia Cave. A) Lamellar anhydrite crystal; B) The tabular to lamellar anhydrite crystals form an elongated area, surrounded by halite, clay minerals, and local patches of antarcticite; C) Detail of the antarcticite crystals (location in B); D) X-ray mapping indicates the presence of Ca and Cl. Manganese peaks are related to Mn oxides or the clay minerals.
Sulphates

Despite the predominant halite lithology of the Atacama host rock, its caves represent a large variety of sulfate minerals probably derived from minor presence of sulphates in the host rock. Among these secondary phases, gypsum, bassanite, and anhydrite have commonly been found in the caves and at the surface (De Waele & Forti, 2010). Gypsum occurs close to the opening of Chulacao Cave where it was partially transformed into anhydrite, and in the entrance part of Zorro Andina Cave associated with tabular to lamellar anhydrite and bassanite. Anhydrite forms relatively pure white, fine powders filling fractures and dissolution pockets far from the entrance in the Arco de la Paciencia Cave, close to the base of an internal 25 m shaft, and in the folded decametric clayey interstratum. Anhydrite has also been sampled in Vicuña Seca and Ventanas caves in similar environmental conditions. This mineral occurs as microscopic tabular and lamellar crystals (Fig. 5A). In many cases the crystals are made of tiny little lamellae (Fig. 5B); otherwise crystals are equant showing large pinacoidal faces (Fig. 5C). Larger anhydrite crystals are often tabular (Fig. 3A and 5A).

Leonite is, together with blödite, a double salt characteristic of marine salt deposits on Earth (Alpers et al., 2000) forming as secondary mineral of metasomatic origin. Its first description as a cave mineral was in Tăuşoare Cave in Romania (Onac et al., 2001) and then in Wooltana Cave in Australia (Snow et al., 2014). In Atacama it occurs together with antarcticite in Arco de la Paciencia Cave, but also in the yellowish crusts over dry lakes in the Cressi cave system, together with other sulphates (blödite and darapskite), and halite (Fig. 6). Leonite is a sulfate mineral subjected to humidity-related phase transition (deliquescence), so secondary reactions in the salt-clay beds can remove it from the evaporite crusts. In this case, at cave temperatures below 25°C, the blödite precipitated first with respect to leonite and forms acicular crystals filling voids in the halite mass (Fig. 7A-B).

The leonite-blödite-darapskite paragenesis has also been found in another spot in the Cressi cave system, upstream of the large salt lake; in this case it occurred in association with anhydrite and celestine. Darapskite, on the other hand, forms microcrystalline tabular aggregates covering halite and blödite (Fig. 7C). Point elemental composition analyses have confirmed the presence of these two rare cave minerals (Fig. 7D-E).

As mentioned above, other two sulfate minerals have been recognised in Atacama caves: barite and celestine that occur as micrometric crystals in halite.
Fig. 6. SEM-EDS analyses for leonite in the Cressi cave system sample (left, yellow rectangle where the EDS analysis has been done) and the elemental composition showing the relative proportion of K, Mg and sulphate, together with halite.

Fig. 7. SEM images of secondary minerals precipitated in the dry lakes of Cressi cave system. A) The occurrence of the minerals halite, darapskite, and blödite; B) Detail of the blödite crystals; C) Detail of the tiny tabular darapskite crystals covering halite. D-E) EDS spectra for the mineral particles show the presence of blödite and darapskite.
Phosphates and organic minerals
Among the animals frequenting the caves, barn owls are the most common birds. Their droppings and pellets can be seen close to the entrances, and these organic products have sometimes transformed into secondary cave minerals. Biphosphammite and guanine are two of these minerals, detected in the Parede de Vidrios Cave. Biphosphammite has often been found in caves (Hill & Forti, 1997), related to the liquid phase of guano and/or bird droppings. This very soluble mineral has been preserved in this very dry environment. Guanine, formed during the early stages of the mineralisation of guano and/or bird droppings, is a much rarer cave mineral, reported from desert caves in Chile, Western Australia, and Mexico (Bridge, 1974; Forti et al., 2004). Also the traces of Cl-apatite found in Arco de la Paciencia Cave are probably related to minor amounts of phosphates (bones) brought into the cave.

Silicates
In a karst pocket close to the entrance of Chulacao Cave, authigenic Na-clinoptilolite has been found, which is a typical zeolite of alkaline saline lakes (Mason & Sand, 1960; Gottardi & Galli, 1985; Coombs et al., 1997). Clinoptilolite is probably a more common phase in evaporites, indicting greater aridity and reflecting the high level of Na in the saline brine.

Other minerals
One sample in Arco de la Paciencia Cave also contained an unknown mineral phase, perhaps a new mineral, composed of Sr, Ca, and Cl (Fig. 8A-C). This unknown mineral phase forms porous aggregates (Fig. 8B) covering compact halite with cubic forms. Unfortunately the amount of material found and its deliquescence has not allowed carrying out more detailed mineralogical investigations.
DISCUSSION

Environmental conditions
Most of the secondary minerals found in the salt caves of Atacama are derived from the evaporation of brines (i.e., halite, gypsum, antarcticite, darapskite, blödite, leonite), or from the dehydration of gypsum (bassanite, anhydrite) induced by the hot, arid climate of the area. The high solubility and the ephemeral nature of most of these phases makes them difficult to preserve in most of the common cave conditions around the world, where atmospheres saturated in water vapour react with the previously formed salt minerals, especially with halite whose deliquescence threshold is 75% of relative humidity at 20°C (Filippi et al., 2011).

In the dry conditions of the Cordillera de la Sal, water penetrating the cave’s host rock along fractures and bedding planes after sporadic rain events leads to the dissolution of primary minerals and allows the formation of seeping brines with dissolved salts. Moreover, the undersaturated flood water that rarely inundates the cave passages dissolves rocks and crusts, thus becoming brines at or near saturation with respect to halite. Both of these processes selectively add solutes to the incoming rain water, changing its chemistry and producing brines very depleted in carbonate due to the scarce presence of limestone in the area. The evaporation of these salt-rich fluids at the cave-atmosphere interface causes secondary minerals such as sulphates and especially halite to precipitate in the form of salt efflorescences and crusts. The specific salts that crystallise at any location within these caves are a function of the local composition of the solution, reflecting dissolved element concentrations, and are controlled by the low relative humidity. In fact, the extremely dry climate of Atacama also permits the preservation of very rare mineral assemblages.

In this dry, evaporative cave environment dominated by chloride and sodium the most common salt is halite. Although smaller in volume, gypsum forms in the initial mixture due to its relatively lower solubility in water compared to other soluble calcium salts and sulfate species. The final brine is rich in sodium, potassium, magnesium, calcium and chloride and the further evaporation leads to an evaporative sequence of sulphates and halides, similar to what happens in a closed basin with very high salinity.

In the element recycling process from the primary minerals, the micrometric crystals of barite and celestine are probably also related to the extreme evaporation of brines in which small amounts of Ba\(^{2+}\) and Sr\(^{2+}\) were present. Barium and strontium are common constituents of sea sediments whose mineralogy is dominated by non-terragenous sources, so these alkaline metals are likely mobilised from the cave host rock.

On the other hand, aragonite and atacamite were probably deposited by hydrothermal fluids rising along faults visible near the entrance of Chulacao Cave. The copper needed to form atacamite probably derived from these fluids while the halite provided the necessary chloride ions. Also clinoptilolite, found in pockets near the atacamite and aragonite location, is probably related to the reworking of Al-Si rich volcanic minerals present in the area.

Other minerals have formed in this extremely dry environment in the presence of nitrogen and phosphates coming from nitrate ores or likely from bird droppings or guano and their dehydration products (guanine, biposphammite, and darapskite).

Of all these minerals, five have a genesis that deserves to be explained in more detail: antarcticite, anhydrite, and the paragenesis blödite, leonite, and darapskite.

Origin of the most interesting minerals

Antarcticite
This mineral, the only one taking its name from a continent, has been reported in only a few places on Earth as a discrete mineral (it is also found in fluid inclusions). It was first discovered in a playa lake in the Mojave Desert, in California (Dunning & Cooper, 1969), although its earliest description and publication is due to Torii & Ossaka (1965) based on the finding of prismatic crystals in a hypersaline lake in Antarctica. Another occurrence is located in the Kuntey playa in the west of the Qaidam Basin, Western China (Bingxiao & Kejun, 1986). Antarcticite has also been found in stratified fresh to salt water columns in onshore “blue holes” on North Andros Island (Bahamas) (Eckstein et al., 1994). In all these places antarcticite forms in hypersaline lakes by extreme evaporation, and is associated with halite and gypsum. In Atacama this calcium chloride mineral was found as pure curly white crystals growing on a fine-grained interbedded layer surfacing a cave wall in the Arco de la Paciencia Cave. The genetic mechanism that has allowed the formation of this mineral in the karst environment is far more complex than the extreme evaporation of the hypersaline lakes of the other locations. In fact, in this cave antarcticite has not crystallised in evaporated pools, but on a dry cave wall. The mineral seems to have formed starting from capillary water seeping out from the fine-grained sediments, probably following exceptional rain and/or snow events that occurred some months before its discovery (Fig. 9).

The calcium necessary for the formation of this chloride derives from the gypsum and/or the silicates that typically fill fractures and/or pockets under the salt crusts at the external surface of the Cordillera de la Sal. The water seeping into the cave through joints and more permeable bedding planes (i.e. the silty and sandy sequence interbedded in the salt units of the Paciencia Group) are able to dissolve primary minerals (halides and sulphates) and bring ions into solution. Also important, often-violent flood events dissolve a large amount of elements, but cannot be considered responsible for the slow capillary movement of interstitial fluids necessary for the formation of antarcticite. See page, on the other hand, is able to keep the fine sediments filling these pockets or the interbedded layers moist over long periods of time (several months), also thanks to the shielding
capacity of the impermeable salt crust that uniformly covers the entire surface of the Atacama Desert.

These sediment-laden pockets constitute a humid micro-environment in which solubilisation occurs during rain events, and evaporation-deposition during most of the year (Fig. 10A). In the intermediate zone, capillary movement of water follows in a downward direction during rainwater seepage and upward when evaporation prevails. Along the downward pathway the fluids enrich in salts (the calcium chloride salt being among the most soluble ones) until saturation occurs. This cyclic process of solubilisation and precipitation in parts closer to the surface is responsible for the increase in the most soluble salts. Antarcticite is a very hygroscopic substance and its very high solubility inhibits the more abundant sodium chloride to go into solution because of the common-ion effect (NaCl is ten times less soluble than CaCl$_2$). This calcium chloride is also a diagnostic mineral produced by the final evaporation of a neutral brine very depleted in sulfate after the early precipitation of anhydrite (Bridge & Demicco, 2008). If the proportion of calcium concentration in the solution exceeds that of magnesium, a further loss of water consumes sulfate with the segregation of leonite, and eventually barite, and the resulting solution is enriched in sodium and calcium chloride (Fig. 10B). In general, however, this salt precipitates as an ephemeral mineral phase inside the sediment itself (Fig. 10C) or, in some cases (e.g. following a period of intense precipitation, such as the one occurred at the end of March 2015, eight months before our fieldwork in the Cordillera de la Sal) this calcium chloride-rich fluid can reach the cave walls. The piston-effect of seepage pushed a very small quantity of these enriched fluids to the surface on the ventilated cave walls, and its complete evaporation allowed antarcticite to precipitate as tiny curly white crystals (Fig. 10D).

**Anhydrite**

Gypsum, bassanite, and anhydrite have commonly been found in the salt caves of Atacama and at the surface of salt karst areas. In the Cordillera de la Sal, gypsum is widespread at the surface, occurring...
as crystals and in veins several decimetres long (De Waele & Forti, 2010). The gypsum outcrops are locally named Hoodoos (De Waele & Forti, 2010) and are only rarely observed inside caves, where anhydrite is by far the most common sulfate mineral. The presence of secondary anhydrite in halite caves has been reported from Mount Sedom (Israel) (Forti & Buzio, 1985; Frumkin & Forti, 1997). These previous studies have also demonstrated that the sulfate that precipitates in pools enriched in NaCl is anhydrite, and not gypsum. In 2015, in the Arco de la Paciencia Cave, a very special occurrence of anhydrite was discovered (Fig. 11). Here, this sulfate outcrops as white powders, filling pockets and fractures in the halite cave walls (Fig. 11A-B). These powders were also found below these deposits, forming half metre high cones or winding traces on the floor (Fig. 11C). Pure anhydrite residues have also been reported in other caves of Atacama, always well above the present cave river thalweg, far out of the flow of waters even during the most extreme flood events. These anhydrite deposits resemble those of gypsum described in caves of Sicily (Italy), New Mexico, Argentina, and Russia (Hill & Forti, 1997). In all of these cave occurrences mentioned in Hill and Forti, these fine gypsum particles form by different mechanisms, including: 1) biological digestion of guano in presence of fluids enriched in calcium and sulphate; 2) rapid evaporation of small water volumes surfacing on permeable cave walls (cfr. antarcticite); 3) segregation processes following the freezing of waters containing calcium sulphate.

However, none of these mechanisms can explain the formation of anhydrite in the halite caves of Atacama. First of all the halite bedrock is not permeable enough (in fact, primary porosity is close to zero), and also freezing in the caves most probably does not occur in this climate. In the Atacama case, anhydrite precipitation can only occur by a fractionated crystallisation starting from a sodium chloride saturated solution with small concentrations of calcium and sulfate through slow evaporation. In this way the forming halite crystals increase their size without taking up impurities, such as small anhydrite crystals. This process cannot explain the small dimension of anhydrite crystals, however, and its powdery appearance instead depends on the very arid atmosphere and the rare rain events. In normal conditions anhydrite would form large crystals similar to those of Liquid Crystal Cave in the Mount Sedom area. During the sporadic rain events water infiltrates into the joints, bedding planes and fractures, bringing into solution calcium and sulfate from the overlying sediments. Penetrating into the halite bedrock through discontinuities, rainwater becomes saturated with respect to sodium chloride very quickly. When this solution reaches the cave voids it rapidly gives rise to the formation of the typical halite stalactites and flowstones. In the case where brine flows through tinier fractures and planes, capillary forces drive the water movement (Fig. 12A). Faster running waters, in fact, would have washed the anhydrite powders easily away, similar to what has occurred for the gypsum powders in New Mexico (Calafarra & Forti, 1994). Also dripping water would have transformed these residues into solid crusts or cemented material, as has happened in Argentinian caves (Forti et al., 1993). The formation of this anhydrite powder is thus controlled by the quantity of water that infiltrates following every rain episode (Fig. 12B). After every precipitation event the water becomes saturated in sodium chloride during its capillary movement and later this film dries up very slowly, allowing the halite to cover the rock salt walls within the fractures and leaving the remaining fluids increasingly enriched in calcium and sulphate. At the end of this process the brine is broken into isolated droplets which evaporate completely and lead to the precipitation of fine particles of anhydrite (Fig. 12C). This dry anhydrite powder can then move through the tiny fractures driven by gravity (Fig. 12D). Because rainfall is very infrequent in Atacama, and the fact that every single rain event probably forms only very small amounts of anhydrite, the rather large deposits found in Arco de la Paciencia Cave are probably the result of hundreds of precipitation events (and thus thousands of years).

Blödite, darapskite, and leonite

As in evaporitic closed basins at the surface, it is quite common within Atacama caves to have ephemeral crusts of gypsum and particularly halite covering dry cave lakes. The sudden rainwater inflow during the rare floods forms small water bodies that slowly dehydrate in depressions along the cave passages. These cave pools correspond to closed basins in which
the brines are concentrated by intense evaporation. The most characteristic mineral paragenesis in these dry lakes is constituted by darapskite, blödite, and leonite, which occur in yellowish crusts well visible in large salt-crusted cave pools in the Cressi cave system (Fig. 13).

Darapskite is widespread in nitrate deposits, filling cavities in saline arid soils, where it is a component of efflorescences and other incrustations. It is a very rare cave mineral and has been reported previously only from a cave in Texas (Hill & Ewing, 1977). In Atacama the nitrate is probably supplied by urine and guano, or recycled from cement in the sediments. Blödite, on the other hand, is a much more common mineral in caves, and has been reported from the Mammoth Cave National Park caves (Freeman et al., 1973), Mount Elgon (Kenya) (Bowell et al., 1996), and the Naica caves in Mexico (Badino et al., 2011). Also leonite precipitates during dehydration of lake water from a magnesium and potassium sulfate mixture. It has been already described in caves (Onac et al., 2001; Snow et al., 2014) even though never in association with darapskite and blödite.

In this salt environment where sodium and chloride are considered ubiquitous, short episodes of flooding provide the major input of dissolved calcium into the system, because during the long dry periods this element is removed by calcium sulfate precipitation. The other chemical components necessary for the formation of these mineral species (magnesium, potassium, sulphate, nitrates) derive in part from the moist sediments underlying the salt crust, containing epiclastic (delivering the potassium and magnesium) and organic (nitrates and phosphates) materials (Fig. 14A). These last are brought into the cave from the entrances, where birds usually build their nests (guano). The slow evaporation of cave pools first allows halite to precipitate as crusts, rafts, and shelfstones that progressively grow on the borders of the pools (Fig. 14B), forming larger salt crystals on the muddy floor. As the relative salt concentration is increased and some small windows through the sodium chloride crust in the central parts of the pools are still open to the cave atmosphere, the rise of capillary water leads to precipitate a polymineral assemblage containing blödite, leonite and darapskite together with halite to form in the middle of the pool (Fig. 14C). Along segregation pathways, potassium or sodium sulphates are the earliest minerals in the precipitation sequence. In sodium chloride saturated brines at temperature below 25°C, leonite will not precipitate because blödite deposits first (Jones & Deocampo, 2005). After the precipitation of the double magnesium salts (blödite and leonite), the proportion of sulfate left in the solution will determine whether the final fluid will allow the formation of darapskite or only of halite. The hydrated sodium nitrate sulfate darapskite, usually unstable below 13.5°C, tends to appear after leonite when the cave atmosphere dries out, and disappears at temperatures below 7.2°C or in presence of moisture.

A potential analogue for Mars subsurface salts
Several of the secondary minerals found in the caves of the Atacama Desert are postulated to be present also in evaporative environments on Mars (Squyres et al., 2004). Among them, antarcticite is the most interesting because, alongside Na- and Mg-brines, CaCl₂-rich brines are expected in the deep subsurface of Mars, although they might be somewhat...
diluted (temperatures permitting) and of variable salt composition (Burt & Knauth, 2003). In this paper we show that the process of formation of antarcticite is not exclusive of extreme evaporative hypersaline freezing lakes like Don Juan pond in Antarctica as proposed by Burt & Knauth (2003). Instead, this mineral can form also from differential enrichment of Ca from gypsum or anhydrite by capillary waters along fractures and clay layers in the underground, without any eutectic freeze crystallization. In addition, the presence of an unknown mineral phase, probably a chloride containing both Ca and Sr, confirms that the formation of minerals with high deliquescence behaviour can easily form in cavities underground in such extremely arid conditions as those of Atacama. These chlorides are highly hygroscopic salts that could result in transient solutions with water activity compatible for life in the Martian subsurface (Davila et al., 2010). A future geomicrobiological study of these minerals in the cave environments of the Atacama Desert could also provide additional hints on how these processes could happen in the deep subsurface even without photosynthetic activity.

Other interesting cave minerals to be considered as potential analogues for the subsurface of Mars are blödite and leonite. On Mars, the interaction of salt brines with basalts (which are the most common substrate on the planet) is expected to have enriched the brine in Na, K, or Mg. Similarly in the Atacama desert caves, sulphates like blödite and leonite are the result of the concentration in these elements sourced by regional volcanic material interlayered with halite deposits. Given the evidence that some evaporitic terrains on Mars have been subject to karst or thermo-karst processes (Baioni et al., 2014; Baioni & Tramontana, 2016) probably resulting in solutional cavities (Boston, 2004; Baioni et al., 2009), the Atacama caves represent a promising analogue to secondary mineral formations and the potential of life in the Martian subsurface.

CONCLUSIONS

A total of sixteen secondary minerals have been described as occurring in the salt caves of Atacama, including very rare minerals (atacamite, darapskite, blödite, and leonite) and a new occurrence of antarcticite, a new cave mineral. An unknown calcium-strontium chloride mineral has also been found, but unfortunately its deliquescence and small quantity have not allowed for its detailed classification and description.

Most of the Atacama minerals are highly soluble in water. However, the extremely dry climate of the area permits the formation and persistence of these mineral assemblages. Salt mineral precipitation is controlled by the temperature-dependent solubility of the species in saline water, so that different secondary minerals occur. Processes that drive the crystallization of these phases include the action of thermal fluid-rock interactions and the loss of water through evaporation. Atacamite and aragonite formed from slightly hydrothermal solutions rising along a fault plane in Chulacao Cave.

The other salt minerals in these caves precipitated by evaporative processes during the persistent dry conditions of the Atacama Desert. In fact, the water penetrating along fractures after sporadic rain events leads to the dissolution of primary minerals in the host rock, and the slow evaporation of these salt-rich seeping fluids at the cave interface produces speleothems, salt efflorescences and crustal precipitation. Conceptual models for the formation of some of these minerals (i.e., antarcticite, anhydrite, and the blödite-darapskite-leonite association) have been proposed and are unique to this extreme karst environment that is here proposed also as a potential analogue for the study of hygroscopic salts in the Martian subsurface.

ACKNOWLEDGEMENTS

This research has been made possible thanks to the Integrated Project “Atacama” co-funded by the “Fondazione della Cassa di Risparmio di Bologna” and to La Venta Esplorazioni Geografiche and the Commissione Grotte “Eugenio Boegan” of Trieste that organised the 2015 expedition. Authors are grateful to the director of Valle de la Luna Natural Park for allowing the access to the caves. Special thanks to Kevin Downey (USA) and Elio Padovan (Italy) for their help in finding caves and giving useful information. A special thanks to Prof. Pier Luigi Fabbi of the Centro
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Diversity and biocide susceptibility of fungal assemblages dwelling in the Art Gallery of Magura Cave, Bulgaria

Milena M. Mitova¹, Mihail Iliev¹, Alena Nováková², Anna A. Gorbushina³,⁴, Veneta I. Groudeva¹, and Pedro M. Martin-Sanchez³*

¹Sofia University "St. Kliment Ohridski", Faculty of Biology, 1164 Sofia, Bulgaria
²Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i, Vídeňská 1083, 142 20 Prague 4, Czech Republic
³Bundesanstalt für Materialforschung und -prüfung (BAM), Department 4 Materials & Environment, Unter den Eichen 87, 12205 Berlin, Germany
⁴Freie Universität Berlin, Department of Biology, Chemistry & Pharmacy and Department of Earth Sciences, Malteserstrasse 74-100, 12249 Berlin, Germany

Abstract: Magura Cave, north-western Bulgaria, possesses valuable rock-art paintings made with bat guano and dated from the period between the Eneolithic and Bronze Ages. Since 2008, the Art Gallery is closed to the general public in order to protect the paintings from vandalism, microclimatic changes caused by visitors and artificial illumination, and the consequent growth of fungi and phototrophs. Nevertheless, some tourist visits are allowed under the supervision of cave managers. This study provides the first scientific report on cultivable fungal assemblages dwelling different substrata in the Art Gallery. A total of 78 strains, belonging to 37 OTUs (Ascomycota 81%, Zygomycota 14%, Basidiomycota 5%), were isolated in the study. This fungal diversity was clearly dominated by Penicillium (50% of strains) and Aspergillus (13%). The most relevant visible fungal colonies were detected in sediments rich in bat guano, where, besides Penicillium, other guanophilic fungi such as Mucor, Mortierella, Trichosporon and Trichoderma were dominant. Conversely, scarce fungi were detected on rock surface of painted walls. Based on the biocide susceptibility assay, octylisothiazolinone (OIT) and benzalkonium chloride (BAC) were effective inhibiting the in vitro growth of dominant fungal species in Magura Cave, when applied at concentrations ranged from 100 to 1,000 mg/L. These data provide a valuable knowledge about Magura fungi, and exemplify a type of preliminary test that may be conducted before planning any biocide treatment. However, considering the irreversible effects of biocides on the ecological balance in caves, and the low fungal contamination in painted walls of Magura Cave, there is no reason to use conventional biocides in this cave. Further studies, monitoring microbial communities and microclimatic parameters, should be conducted to improve the knowledge on microbial ecology in Magura Cave and possible human impacts, as well as to allow the early detection of potential microbial outbreaks.

Keywords: fungi, cultivable microorganisms, rock-art caves, bat guano, biocides


INTRODUCTION

Magura Cave, located in Bulgaria, contains more than 700 prehistoric paintings depicting a variety of anthropomorphic figures, animals and signs that resemble letters from an unknown alphabet. These multi-layered paintings were created using bat guano, similar to those from Baylovo Cave in Bulgaria (Stoytchev, 2005) and Grotta dei Cervi in Italy (Groth et al., 2001), and dated from the period between the Eneolithic and Bronze Ages (Stoytchev, 1994). In 1984, Magura Cave was placed on the Tentative List for consideration as World Heritage Site by the United Nations Educational, Scientific and Cultural Organization (UNESCO, Tentative list 45). In 2008, sections of the cave containing the rock art (the Art Gallery) were closed to the public in order to protect the paintings from deterioration due to (i) vandalism, (ii) microclimate warming caused by visitors and artificial lighting installed in 2001, and (iii) the consequent growth of phototrophs and fungi on the walls and sediments (Arcá, 2014). In particular, the main concern was the presence of fungal colonies on paintings in the Art Gallery (Stavreva, 2012). Despite
the closure of the Art Gallery, some guided tourist visits are allowed under the supervision of cave managers. Human presence in caves always leads to alternations of the microclimate, the biogeochemical cycles, and the balance of organic matter. These parameters have enormous impact on native microbial populations. Some fast growing heterotrophic microorganisms may be favored by the new conditions, and cause microbial outbreaks. However, the presence and role of microorganisms in caves is a topic that is often ignored in cave management unless any microbial crisis arises (Saiz-Jimenez, 2012). Furthermore, the decision of closing a cave to the public for conservation reasons is always a controversial topic. Sometimes economic interests prevail over conservation leading to deterioration of cultural heritage (Saiz-Jimenez et al., 2011). In rock-art caves, when visits cannot be prevented, cave management should include ways to control organic matter inputs by visitors, and periodic monitoring of microclimate and microorganisms present in all cave compartments. Microbial ecological studies can provide useful information for early detection of dangerous microbial outbreaks (Saiz-Jimenez, 2012, 2013). Knowledge about the most relevant microorganisms, such as their colonization patterns, dispersion mechanisms, and potential adverse effects on human health and rock art (if present), can help cave managers to adopt conservation measures.

A variety of studies have demonstrated that the use of biocides in caves is a dangerous choice to control microbial outbreaks (Boston et al., 2006; Saiz-Jimenez, 2013). Boston et al. (2006) discussed the effect of different cleaning chemicals traditionally used in caves, such as chlorine bleach, soaps and solvents, on microbial communities and mineral formations. Mulec and Kosi (2009) reviewed the pros and cons of physical, chemical, and biological methods to control the phototrophic biofilms associated with artificial illumination of caves, demonstrating that all evaluated control methods have relevant weaknesses. Biocides completely change the original microbial communities, altering the natural ecological balance of the cave, and subsequent recolonizations can provoke further microbial outbreaks. In addition, when biocides are applied without the appropriate previous efficacy tests against the specific cave microbiota, the ecological effects of such treatments are totally unpredictable. Although there is no clear report about biocide treatments in Magura Cave to date, some authors mentioned that a small fraction of the paintings was treated in 1983 by Aneta Slavova, from the National Institute for Cultural Monuments, with an unknown solution which prevented fungal growth on the treated surfaces (Stavreva, 2012; Arcà, 2014).

The presence of bats is a critical challenge for conservation of rock-art caves. Bat colonies significantly affect the microbial diversity in caves due to input of organic matter through deposition of guano and carcasses, and its influence on bacterial and fungal dispersion (Vanderwolf et al., 2013; Kokurewicz et al., 2016; Ogórek et al., 2016). Bat-inhabited caves can be reservoirs of human pathogenic fungi such as *Histoplasma capsulatum*, causative agent of histoplasmosis (Cano & Hajeh, 2001), which is frequently isolated from warm caves in tropical areas. In addition, the sudden and catastrophic appearance of the White Nose Syndrome (WNS), caused by *Pseudogymnoascus destructans*, in cave-dwelling North American bats in 2006 has stimulated interest in cave fungi in the last few years (Blebert et al., 2009; Lorch et al., 2013). A huge bat colony of more than 2,000 individuals spends the winter in Magura Cave. A total of eight bat species with priority conservation status in Europe have been identified in Magura Cave (Bulgarian Biodiversity Foundation, “See More: FOR the Bats” project).

The bacterial assemblages inhabiting the Art Gallery of Magura Cave were recently studied by Tomova et al. (2013a, b), and Ivanova et al. (2013). However, despite the relevance of fungi in cave environments and their well-known role in rock-art deterioration, no fungal studies have been conducted to date in Magura Cave. Among other reasons, the need of a fungal study in this cave is supported by the abundance of bat guano in the whole cavity, as well as the fact that fungal colonization of paintings was reported as one of the key motivations for closing the Art Gallery to the public in 2008. Considering this lack of knowledge, the goals of this study are (i) to characterize the fungal assemblages that colonize different substrata in the Art Gallery of Magura Cave by cultivation as well as culture-independent analyses, and (ii) to evaluate the susceptibility of the most abundant cultivable fungi to different biocides.

**MATERIALS AND METHODS**

**Sampling**

Magura Cave is located near Rabisha village in the Vidin district at north-western Bulgaria. The cave has an area of 28,600 m², 2.5 km of underground passages, and its entrance is situated 375 m above sea level (43°43'41.16"N, 22°34'54.84"E). This study focused on the Art Gallery (Fig. 1a), placed in a left branch of the cave which can be accessed through a narrow corridor around 200 m from the entrance. The mean air temperature and relative humidity in this gallery is 12°C and ~94%, respectively.

Surveys were carried out 2011-2015, and samples were collected from 15 different points along the Art Gallery (Fig. 1a). A total of 35 samples were analyzed, 24 from the rock surface close or on the paintings (hereafter “painting samples”, Fig. 1b-d), and 11 from cave sediments rich in bat guano (hereafter “sediment samples”, Fig. 1e-g) which frequently showed growth of fungal mycelia. Painting samples were aseptically collected using cotton swabs, and sediment samples were collected in 50 ml Falcon tubes using sterile scalpels. All samples were kept on ice during transport to the laboratory.

**Isolation of fungi**

Swabs of painting samples were transferred to 15 ml Falcon tubes containing 2 ml of sterile saline solution (0.9% NaCl). Similarly, 2 g of sediment samples were
transferred to 15 ml Falcon tubes containing 10 ml of sterile saline solution. Afterwards all samples were homogenized in the orbital shaker at 150 rpm for 30 min. Isolation of fungi was carried out by plating in triplicate 100 µl aliquots of the resulting suspensions, and three decimal serial dilutions from them, on the following culture media – potato dextrose agar (PDA), malt extract agar (MEA), Sabouraud’s glucose agar (SGA) and dichloran rose bengal chloramphenicol agar (DRBC). Culture plates were incubated at 25°C in darkness for one month, checked every two days during the first two weeks, and once per week later. This standard incubation temperature, higher than air temperature in this cave, was chosen in order to (i) save time of analysis, because the majority of fungi grow quicker at 25°C than 14°C, and (ii) compare the results to most of fungal cave studies, with similar culture conditions. Based on colony morphology, the most abundant cultivable fungi, as well as some further representative strains, were isolated in pure cultures on the same culture media. Based on colony morphology, the most abundant cultivable fungi, as well as some further representative strains, were isolated in pure cultures on the same culture media. Molecular identification

Molecular identification of all fungal strains was performed by PCR and sequencing of the rDNA internal transcribed spacers (ITS) or a fragment of the 18S rRNA gene (18S). The 18S marker was alternatively analyzed for some fungal strains in which the ITS analysis failed. Genomic DNA of each strain was extracted from its biomass grown on MEA. Biomass was collected and transferred to a 2 ml Eppendorf tube containing 500 µl TNE buffer.
(10 mM TrisHCl, 100 mM NaCl, 1 mM EDTA; pH 8) and glass beads of three different diameters (0.5, 2, and 5 mm). The mixture was shaken at 4.5 ms-1 for 1 min in a Fast Prep RiboLyser cell disrupter (Thermo Hybaid GmbH, Ulm, Germany). The DNA was purified through phenol/chloroform extraction and isopropanol precipitation. The extracted DNA was resuspended in 100 µl sterile ultrapure water, and its DNA concentration was quantified using NanoDrop 2000C (Thermo Fisher Scientific, Wilmington, DE, USA), following the manufacturer’s instructions.

The corresponding molecular marker was analyzed by conventional PCR for each strain, the whole ITS region using the primers ITS1 and ITS4 (White et al., 1990) or a fragment of 18S using the fungal primers 0817F (Borneman & Hartin, 2000) and 1750-3’ (Gargas & Taylor, 1992). PCR reactions were performed in a BioRad C1000 Thermal Cycler (BioRad, Hercules, CA, USA). Cycling parameters for both markers were 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min (ITS) or 2 min (18S), with a final extension at 72°C for 10 min. Reactions were performed in duplicate, and negative controls (containing no DNA) were included in each PCR trial. All PCR products were checked by electrophoresis in 1.5% (w/v) agarose gels stained with GelRed dye (Genaxxon Bioscience GmbH, Ulm, Germany) and visualized under UV light. Positive amplification products were purified by using a QIAquick PCR Purification Kit (Qiagen GmBH, Hilden, Germany) and sequenced in duplicate by Macrogen Europe Company (Amsterdam, The Netherlands) with the same primer sets used for PCR. After edition, final sequences were submitted to the European Nucleotide Archive (ENA, EMBL-EBI) under the accession numbers LT623959 - LT623995. Identification of strains was based on comparison of their rDNA sequences with the GenBank database using the BLAST algorithm from the NCBI.

**Phylogenetic relationships**

Based on previous molecular identification, representative strains of the different fungal taxa isolated from Magura Cave were selected (Table 1). Phylogenetic relationships between such representative strains and the closest related sequences of reference strains, extracted from GenBank, were estimated. The ITS sequences were aligned using MUSCLE web service (EMBL-EBI), and the phylogenetic analyses were conducted using MEGA 5.2 with the Neighbor-Joining method. Gaps were treated as missing data, the Kimura 2 parameter substitution model was used, and bootstrap values were generated using 10,000 replicates. Additionally, the resulting topology was compared with results from other treeing algorithms, including the Maximum-Likelihood and Maximum-Parsimony methods.

**Morphological studies**

Morphological characteristics of representative strains (Table 1) were additionally characterized. For such purpose all fungal strains were cultivated on MEA under standard incubation conditions (25°C in darkness) for seven to ten days. If poor sporulation was achieved, longer incubation time and other nutrient media were used – e.g., PDA, carrot agar (CA) or potato carrot agar (PCA) (Atlas, 2010). In the case of Penicillium and Aspergillus strains, additional standard nutrient media were needed for the identification process – Czapek yeast autolysate agar (CYA), Blakeslee malt extract agar (BMEA), yeast extract sucrose agar (YES), and creatine sucrose agar (CREA) (Samson & Frisvad, 2004).

The morphological identification was based on macro- and micro-morphological properties (phenophytic and microscopic characters), i.e. the assessment of growth rate, colony morphology, production of exudate and soluble pigment, sporulation type, size and shape of all components of conidiophore or sporophore, type of conidiogenous cells, production of ascocarps, size and shape of ascocarps, asci, and ascosporas; according to taxonomic literature and compendia (Domsch et al., 2007; de Hoog et al., 2000).

If necessary, simple physiological tests, such as Ehrlich test of color reactions and estimation of acid and base production on CREA, were also used for Penicillium and Aspergillus identification. All species were examined using a Zeiss Axio Scope.A1 microscope with Nomarski interphase contrast. Microscopic slides were prepared from cultures showing good sporulation using 60% lactic acid with fuchsin as mounting medium (Kreisel & Schauer, 1987).

**Denaturing Gradient Gel Electrophoresis**

Fungal assemblages of environmental samples collected from paintings and sediments were characterized by denaturing gradient gel electrophoresis (DGGE). For comparison, the DGGE profiles of all fungal strains were also analyzed. DNA from environmental samples was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, USA) following the manufacturer’s instructions. DNA extraction protocol for fungal strains was detailed above. After PCR amplification of ITS marker, as previously described, for DGGE analysis a second round of PCR is needed, using the primers ITS1-GC (containing a GC clamp on its 5’ end) and ITS2 (White et al., 1990), and 1 µl of the first PCR product as DNA template. The cycling parameters in the second PCR were 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min.

For DGGE fingerprint of samples, 20 µl of positive PCR products (containing the ITS1 region) were analyzed. Gel electrophoresis was performed as previously described by Muyzer et al. (1993) in 0.5X TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na₂EDTA; pH 7.8), with acrylamide gels (195+160×1 mm), using 10% (v/v) Rotiphorese Gel 30 (30% acrylamide, 0.8% bisacrylamide; Carl Roth GmbH, Karlsruhe, Germany) containing a gradient of denaturants (formamide and urea) of 25-45%. The gels were run in a D-Code System (BioRad) for 3.5 h in TAE 0.5X buffer at 60°C and constant voltage of 200 V. After electrophoresis, gels were stained in GelRed dye and visualized under UV light. A marker, containing the PCR products
Table 1. Representative fungal strains isolated from Magura Cave.

<table>
<thead>
<tr>
<th>Representative strain</th>
<th>Accession No.</th>
<th>Number of strains</th>
<th>Source</th>
<th>Molecular identification</th>
<th>Closest related species from GenBank</th>
<th>Similarity (%)</th>
<th>Morphological identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>P5*</td>
<td>LT623959</td>
<td>12</td>
<td>painting / sediment</td>
<td>Penicillium sp.</td>
<td>P. cordaense, P. palonheim, P. lipiodosum, P. thomii</td>
<td>100</td>
<td>Penicillium commune</td>
</tr>
<tr>
<td>P7</td>
<td>LT623995</td>
<td>6</td>
<td>painting / sediment</td>
<td>Eurotiales**</td>
<td>P. commune, P. oxalicum, P. camemberti</td>
<td>100</td>
<td>Penicillium commune</td>
</tr>
<tr>
<td>P31.1</td>
<td>LT623960</td>
<td>4</td>
<td>painting</td>
<td>Penicillium sp.</td>
<td>P. lanosum, P. halotolerans</td>
<td>99</td>
<td>Penicillium aurantiogriseum</td>
</tr>
<tr>
<td>S0-3</td>
<td>LT623961</td>
<td>1</td>
<td>sediment</td>
<td>Penicillium sp.</td>
<td>P. janthinellum, P. ochrochloron</td>
<td>99</td>
<td>Penicillium janthinellum</td>
</tr>
<tr>
<td>S1-4</td>
<td>LT623962</td>
<td>1</td>
<td>sediment</td>
<td>Penicillium sp.</td>
<td>P. echinulatum, P. commune, P. solitum, P. caesareum</td>
<td>100</td>
<td>Penicillium solitum</td>
</tr>
<tr>
<td>2-1</td>
<td>LT623963</td>
<td>1</td>
<td>painting</td>
<td>Penicillium sp.</td>
<td>P. panosporum</td>
<td>99</td>
<td>Penicillium coprophilum</td>
</tr>
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<td>2-2</td>
<td>LT623964</td>
<td>1</td>
<td>painting</td>
<td>Penicillium sp.</td>
<td>P. brevicaespunctum</td>
<td>100</td>
<td>Penicillium brevicompactum</td>
</tr>
<tr>
<td>2-3</td>
<td>LT623965</td>
<td>6</td>
<td>painting</td>
<td>Penicillium sp.</td>
<td>P. coprophilum, P. chrysogenum, P. concentricum</td>
<td>100</td>
<td>Penicillium coprophilum</td>
</tr>
<tr>
<td>2-7</td>
<td>LT623966</td>
<td>1</td>
<td>sediment</td>
<td>Penicillium sp.</td>
<td>P. echinulatum, P. commune, P. solitum, P. caesareum</td>
<td>100</td>
<td>Penicillium solitum</td>
</tr>
<tr>
<td>SF20-3</td>
<td>LT623967</td>
<td>1</td>
<td>sediment#</td>
<td>Penicillium sp.</td>
<td>P. concentricum</td>
<td>98</td>
<td>Penicillium concentricum</td>
</tr>
<tr>
<td>P3*</td>
<td>LT623968</td>
<td>8</td>
<td>painting / sediment</td>
<td>Aspergillus sp.</td>
<td>A. versicolor</td>
<td>100</td>
<td>Penicillium versicolor</td>
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<td>P28</td>
<td>LT623969</td>
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<td>painting / sediment</td>
<td>Aspergillus sp.</td>
<td>A. versicolor</td>
<td>100</td>
<td>Penicillium versicolor</td>
</tr>
<tr>
<td>P3*</td>
<td>LT623970</td>
<td>6</td>
<td>painting / sediment</td>
<td>Simplicillium sp.</td>
<td>S. cylindrosporum, S. miniatense</td>
<td>99</td>
<td>Penicillium cylindrosporum</td>
</tr>
<tr>
<td>5-1</td>
<td>LT623971</td>
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<td>painting</td>
<td>Chaetomiaceae</td>
<td>Chaetomium arcticum</td>
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<td>Chaetomium arcticum</td>
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<tr>
<td>SF15-4</td>
<td>LT623972</td>
<td>2</td>
<td>sediment§</td>
<td>Chaetomiaceae</td>
<td>Chaetomium piluliferum</td>
<td>99</td>
<td>Chaetomium piluliferum</td>
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<tr>
<td>S1-8*</td>
<td>LT623973</td>
<td>2</td>
<td>sediment</td>
<td>Trichosporon sp.</td>
<td>T. porosum</td>
<td>100</td>
<td>Penicillium porosum</td>
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<tr>
<td>SF19-2</td>
<td>LT623974</td>
<td>2</td>
<td>sediment§</td>
<td>Trichosporon sp.</td>
<td>T. jirovecii</td>
<td>98</td>
<td>Penicillium jirovecii</td>
</tr>
<tr>
<td>5-2*</td>
<td>LT623975</td>
<td>3</td>
<td>painting</td>
<td>Gymnascococcus sp.</td>
<td>G. reessii</td>
<td>99</td>
<td>Gymnascococcus reessii</td>
</tr>
<tr>
<td>S1-3</td>
<td>LT623976</td>
<td>2</td>
<td>sediment</td>
<td>Mortierella sp.</td>
<td>M. parvisporis, M. jenkinsii</td>
<td>99</td>
<td>Penicillium parvisporis</td>
</tr>
<tr>
<td>S1-7</td>
<td>LT623977</td>
<td>1</td>
<td>sediment</td>
<td>Mortierella sp.</td>
<td>M. verticillata, M. epichadua</td>
<td>99</td>
<td>Penicillium verticillata</td>
</tr>
<tr>
<td>S2-6*</td>
<td>LT623978</td>
<td>1</td>
<td>sediment</td>
<td>Mucor sp.</td>
<td>M. circinelloides</td>
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<td>Mucor circinelloides</td>
</tr>
<tr>
<td>SF15-7</td>
<td>LT623979</td>
<td>1</td>
<td>sediment§</td>
<td>Mucor aligarensis</td>
<td>M. aligarensis</td>
<td>100</td>
<td>Mucor aligarensis</td>
</tr>
<tr>
<td>SF20-2</td>
<td>LT623980</td>
<td>1</td>
<td>sediment§</td>
<td>Mucor sp.</td>
<td>M. plumbeus</td>
<td>100</td>
<td>Mucor plumbeus</td>
</tr>
<tr>
<td>P16</td>
<td>LT623981</td>
<td>2</td>
<td>painting</td>
<td>Cladosporium sp.</td>
<td>C. variabile</td>
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<td>Cladosporium variabile</td>
</tr>
<tr>
<td>S1-5*</td>
<td>LT623982</td>
<td>2</td>
<td>sediment</td>
<td>Ascomycota</td>
<td>Humicola grisea, Trichocladium asperum</td>
<td>99</td>
<td>Humicola grisea var. grisea</td>
</tr>
<tr>
<td>S0-9*</td>
<td>LT623983</td>
<td>1</td>
<td>sediment</td>
<td>Trichoderma sp.</td>
<td>T. koningii, T. hispanicum, T. viride</td>
<td>100</td>
<td>Trichoderma koningii</td>
</tr>
<tr>
<td>S1-1</td>
<td>LT623984</td>
<td>1</td>
<td>sediment</td>
<td>Trichoderma sp.</td>
<td>T. harzianum</td>
<td>100</td>
<td>Trichoderma harzianum</td>
</tr>
<tr>
<td>P18*</td>
<td>LT623985</td>
<td>1</td>
<td>painting</td>
<td>Hypocreales</td>
<td>Sarocladium zeae, Sarocladium strictum</td>
<td>94</td>
<td>Acrocornium zeae</td>
</tr>
<tr>
<td>S0-12*</td>
<td>LT623986</td>
<td>1</td>
<td>sediment</td>
<td>Exophiala sp.</td>
<td>E. salmonis</td>
<td>100</td>
<td>Acrocornium salmonis</td>
</tr>
<tr>
<td>S0-2*</td>
<td>LT623987</td>
<td>1</td>
<td>sediment</td>
<td>Bionectria sp.</td>
<td>B. rossmaniae</td>
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<td>B. rossmaniae var. B. sesquispili</td>
</tr>
<tr>
<td>S1-6*</td>
<td>LT623988</td>
<td>1</td>
<td>sediment</td>
<td>Arthrinium sp.</td>
<td>A. arundinis</td>
<td>100</td>
<td>Arthrinium arundinis</td>
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<tr>
<td>S1-9*</td>
<td>LT623989</td>
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<td>sediment</td>
<td>Torulaspora sp.</td>
<td>T. delbrueckii</td>
<td>99</td>
<td>Torulaspora delbrueckii</td>
</tr>
<tr>
<td>S2-2*</td>
<td>LT623990</td>
<td>1</td>
<td>sediment</td>
<td>Pochonia sp.</td>
<td>P. rubescens, P. suchlasporia</td>
<td>98</td>
<td>Pochonia rubescens</td>
</tr>
<tr>
<td>S2-4*</td>
<td>LT623991</td>
<td>1</td>
<td>sediment</td>
<td>Doratomyces sp.</td>
<td>D. stemonitis, D. purpureofuscos, D. castaneus</td>
<td>99</td>
<td>Doratomyces purpureofuscos</td>
</tr>
<tr>
<td>SF3-4</td>
<td>LT623992</td>
<td>1</td>
<td>sediment§</td>
<td>Debaryomyecetaceae</td>
<td>Debaryomyces sp.</td>
<td>100</td>
<td>Debaryomyces sp.</td>
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<tr>
<td>S2-7</td>
<td>LT623993</td>
<td>1</td>
<td>sediment</td>
<td>Bionectriaceae</td>
<td>Glomastix muscorum</td>
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<td>Glomastix muscorum</td>
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<tr>
<td>P31.2*</td>
<td>LT623994</td>
<td>1</td>
<td>sediment</td>
<td>Pseudogymnascus sp.</td>
<td>P. panosporum</td>
<td>99</td>
<td>Pseudogymnascus panosporum</td>
</tr>
</tbody>
</table>

*Representative strains of taxonomical groups showing identical sequences in the molecular analysis. †Strains included in the biocide susceptibility assay (Table 2). **Isolation source in Magura Cave, samples from the rock surface close or on the paintings collected using cotton swabs (painting), or samples from cave sediments rich in bat guano (sediment). §Sediment samples showing mycelial growth visible to the naked eye.

- Molecular identification based on comparison of their ribosomal ITS sequences, except some strains (**) for which the 18S rDNA gene was used because the ITS primers failed, with GenBank by BLAST algorithm from NCBI. The closest relative species are detailed.
- Some strains could not be morphologically identified because they produced sterile colonies or additional studies in detail are need.
Biocide susceptibility of fungal strains

Biocide susceptibility of major fungi isolated from Magura Cave was tested by using the agar disc-diffusion test. Two biocidal substances were evaluated, benzalkonium chloride (BAC) and 2-octyl-3-isothiazolinone (OIT), which have a broad antimicrobial activity including against fungi. BAC, composed by mixtures of n-alkyldimethylbenzyl ammonium chlorides, belongs to the group of surface-active quaternary ammonium compounds and is active against bacteria, fungi, some viruses and protozoa. BAC biocidal action is due to disruption of intermolecular interactions causing dissociation of cellular membrane lipid bilayers, which compromises cellular permeability controls and induces leakage of cellular contents (Gilbert & Moore, 2005). OIT is an isothiazolinone derivative with broad-spectrum biocidal action used to control the growth of bacteria, fungi and algae in cooling water systems, storage tanks, emulsions and paints (Kramer et al., 2008). Both compounds are widely used for conservation of many materials including culture heritage assets.

In order to evaluate accurate concentrations of biocides, high-purity active ingredients were used for both biocides, BAC at ≥ 95.0% (Sigma-Aldrich Chemie GmbH, Munich, Germany; product number 12060) and OIT at 97.8% (non-commercial product kindly provided by Thor GmbH, Speyer, Germany). According to similar previous studies, three different biocide concentrations (100, 500 and 1,000 mg/L) were evaluated. For each strain the test was conducted on MEA plates in triplicate plus a control plate without biocide. All plates were initially inoculated with 100 µl of the fungal suspension (described below). Afterwards three sterile Whatman paper discs (5 mm in diameter) were placed on the medium and subsequently soaked with 15 µl of the corresponding biocide concentration. Plates were incubated at 25°C in darkness for 10 days and measurements of the inhibition zones around the discs were recorded.

For fungal inoculation, cell suspensions were prepared in sterile phosphate-buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄; pH 7.4), from 2-week-old MEA cultures of selected strains, following two different procedures depending on sporulation rate. For fungi with high sporulation, conidia were collected washing the colony surface with 5 ml PBS using a sterile pipet. For slow growing fungi or those with poor sporulation, mycelia were mixed with 10 ml PBS and 5 mm metal beads, and subsequently homogenized in a Retsch MM400 mill (Retsch GmbH, Haan, Germany) for 10 min at 30 Hz. The cell concentration of all resulting suspensions was very high, developing a homogeneous growth over the whole surface of culture plate in a few days (2-7) after inoculation with 100 µl.

RESULTS AND DISCUSSION

Fungal assemblages inhabiting Magura Art Gallery

A total of 78 strains, belonging to 37 operational taxonomic units (OTUs; whose members show identical sequences), were isolated during the study (Table 1). According to the phylogenetic study (Fig. 2 and 3), the majority of OTUs belong to the phylum Ascomycota (30 OTUs, 81%), in contrast to fewer Basidiomycota (two Trichosporon species) and Zygomycota (five species of the genera Mucor and Mortierella). Among the ascomycetes, fourteen OTUs were from class Eurotiomycetes, with the major genera Penicillium (10 OTUs, 39 strains) and Aspergillus (2 OTUs, 10 strains), and twelve OTUs were included in class Sordariomycetes, with a more diverse representation of taxa.

All OTU-representative strains were further morphologically characterized to be identified at the species level. However, only twelve strains (32%) have been identified using this approach (Table 1) due to the well-known limitations of such studies. For some studied strains, such as Aspergillus sp. P23, additional morphological, physiological and/or phylogenetic studies would be needed for reliable identification. Other strains, such as Mortierella sp. S1-3, only produced sterile colonies which prevented their characterization. The phylogenetic trees (Fig. 2 and 3) show the final consensus identification of strains using both molecular and morphological data.

Regarding DGGE characterization of Magura strains, in general the OTUs detailed in Table 1 showed distinctive patterns, like those Penicillium strains belonging to the most abundant OTU (Fig. 4a). As demonstrated in previous studies (Martin-Sanchez et al., 2012), the fungal strains in pure culture frequently develop characteristic multiband DGGE profiles, due to PCR artifacts, which complicates the interpretation of DGGE results from diverse environmental samples. In this sense, Neilsen et al. (2013) concluded that DGGE provides an excellent tool for comparative community structure analysis, but such method-specific artifacts preclude its use for accurate quantitative diversity analysis.

In general, the fungal diversity found in the Art Gallery was rather low with clear dominance of a few genera. However, as well known in literature, cultivable populations do not represent the true diversity of the ecosystem. Less than 1% of the estimated microbial diversity is thought to be cultivable in laboratory conditions due to the very low growth rate of many environmental microorganisms (Aman et al., 1995). In addition, the selected culture methods, media and incubation conditions, have most likely favored these genera against the rest of microbes. In accordance with many previous cultivation-based studies in other caves (Vanderwolf et al., 2013), the fungal community in Magura Cave was clearly dominated by Penicillium and Aspergillus species (Fig. 3). Most of Penicillium strains (59%) were taxonomically placed in the subgenus Penicillium, section Fasciculata, according to the classification of Houbraken and Samson (2011) and Visagie et al. (2014a), including the species...
P. commune and P. solitum. Six Eurotiales strains, with P7 as representative, were closely related to *Penicillium malachiteum* (subgenus *Aspergilloides*, section *Sclerotiora*) based on the analysis of their 18S rRNA gene sequences (Table 1). However, they could not be included in the phylogenetic analysis focused on ITS region. Members of Chaetomiaceae family and genera such as *Simplicillium*, *Trichosporon*, *Gymnoascus*, *Mortierella*, *Mucor*, *Cladosporium* and *Trichoderma* were repeatedly isolated in the studied samples. Most of genera isolated from Magura Cave were frequently found in previous studies of air samples from indoor environments, and either in sediment or rock surface samples from other caves in Europe (Vanderwolf et al., 2013). This is logical considering the high concentration of airborne fungal spores expected in caves like Magura, populated by bat colonies and accessible to tourists, with the consequent inputs of organic matter.

Ascomycota appears to be the most abundant phylum in any natural environment irrespective of whether culture dependent or independent approaches are used. According to previous studies (Nováková et al., 2009; Docampo et al., 2011; Vanderwolf et al., 2013; Man et al., 2015), the most abundant fungal genera in caves are *Penicillium*, *Aspergillus*, *Cladosporium*, *Mortierella*, *Mucor*, and *Cladosporium*.
Fig. 3. Eurotiales phylogeny of the ITS region showing the placement of representative Penicillium and Aspergillus strains from Magura Cave (in bold) and the closest reference strains, including type strains (‘) and isolates from caves (‘). Penicillium sections according to Houbraken and Samson (2011) are shown. Numbers of strains higher than one are presented in brackets. The tree was constructed using the Neighbor-Joining method applying the Kimura 2 parameter model, and rooted with Gymnoascus reessii 5-2 as outgroup. All positions containing gaps and missing data were eliminated. There were a total of 445 positions in the final dataset. The tree was bootstrapped 10,000 times and values above 50% are indicated at nodes. The asterisks indicate branches of the tree that were also recovered using Maximum-Likelihood and Maximum-Parsimony treeing algorithms. Bar, 0.02 substitutions per nucleotide position.

Fig. 4. DGGE profiles of fungal strains and environmental samples from Magura Cave. a) Strains Penicillium sp. P5 (= P1.1, P1.2, P4, P12, P13, P14, P15), Simplicillium sp. P3 (= P6), Aspergillus sp. P9, Cladosporium sp. P16 (= P17) and Acremonium zeae P18; marker composed by the strains P4, P9 and P16; b) Sediment samples (S) rich in bat guano, wall painting samples (W) collected from rock surface close or on the paintings, and some strains from samples W2 (Penicillium sp. 2-1, Penicillium coprophilum 2-3 and Penicillium solitum 2-7) and W5 (Chaetomiaceae 5-1 and Gymnoascus reessii 5-2).
Mucor, Fusarium and Trichoderma, which may be due to their ubiquitous distribution in nature. Aspergillus/ Penicillium was the most abundant spore type found in the air of Nerja Cave, Spain, which represented 50% of the total spores, followed by Cladosporium with 17% (Docampo et al., 2011). Penicillium and Aspergillus species grow better in warm and humid environments but can tolerate wide ranges of temperature and pH. They are ubiquitous molds that grow on organic matter producing abundant conidia which enhance their fast spreading (Visagie et al., 2014b).

Rock surface of painted walls

As described by Man et al. (2015) in Heshang Cave, China, differences in abundance and composition of fungal assemblages were detected comparing different substrata of Magura Cave. First, it is noteworthy to mention than we did not detect relevant in situ fungal growth on the rock surface of painted walls in the different sampling campaigns. Evident fungal mycelia were only observed in a few small areas near the paintings, which were especially targeted for sampling. This fact could explain the low number of colonies grown on culture media from the painting samples collected using swabs. For instance, cultivable fungi were only isolated from three (W2, W5, and W11) of twelve painting samples collected in the last sampling campaign in 2015. However, the DGGE patterns corresponding to nine of these samples showed more diverse assemblages (Fig. 4b), remarking the prevalence of non-cultivable fungi on this substratum. Despite the commented limitations of DGGE (Neilson et al., 2013), which hinder the comparison between environmental samples and isolated strains, results show that structure of fungal assemblages on rock surfaces is rather different depending on studied location. Samples W1 and W2, collected from nearby areas in the entrance of Art Gallery, showed similar patterns between them, and different to samples collected from other locations in the gallery. Moreover, the main members of these assemblages most likely correspond to non-cultivable fungi under the selected media and incubation conditions. Penicillium, Aspergillus, Simplicillium, Gymnoascus and Cladosporium were the most representative genera cultivated from these samples. The genera Aspergillus and Penicillium belong to the group of fermentative microorganisms which excrete a variety of organic acids that can cause biodeterioration of rock substratum (Sterflinger, 2000).

Bat guano-rich sediments

On the other hand, in sediment samples rich in bat guano, high abundance of colonies and diversity were found by culturing, and subsequently confirmed by DGGE (Fig. 4b). Although Penicillium remains the major group in this substratum, other genera such as Trichosporon, Mortierella, Mucor, Humicola and Trichoderma were also abundant there, while none of them were detected on rock surfaces. In particular, for those sediment samples showing mycelial growth visible to the naked eye (Fig. 1g), members of genera Mucor, Penicillium and Trichosporon, and families Chaetomiaceae and Debaryomycetaceae, were identified (Table 1). Most likely, based on morphology, such fungal growth was mainly caused by Mucor species. Jurado et al. (2010) reported similar mycelial growth on sediments of Castañar de Ibor Cave, Spain, during a fungal outbreak associated with the species Mucor circinelloides and Fusarium solani.

Novákova (2009) reported a comprehensive fungal inventory (195 taxa) from Domica Cave system, Slovakia. The highest number of taxa (92) was found in bat guano samples, where species of genera Penicillium, Mucor, Doratomyces and Trichoderma were prevalent. Furthermore, Mucor species often formed visible colonies on bat droppings and surface of guano heaps from these caves. Bat guano samples from other Slovakian caves were recently studied by Ogorek et al. (2016), with Penicillium, Aspergillus, Mucor and Rhizopus as dominant genera. Trichosporon species are widespread in cave sediments, especially in those rich in bat guano, which highlights the relevance of this cave substratum as reservoir of potentially pathogenic fungi (Saiz-Jimenez, 2012). This basidiomycetous yeast-like genus has frequently been reported as causal agent of superficial infections, as well as opportunistic agent of invasive infections (Colombo et al., 2011). Different studies have reported novel Trichosporon species isolated from sediments of bat-inhabited caves in Japan and Slovakia (Sugita et al., 2005; Novákova et al., 2015). Likewise, many Mortierella species were usually isolated from bat dung samples collected in caves in Japan (Degawa & Gams, 2004). Species of the genera Mucor, Mortierella, Trichoderma and Pseudogymnoascus were cultured from dead bats in Berryton Cave, Canada, being the fast growing Zygomycetes, Mucor and Mortierella, particularly abundant on freshly dead bats (Vanderwolf et al., 2016). The results confirm that members of genera found in Magura Cave are significant constituents in many karstic caves. Their ecological function and possible impact on the paintings are not well understood yet.

The main input of organic matter in Magura Cave is clearly from the bat colony inhabiting this cavity long time ago, with the consequent accumulation of bat guano in sediments of the whole Art Gallery, and recurrent presence of visible fungal colonies covering these sediments. However, according to our observations and results, the fungal growth on rock surface of painted walls has been quite scarce up to date. As expected and demonstrated in this study, the current fungal community in Magura Cave is mainly composed by guanophilic species. Considering that Magura paintings were made using bat guano, this cave has most likely been inhabited by bats and their associated guanophilic microbiota since the origin of paintings thousands of years ago. Therefore, any conservation measure addressed to reduce organic matter inputs by controlling bat populations, and/or cleaning bat guano, does not make sense. Besides, such proposals would not be conceivable with current bat protection rules. The potential input of organic matter coming from tourists may be considered insignificant in comparison to bat guano contribution.
However, humans can critically affect environmental conditions in caves, e.g. increasing variables such as temperature and carbon dioxide concentration, and lead to an ecological imbalance that stimulates the growth of certain microorganisms causing microbial outbreaks.

**Fungal susceptibility to biocides**

The susceptibility of Magura fungi to two of the most commonly used biocides, BAC and OIT, was additionally evaluated by agar disc-diffusion test. A total of seventeen strains, representative of the major phylotypes dwelling in the Art Gallery, were studied (Table 2). In general, both biocides were able to inhibit the fungal growth on culture plates when applied at described concentrations (100, 500 and 1,000 mg/L). These concentrations are significantly lower than those specified in manufacturers’ instructions of commonly used commercial products, in general ranged from 1 to 25 g/L. Only *Trichoderma* sp. strain S0-9 was resistant to all tested BAC doses. The majority of studied strains showed much higher susceptibility to OIT in comparison to BAC (Fig. 5a-c), except for yeast species. *Torulaspora* sp. S1-9 showed the opposite trend (Fig. 5d-f) and *Trichosporum* sp. S1-8 showed similar susceptibility to both biocides. The lowest OIT concentration tested (100 mg/L) inhibited all fungi, except *Trichoderma* sp., causing a significant inhibition zone diameter (> 16 mm) in 13 of 17 strains. In this sense, it is noteworthy to mention the high inhibition degree of some fungi, such as *Penicillium* sp., *Exophiala* sp., *Doratomyces* sp. and *Pseudogymnoascus* sp., to 500 and 1,000 mg/L OIT, which could not accurately be assessed because of their great inhibition zones (diameters > 50 mm; ++++).

The most susceptible fungi to BAC were the mentioned yeasts, *Trichosporum* sp. and *Torulaspora* sp., as well as *Exophiala* sp., with inhibition zone diameters lower than 30 mm (Table 2). Growth inhibition by BAC was very limited (5-15 mm diameter) or non-existent in 9 of 17 evaluated species, including the prevalent *Simplicillium* sp., *Mucor circinelloides*, and *Trichoderma* sp.

These results provide valuable knowledge about the susceptibility of cave fungi to common biocides. According to these data, in general, one may think that a biocide treatment based on both active substances (especially OIT), could be a useful tool to control the fungal growth in Magura Cave. However, biocide efficacy is questionable in cave ecosystems because of the presence of complex biofilms, inaccessible to biocides, and the fast inactivation of biocides through biotic and abiotic factors (Martin-Sanchez et al. 2012). In this sense, about the in vitro biocide assay included in this study, it should be noted that inhibition zones shown in Table 2 were recorded after a few days of incubation (ranged from 2 to 7 days, mostly 3 days), during the initial phase of fungal growth. However, afterwards, fungal colonies began to colonize the inhibition zone progressively reducing its area with the time. Hence, it is expected that efficacy of biocides will be limited to a time period after application, especially under natural cave environmental conditions.

Some cave restoration efforts in the last decades applied conventional biocides leading to detrimental effects on the complex microbial communities inhabiting caves (Saiz-Jimenez, 2013). Intense biocide treatments were applied in Lascaux Cave

Table 2. Biocide susceptibility of fungi isolated from Magura Cave.

<table>
<thead>
<tr>
<th>Strains a,b</th>
<th>Identification</th>
<th>Benzalkonium chloride Growth inhibition c</th>
<th>2-Octyl-3-isothiazolinone Growth inhibition c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100 mg/L</td>
<td>500 mg/L</td>
</tr>
<tr>
<td>P14*</td>
<td><em>Penicillium</em> sp.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2-7</td>
<td><em>Penicillium solitum</em></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>P38*</td>
<td><em>Aspergillus</em> sp.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3-7</td>
<td><em>Simplicillium</em> sp.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S1-8</td>
<td><em>Trichosporon</em> sp.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5-2</td>
<td><em>Gymnoascus reessii</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S2-6</td>
<td><em>Mucor circinelloides</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S1-5</td>
<td><em>Humicola grisea var. grisea</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S0-9</td>
<td><em>Trichoderma</em> sp.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S1-7</td>
<td><em>Acremonium zeae</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S0-12</td>
<td><em>Exophiala</em> sp.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S0-2</td>
<td><em>Bionetria rossmaniae</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S1-6</td>
<td><em>Arthrinium arundinis</em></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S1-9</td>
<td><em>Torulaspora</em> sp.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S2-2</td>
<td><em>Pochonia</em> sp.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S2-4</td>
<td><em>Doratomyces</em> sp.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>P31.2</td>
<td><em>Pseudogymnoascus</em> sp.</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

a: All selected strains are representative ones included in Table 1, except (*) P14 and P38, whose representative strains are P5 and P23, respectively.
b: Inhibition zones were measured after 3 days of incubation (25°C, darkness), except for the strains S2-6 (after 1 day), 5-2 and S2-2 (after 2 days), P3 (after 4 days), and S0-12 (after 1 week).
c: Scale according to inhibition zone diameter: no inhibition (-), 5 - 15 mm (+), 16 - 30 mm (++), 31 - 50 mm (+++), and >50 mm (+++). Values of inhibition zone diameter were average of three replicates.
Fungal assemblages in Magura Cave

Fig. 5. Inhibition of fungal growth by the biocides benzalkonium chloride (BAC) and 2-octyl-3-isothiazolinone (OIT) in the agar disc-diffusion test. a-c) Cultures of Aspergillus sp. P38 on MEA after 3 days, control without biocide (a) and effect of different concentrations of BAC (b) and OIT (c); d-f) Cultures of Torulaspora sp. S1-9 on MEA after 3 days, control (d), BAC (e) and OIT (f). Biocide concentration in the discs: 100 mg/L (bottom right), 500 mg/L (bottom left) and 1,000 mg/L (top).

during several periods over years, mainly based on BAC but including other compounds such as OIT, formaldehyde, streptomycin sulphate and polymyxin sulphate. In particular, BAC concentrations used were ranged from 5 to 25 g/L, and OIT concentrations around 1 g/L (Martin-Sanchez et al., 2015). Such prolonged chemical treatments caused negative effects to the cave environment, and selected resistant microorganisms which later played a key role in the black stain outbreak (Bastian et al., 2009; Martin-Sanchez et al., 2012, 2015). Akatova et al. (2009) evaluated the efficiency of different cleaning protocols on phototrophic biofilms of Salpetre Cave (Collbató, Spain), including mechanical removal using 70% ethanol and subsequent treatment with BAC biocides. The treated areas of speleothems were monitored under white or green light. After one year, none of the treatments was fully effective to control the recolonization by phototrophic microorganisms in the stalactite illuminated with white light. Recently, Urzì et al. (2016) reported the changes of the microbial assemblages in green/greyish phototrophic biofilms from Domitilla Catacombs (Rome, Italy) caused by a biocide treatment with the same compounds, BAC (8-12 g/L) and OIT (1.4-2 g/L). In this study, such biocides had little effect on cyanobacteria, but caused significant changes on bacterial population increasing its number and diversity.

Some authors have proposed the use of hydrogen peroxide as an environmental friendly alternative to control microbial outbreaks (Faimon et al., 2003; Boston et al., 2006). Jurado et al. (2010) reported satisfactory results using this product to control the fungal outbreak in Castañar de Ibor Cave, Spain, mainly associated with Mucor species. They proposed carrying out mechanical removal of visible fungal colonies followed of disinfestation of affected areas by hydrogen peroxide application. The main advantage of hydrogen peroxide is its degradation in contact with organic matter towards harmless molecules for the cave environment such as water and free oxygen gas.

CONCLUSIONS

Rock-art caves, like Magura Cave, are one of the most valuable properties of our ancient cultural heritage which attract massive tourism. For an appropriate cave management, especially in rock-art caves opened for visitors, it is essential to investigate the diversity and ecological role of microorganisms inhibiting there. This study provides the first scientific report on cultivable fungal assemblages dwelling different substrata in the Art Gallery of Magura Cave. As typical for caves, the fungal diversity in this gallery was clearly dominated by Penicillium and Aspergillus species which might due to their ubiquitous distribution in the nature. The most obvious growth of fungal mycelia in this gallery was observed in sediments rich in bat guano. These samples showed much higher number and diversity of colonies grown on culture plates than samples collected from rock surface of painted walls. Besides Penicillium, the dominant phytopotypes in these sediments were guanophilic fungi such as Mucor, Mortierella, Trichosporon and Trichoderma. Conversely, on the rock, a few small areas near the paintings showed evident fungal mycelia, and scarce fungi were isolated from some of these samples.
Based on biocide susceptibility assay, the active compounds BAC and OIT were effective inhibiting the in vitro growth of dominant species from Magura Cave when applied at concentrations ranged from 100 to 1,000 mg/L. These data provide a valuable knowledge about Magura fungi, and exemplify a kind of preliminary test that may be conducted before planning any biocide treatment. However, considering the irreversible effects of biocides on the ecological balance in cave environments, multiple factors should carefully be evaluated before taking such critical decision in caves with rock-art paintings. As described by Martin-Sanchez et al. (2012), preliminary studies on possible advantages and disadvantages of applying biocides in each particular case are required. Any biocide treatment should be planned after testing the active compounds under different conditions, including (i) laboratory assays like in this study, but also (ii) field assays in real conditions, which are very difficult to design properly. In Magura Cave, considering the low fungal contamination in rock surfaces of painted walls, and the previously mentioned risks, there is no reason to use conventional biocides. These treatments should be avoided in order to preserve the relatively stable current balance, which of course is already altered compared to its original natural condition. Alternatively, if a more significant fungal outbreak arises, cleaning protocols combining careful mechanical removal of fungal biomass and subsequent disinfection of affected areas using hydrogen peroxide could be conducted, as long as this cleaning treatment does not lead to damages in rock-art paintings.

Human visitors can influence the quantity and diversity of microbiota in caves through of microclimate changes, organic inputs as well as lighting systems. Such human impacts only could partially be predicted by periodic microclimatic and microbiological studies. To the best of our knowledge, in Magura Cave, there was no microclimatic study up to date, and the microbiological reports are still scarce to shed light on its microbial ecology. Hence, further studies, monitoring of microbial communities and microclimatic parameters in the different cave substrata, should be conducted in order to improve the knowledge on microbial ecology in Magura Cave and to allow the early detection of potential microbial outbreaks.

ACKNOWLEDGEMENTS

The study was supported by the National Science Fund of Ministry of Education and Science, Bulgaria (Project №ДДБУ/02-73/2010), Project 13/2013/ SU, and the Bundesanstalt für Materialforschung und -prüfung (BAM), Berlin, Germany. MM thanks to the Erasmus+ Programme of the European Commission which funded her scholarship at the BAM. PMMS thanks to the Adolf Martens Fellowship Programme of the BAM. The authors are especially grateful to Dr. Jörg Toepel for scientific advices and support during laboratory works, and to Dr. Ute Schoknecht for providing the biocides used in this study. Comments and corrections of three anonymous reviewers were extremely valuable to improve this article.

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https://doi.org/10.1007/s00114-009-0540-y


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https://doi.org/10.1016/j.scitotenv.2010.10.048

Fungal assemblages in Magura Cave


International Journal of Speleology, 46 (1), 67-80. Tampa, FL (USA) January 2017


Bat urea-derived minerals in arid environment.
First identification of allantoin, C₄H₆N₄O₃,
in Kahf Kharrat Najem Cave, United Arab Emirates

Philippe Audra¹, Pavel Bosák²-⁶, Fernando Gázquez³, Didier Cailhol⁴, Roman Skála², Lenja Lisá², Šárka Jonášová², Amos Frumkin⁵, Martin Knez⁶, Tadej Slabe⁶, Nadja Zupan Hajna⁶, and Asma Al-Farraj⁷

¹University of Nice Sophia-Antipolis, Polytech’Lab, 930 route des Colles, 06903 Sophia-Antipolis, France
²Institute of Geology of the CAS, v.v.i., Rozvojová 269, 165 00 Praha 6, Czech Republic
³Laboratoire EDYTEM, University Savoie – Mont-Blanc, CNRS, Pôle Montagne, 73376 Le Bourget-du-Lac, France
⁴Institute of Earth Sciences, The Hebrew University, 91905 Jerusalem, Israel
⁵Institute of Geology and Palaeontology, University of Cambridge, CB2 3EQ, United Kingdom
⁶Karst Research Institute ZRC SAZU, Titov trg 2, 6230 Postojna, Slovenia
⁷Emirates Geographical Society, P.O. Box 4368, Ras Al-Khaimah, United Arab Emirates

Abstract: Kahf Kharrat Najem Cave is a small cave in United Arab Emirates (UAE) that hosts a bat colony which is the source of guano deposits and peculiar centimeter-long yellowish stalactites. The mineralogy and geochemistry of these deposits were analyzed using powder X-ray diffraction (XRD), energy dispersive X-ray spectroscopic microanalysis (EDX), scanning electron microscope (SEM), and stable isotope composition (δ¹³C and δ¹⁵N). Urea CO(NH₂)₂ was found to be the main compound of these stalactites, while allantoin C₄H₆N₄O₃ was found to be an accessory urea byproduct. This paper is the first to mention allantoin in a cave environment. We also identified rare sulfate minerals (aphthitalite, alunite) and phosphates that probably correspond to the archerite-biphosphammite series. The occurrence of these rare bat-related minerals is due to the extremely dry conditions in the cave, which accounts for the extraordinary preservation of the guano deposits and allows for the crystallization of these very soluble minerals.

Keywords: cave minerals, allantoin, bat guano, bat urea, Kahf Kharrat Najem Cave, United Arab Emirates


INTRODUCTION

Caves in the Arabian Peninsula, and in particular in the United Arab Emirates (UAE), have been poorly studied. Speleological surveys in this region started in the 1980’s (Edgell, 1990; Waltham & Fogg, 1998; Waltham & Jeannin, 1998; Fogg et al., 2002). In the Oman Range, and especially on the Oman side, large epigenic cave systems (i.e., those originating from the infiltration of rain water) exist. They are generally developed where relief favors high slope gradients and large catchment areas, which when combined with extreme storm events leads to the formation of large subterranean systems (Waltham et al., 1985). However, in the UAE epigenic caves are generally of limited extent because of the scarce surface runoff and the relatively fast filling of these caves with wind-blown deposits. In contrast, volcanic and hypogenic caves, which are generally less frequent on Earth, are significantly more common in the Arabian Peninsula (Pint, 2003). Hypogenic caves form from the rising of deep-seated, oftentimes hydrothermal waters, which are usually rich in dissolved CO₂ and/or H₂S (Klimchouk, 2007; Audra & Palmer, 2015). In the caves of the UAE evidence for speleogenesis by deep-seated fluids and rising thermal water includes typical morphologies and deposits such as a high density of cupolas, condensation-corrosion features, rift-and-tubes mazes, feeders, and hydraulic breccia, together with iron oxy-hydroxides deposits as well as thick crusts of calcite or massive gypsum (Jeannin, 1990; Waltham and Jeannin, 1998; Fogg et al., 2002; Zupan et al., 2016).
To date only a few studies focusing on the Arabian Peninsula karst and caves have been published. This includes surveys on karst surface features in Qatar (Sadiq & Nasir, 2002); hypogenic caves in Saudi Arabia (Kempe & Dirks, 2008); a preliminary study of small 3-D maze cave systems, presumably of hypogene origin, which occur on wadi slopes and which are filled with past fluvial sediments (Al-Farraj et al., 2014; Zupan Hajna et al., 2016); several climatic reconstructions from speleothems of Oman Caves (Burns et al., 2001; Fleitmann & Matter, 2009); and finally, a couple of studies related to engineering issues of collapse in urban areas (Amin & Bankher, 1996; Gao et al., 2015). Studies on the mineralogy of these caves are even scarcer (Forti et al., 2005; Zupan Hajna et al., 2016). However, they are of outstanding mineralogical interest due to the extremely arid climate and geological environment, with some caves ranking among the richest in the world for their mineralogical diversity (Forti et al., 2004). In addition, the extreme arid conditions enable specific cave biota to develop; for example, different bat species which are among the highlighted symbol of Arabian fauna (Nader, 1976; Davis, 2007 and references therein).

The arid climate and low elevation of the UAE area make these caves hotter than caves found in temperate or tropical latitudes. Cave air temperature normally ranges from 27°C in the lowlands to 20°C at higher elevations, and up to 38°C in caves influenced by geothermal heating that is related to the hypogenic origin of these karstic systems (Jeannin, 1990). Correlating with temperature, extremely dry conditions frequently prevail, and relative humidity (RH) ranges from 28 to 72%, averaging ~50-60% (Jennings, 1983). Surprisingly, some of these caves show moisture saturation under wet conditions, especially where the cave atmosphere is influenced by a strong geothermal gradient responsible for strong air convection, and where condensation occurs after the temperature decreases from 38°C at depth to 24°C close to the surface (Jeannin, 1990). Such special hot microclimates, either extremely dry or sometimes relatively wet, provide the environment necessary for the genesis of rare evaporite cave minerals such as sulfates or chlorides. Additionally, the frequent presence of bat colonies (Davis, 2007) and thus guano accumulations, cause cave mineralization rich in elements that are normally allochthonous to the host rock, giving rise to phosphate and nitrate cave mineral deposits. These phosphates and nitrates often combine with the above mentioned evaporites (Forti et al., 2004).

In this paper, we investigate the mineralogy and the isotopic composition (δ13C and δ15N) of guano deposits and related speleothems in Kahf Kharrat Najem Cave (Northern UAE), including phosphates, sulfates and urea-derived byproducts related to the presence of a small bat colony therein. Despite the small extent of the cave and the hot-dry microclimate, the influence of the external conditions combined with the presence of bats provied specific environmental conditions favorable to the mineralogenesis of uncommon cave minerals and degradation products from bat excreta.

**GEOLOGICAL SETTING**

The UAE is composed of three main physiographic domains: (1) a sandy desert belonging to the Arabian sedimentary basin that covers most of the country, (2) coastal areas and mangroves along the Persian Gulf shores, and the (3) Oman Mountains along the northeastern part (Fig. 1). These mountains mainly extend along Oman coast, but in its northern part the Musandam Mountain is split between the UAE and Oman, where it reaches 2087 m at Ru’us al-Jibal. The relief displays narrow crests cut by more than 1,000 m deep wadis (canyons) and some dissected plateaus in-between. To the northwest in the UAE, the steep slopes of the mountain make an abrupt contact with the desert plain. This contact is only softened by stepped marine terraces on the slopes (Ricateau & Richié, 1980; Kusky et al., 2005) and by alluvial fans extending from the mouths of the wadi to the plain (Al-Farraj & Harvey, 2000).

In the Oman Mountains, the renowned ophiolitic series that obducted over the Arabian shield during the Late Cretaceous, crop out (Searle et al., 1983; Searle, 1988, Fig. 2). However, to the north, the Musadam Mountains greatly differ, since they are built of the (par)autochthonous 3.5 km-thick shelf carbonate sequence of the Hajar Supergroup, which ranges from Permian to Cenomanian (Fontana et al., 2014). The upper part corresponds to the Musadam Group, which comprises 1,800 m of massive shelf carbonates, mainly limestones and dolomites, with ages ranging from Middle Lias to Lower Cretaceous (Ricateau & Richié, 1980). To the south, the Dibba zone is a transition between the Musadam shelf sediments to the north and the Hajar ophiolites to the south. The boundaries between these zones correspond to

![Fig. 1. Map of the UAE with location of Kahf Kharrat Najem in Fujairah Emirate.](image-url)
NE-SW transcurrent faults. The Dibba zone is built with the allochthon Hawasina nappes. Their colorful sediments correspond to the accretionary prism thrust ahead the ophiolites nappes.

The Musadam area evolved under successive major uplifting and quiescence events (Ricateau & Richié, 1980; Kusky et al., 2005; Lacinska et al., 2014). The obduction chain was entirely eroded after its building at Late Cretaceous. Major uplifts eventually occurred at the end of Oligocene and in Upper Miocene, altogether estimated to be at about 3,000 m elevation. Tectonic deformation is still actively ongoing. Northeast tilting is shown in Fig. 2 on the western edge in Ras Al Khaimah by the development of up to 30 m-thick fluvial terraces, by the uplifting of peneplanation surfaces over 1,500 m and of Quaternary marine terraces up to 190 m (Kusky et al., 2005), and to the north by the submersion of the tip of the peninsula, which displays “fjord-like” submerged landscapes (Ricateau & Richié, 1980). Regarding hypogene karstification, several phases of deep fluid migration are associated with successive tectonic phases, such as early dolomitization, hydraulic fracturing, and karstification during emersion phases filled with microsparite and late silicification (Callot et al., 2010; Fontana et al., 2014). Presently, shallow loops of meteoric fluids rising at moderate temperature are testified by hot springs (e.g., Khatt spa, 38.5°C); these are responsible for karstification and associated calcite-cloud speleothem (Callot et al., 2010; Al-Farraj et al., 2014).

CAVE DESCRIPTION

Geology and geomorphology

Kahf Kharrat Najem Cave (“Cave of the Shooting Star”) is a small, shallow cavern roughly 10 m deep and 30 m long located in Fujairah Emirate between Khatt and Idhn cities (Northern UAE), about 10 km to the southeast of Ras Al-Khaimah airport at the mouth of Wadi Taweeyeen (Fig. 1). The cave is developed in the Jurassic to Lower-Cretaceous limestones of the Musadam Group. In the vicinity, the limestone contains chert beds, diagenetic dolomite, and significant hypogene iron-oxide deposits. The cave is located at the southern end of an anticlinal structure along its outer limb, as shown by the 15 to 25° dip of the rocks to the west (Fig. 2). The anticline is cut in the south by the main regional transcurrent Dibba fault, which separates the Musadam Mountains and the Dibba zone (Searle et al., 2014). The southern side of the wadi displays colorful marly sediments from Hawasina nappes of the Dibba zone (Fig. 2). The wadi has enlarged this contact zone to the detriment of the Hawasina marls.

The entrance to Kahf Kharrat Najem Cave opens at 57 m a.s.l., 25 m above the wadi northern slope and 1 km from the plain (Fig. 3). Several levels of terraces, glacis, or pediments, are present above the cave; these demonstrate the active uplift and the recent opening of the cave by denudation. The cave passages follow the dip and strike of the inclined bedding planes, whereas the entrance shaft has developed along a master sub-vertical fault. A first 6 m shaft in two steps leads to a small chamber, which splits into two passages (Fig. 4). To the south, a descending low passage leads to the lower part of the cave at 10 m depth. Another passage runs to the north, first horizontally, then slightly descending. The bottom of both passages is filled with fine clay sediments. The cave walls lack conventional calcite flowstone and dripstone deposits.

Fig. 2. Simplified geological map and profile of the Musandam Peninsula mainly built of the thick shelf carbonate sequence of the Hajar Supergroup (after Fontana et al., 2014, redrawn from Searle, 1988).

Fig. 3. The narrow entrance of Kahf Kharrat Najem Cave leading to the small shaft that gives access to the main cave chamber (photo G. Pucelj).
The cave was formed under phreatic conditions by ascending hypogene water originating from the extremity of both passages and proceeding upwardly through the shaft, as evidenced by rising phreatic morphologies associated with iron oxide deposits in the cave and outside. Thus, the cave was not formerly directly connected to the surface, and the current entrance resulted from surface denudation that finally intersected the cave passage. After intersection, the cave became partially filled with fine and coarse sediments brought by surface runoff during storms. Also, bats were able to colonize the cave after it was opened.

General microclimate and cave fauna

Mean cave temperature oscillates around 27°C at these altitudes and latitude, which corresponds to the mean temperature at Ras Al-Khaimah (mean: 27.1°C; coldest month: 14.1°C in January; warmest month: 38.8°C in July). However, due to the cave morphology and to the exchange of cave air with the surface, some significant differences were observed. The chamber at the bottom of the shaft is under the influence of sinking outer cool air during winter nights, which was below 14°C at the time of measurement. During our visit (January 2016), the outside temperature was 22.3°C, whereas the cave air temperature was 26.2°C at the bottom of the shaft. The south descending passage is also under the influence of this seasonal cooling process. In contrast, the north passage that opens on top of the chamber is isolated from this cooling air flow (Fig. 4). Its temperature was 30.2°C on that same day. Bats possibly contribute to the warming of the small volume of this passage. Also, thermal groundwater (38.5°C), pouring out 10 km to the north at Khatt spa and located at shallow depth below the cave, may contribute to the significant warming.

The cave atmosphere is extremely dry. This is not only because of the arid climate, but it is also related to the vertical development of the cave that favors the sinking of cool air in winter, which subsequently warms and dries up the cave passages, whereas in summer hot outer air maintains stable conditions without airflow. Altogether, these factors contribute to maintain permanently dry conditions, with a very low RH (not measured), probably similar to other caves in the UAE (~50-60%; Jennings, 1983).

About 10 to 20 mouse-tailed bats reside in the cave. The species most likely corresponds to the insectivore small mouse-tailed bat, *Rhinopoma muscatellum*, Thomas 1903 (Nader, 2003) (Fig. 5), which were actively moving about in the cave during our visit. This bat colony is responsible for the guano deposits and yellowish stalactites examined in this study. The mineralogical and geochemical compositions of the stalactites are the main topic of this paper.

MATERIALS AND METHODS

In January 2016, field work consisted of the cave survey, geological and geomorphological description, photographic documentation, and sampling of the guano deposits (sample RAK 2) and yellowish stalactites (sample RAK 3) for mineralogical and geochemical analyses. About ~20 square meters of guano accumulations are present in both the South and the North passages as 10 to 15 cm thick deposits.
Guano entirely covers the bottom of the North passage (Fig. 6C). The guano deposits are soft and dry, and poorly mineralized. In the North Passage, the flat ceiling is decorated by yellowish stalactites, crusts and drapery-like speleothems 1 to 4 cm long. These speleothems are not associated with bedrock fissures or preferred infiltration points (Fig. 6A, B).

Mineralogical investigations comprise the petrographic description of bulk sample and thin sections, phase identification using powder X-ray diffraction (XRD) technique and energy dispersive X-ray spectroscopic microanalysis (EDX). First, bulk guano and stalactite samples were analyzed for XRD and EDX microprobe. Subsequently, light- and dark-colored crystals were separated under binocular microscope for more detailed identification using the same techniques.

Phase identification by XRD was carried out at the X-ray service, CEREGE, Aix Marseille University (France). XRD patterns were recorded on a Panalytical X’Pert Pro MPD θ – θ diffractometer using cobalt radiation with a secondary graphite monochromator. The X-ray tube operating conditions were 40 kV and 40 mA and the step-scan data were continuously collected over the range 3.5 to 78° of 2θ. Additional XRD analyses were performed at the Laboratory of Analytical Methods of the Institute of Geology of the Czech Academy of Sciences at Praha by a Bruker D8 DISCOVER diffractometer. The data were collected in the angular range 4–80° (2θ) with a step of 0.014° (2θ) and cumulative exposure time for 1 detector channel of 95 second. To collect the data, a copper radiation was used and the X-ray tube was operated at 40 kV and 40 mA. The Bruker proprietary software DIFFRAC.EVA with an ICDD PDF2 database 229 (release 2011) was used for phase identification.

The surface morphology of the material was observed with a variable pressure scanning electron microscope (SEM) TESCAN VEGA 3XMU (Laboratory of the Analytical Methods of the Institute of Geology of the Czech Academy of Sciences in Praha). Samples were placed on carbon adhesive tape attached to aluminum stubs and observed with back-scattered electron (BSE) detector in low-vacuum mode without coating with any conducting medium. The chemical composition was determined qualitatively using an energy-dispersive spectrometer (EDX) Bruker QUANTAX 200. Accelerating voltage was set to 20 kV and current optimized to yield optimum conditions for chemical analysis.

Stable isotopes of guano and urea samples were analyzed for δ^{13}C and δ^{15}N using a Costech (Valencia, CA) automated elemental analyzer coupled in continuous-flow mode to a Thermo Finnigan MAT253 (Bremen, Germany) mass spectrometer at the Godwin Laboratory, Department of Earth Sciences, University of Cambridge (United Kingdom). Previous to the analysis, samples were dried overnight at 45°C and then encapsulated in tin capsules. Stable isotope concentrations are measured as the ratio of the heavier isotope to the lighter isotope and expressed as per mil (%o) relative to internationally standard materials, VPDB for carbon and AIR for nitrogen. Based on replicate analyses of international and laboratory standards (Caffeine δ^{15}N = 1.0‰, δ^{13}C = -27.5‰; and USGS40 δ^{15}N = -4.5‰, δ^{13}C = -26.2‰), the measurement errors (1σ) are less than ±0.2% for δ^{13}C and δ^{15}N.
RESULTS

The yellow stalactite (sample RAK 3; Fig. 7A) in thin section has prismatic microstructure composed of crystals of different shape (Fig. 7B). Most crystals are present in the form of druses with radiating bladed crystals as well as single thin tabular structures. Their color is pale yellow or colorless with pleochroism varying from absent to pale yellow hues. The length of crystals usually varies between 500–1,000 µm; their width is approximately 50–100 µm. The druses are overgrown by a next generation of druses and the center of these features is more or less empty, containing just small single crystals. The matrix of sample RAK 3 (preserved occasionally within the clusters of crystals) is composed of decomposed fine-grained organic matter.

The XRD analyses of the bulk RAK 3 sample show the presence of urea, CO(NH$_2$)$_2$ and allantoin, C$_4$H$_6$N$_4$O$_3$ (Fig. 8A). The reference intensity ratio method was applied to determine quantitative distribution of minerals and organic phases from XRD data. The presence of minor phases, which would otherwise remain undetermined, is supported by EDX spectroscopic data. The major compound of the yellowish stalactites is urea (94%), whereas minor compounds include tripotassium sodium sulfate, K$_3$Na(SO$_4$)$_2$, potassium sodium sulfate, and allantoin (about 2.5%) and, as trace phases, alunite, KAl$_3$(SO$_4$)$_2$(OH)$_6$ and potassium hydrogen phosphate hydrate, H$_3$KP$_2$O$_7$·H$_2$O, are present in the sample (Fig. 8B, C).

The SEM images illustrate the mineral assemblage organization; in back-scattered electron (BSE) images, the zones composed of lighter elements look darker, whereas zones with heavier elements are brighter (lighter) in color (Fig. 8D). There are large (100 µm in length) dark platy tetragonal crystals of urea corresponding to the bulk of the sample. Smaller (10 µm in length) lighter colored crystals correspond to K-Na phosphates and sulfates (Fig. 8D). A bright prismatic hexagonal crystal (25 µm in length) is aphthitalite, K$_3$Na(SO$_4$)$_2$. After manual separation, dark and light crystals were again analyzed by EDX technique. Dark-colored (in BSE) crystals show only C, N and O lines corresponding to urea, and possibly allantoin (Fig. 8E). Light-colored crystals are devoid of C, corresponding to potassium-ammonium phosphates and potassium-sodium sulfates (Fig. 8F). The guano (RAK 2) and the yellow stalactites (RAK 3) were analyzed for δ13C and δ15N. Results are reported in Table 1 and discussed below.

DISCUSSION

Precipitation of bat waste-derived minerals (allantoin and urea)

The organic degradations of cellular molecules (catabolism) ultimately produce water, carbon dioxide, and diverse nitrogenous waste, which are excreted in urine. Amino acids and pyrimidic bases mainly breakdown into ammonia (NH$_3$). Subsequently, this is converted by an addition of carbon dioxide into urea and uric acid, C$_5$H$_4$N$_4$O$_3$ (Fig. 9). A much smaller part of byproducts originate from the catabolism of purine bases in excess (adenine and guanine) is ultimately converted into uric acid by action of enzymes (Ngo & Assimos, 2007). For higher primates, including humans, uric acid is directly excreted in urine. For lower mammals, to avoid water losses, uric acid is first oxidized by uricase enzyme into allantoin before excretion in urines.

Both compounds (allantoin and urea) are present in the yellowish stalactites examined in this study. Allantoin is a common compound used in cosmetology and dermatology as a skin protector. When crystallized, allantoin occurs as white powder of colorless orthorhombic prismatic crystals (Mootz, 1965). It is highly soluble in water (5 g/L at 25°C) and consequently its solid crystalline state can only occur in extremely dry conditions. It has never been mentioned as occurring in caves before.
Bat urea minerals in Kahf Kharrat Najem Cave, UAE

Fig. 8. Analytical results of the yellow stalactite (sample RAK 3). A) XRD spectrum (Panalytical) of the bulk sample, showing the presence of mainly urea and allantoin; B) XRD spectrum (Brucker) (see text for details); C) EDX spectrum of bulk sample, showing the presence of P, S, Si, Al, Mg, Na, and Cl, for K-phosphates, K-Na sulfates, and alunite; D) BSE image of bulk sample. Dark-colored crystalline mass corresponds to urea and light-colored are K-Na phosphates and sulfates; the hexagonal light-colored mineral in center is aphthitalite; E) EDX spectrum of in BSE image dark-colored crystals. The C, N, and O lines of the spectrum correspond to urea only; F) EDX spectrum of in BSE light-colored crystals. Lines of K, N, and P correspond to K-NH₃ phosphates; Lines of K, Na, and S correspond to K-Na sulfates.

Table 1. Stable isotopes values of the guano and urea-allantoin stalactites.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Description</th>
<th>δ¹³C (‰)</th>
<th>δ¹⁵N (‰)</th>
<th>C/N (atoms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAK 2</td>
<td>Guano</td>
<td>-18.6</td>
<td>+7.5</td>
<td>2</td>
</tr>
<tr>
<td>RAK 3</td>
<td>Yellowish stalactites (urea-allantoin)</td>
<td>-16.0</td>
<td>+7.8</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Fig. 9. Chemical structures of the organic minerals urea and allantoin.
Urea in caves generally occurs as colorless, pale yellow or brown tetragonal crystals. It derives from bat guano and urine, and it is stable only under very dry conditions, i.e., in caves of arid regions (Hill & Forti, 1997). It is a relatively rare cave mineral, occurring as crusts, stalagmites and acicular crystals. Urea was first described in Toppin Hill Caves, Australia (Bridge, 1973, 1975). It is also reported from Wilgie Mia Cave, Australia (Bridge, 1975), in Arnhem, Temple of Doom, Nooitgedachtgrot, Leeurantegrot, Hermitage, and Gåub Aas Caves, Namibia (Irish et al., 1991; Marais et al., 1996; Martini & Marais, 1997), in Hibashi Cave, Saudi Arabia (Pint et al., 2005), in Israel in several Judean Desert caves, including Kanaaim, Hitchcock and Sela Caves (Buzzio & Forti, 1985; Lisker, 2007; Frumkin & Langford, 2012; Porat & Frumkin, 2012), and in Colossal Cave, USA (Rogers, 1981; Brod, 1989).

**Sulfate minerals in urea-derived minerals (aphthitalite and alunite)**

Aphthitalite has been found in the yellowish stalactites subject to study (RAK 3). This mineral generally occurs as white or colorless hexagonal crystals. It is highly soluble in water and has been found to be present in volcanic fumaroles, evaporite deposits, and guano deposits (Anthony et al., 2005). Aphthitalite is a relatively rare cave mineral mentioned in arid areas where it derives from bat guano and urine, and occurs as component of crystalline crust, efflorescences on guano, stalactites, and flowstones, generally associated with sulfates, phosphates, halides, or urea (Hill & Forti, 1997). Aphthitalite was reported as occurring in Al Hibashi Cave, Saudi Arabia (Forti et al., 2004; Pint et al. 2005), Murra-el-elevyn, Petrogale, and Toppin Hill Caves, Australia (Bridge, 1973, 1977; Gillieson & Bridge, 1973); and in the Temple of Doom, Arnhem Hermitage, and Gåub Aas Caves, Namibia (Marais et al., 1996; Martini & Marais, 1997). Also, it was reported from lava tubes in Island (Jacobsson et al., 1992), in Hawaii, USA (Hon et al., 2009), and in Etna, Italy, where it is related to the lixiviation of basalts by seepage water (Hill & Forti, 1997).

Alunite has been detected by XRD in the yellowish stalactites. Peak shifts in XRD patterns indicate the presence of minor substitution of sodium in, otherwise, nominally potassium mineral (Fig. 8B). According to the relative high content of sodium and potassium, the occurrence of alunite in Kahf Kharrat Nejem Cave could be possibly to be linked to that of natroalunite, NaAl₅(SO₄)₄(OH)₁₂, as an accessory mineral. The origin of both sulfate minerals in caves is generally associated to sulfur, either from sulfuric acid speleogenesis (SAS) or from sulfuric ores (Hill & Forti, 1997), or to sulfates derived from guano. In our case study, iron oxihydroxides are present as thick crusts in the immediate area surrounding of the cave; iron oxihydroxides and (n)atroalunite could represent the byproducts of the oxidation of iron sulfides deposits and their reaction with clays, respectively, as proposed for the origin of this minerals in other hypogene caves (Polyak & Güven, 1996). Otherwise, (n)atroalunite could be the result of sulfuric reactions derived from guano mineralization at the contact of clay deposits. Current data do not allow discriminating between the two pathways, thus both are possible in this environment.

**Phosphates associated with bat wastes-derived minerals**

The only undoubtedly identified phosphate mineral occurring in the sample RAK 3 is a member of biphosphammite (NH₄,K)H₂(PO₄) – archerite H₃K(PO₄) solid solution series (Frost et al., 2011). Its presence is also supported by detection of phosphorus by EDX spectroscopy (Fig. 8B). The identity of this mineral is also substantiated by comparing the calculated theoretical diffraction pattern for the (NH₄)₃-end member from the crystal structure determined by Khan & Baur (1973). However, based solely on powder diffraction and rather qualitative EDX spectral data, we cannot resolve which of the two minerals is actually present in the sample. Such ammonium-potassium phosphates have only been described in caves of arid areas such as Arizona, Australia (Bridge, 1974, 1977; Gillieson & Bridge, 1973; Snow et al., 2014), Bahamas (Onac et al., 2009), Saudi Arabia (Pint et al., 2005), Chile (De Waele et al., 2009), Botswana and Namibia (Martini, 1994), always associated with bat guano and urea deposits, or with very soluble sulfates such as aphthitalite (Hill & Forti, 1997 and references therein).

Finally, identification of monopotassium trihydrogen pyrophosphate monohydrate (KH₃P₂O₇·H₂O) in the yellowish stalactites is ambiguous due to its low content.

**Stable isotopes in guano and urea-derived minerals**

Several studies have shown δ¹³C and δ¹⁵N values of bat guano to be faithful tracers of dietary sources for bats (e.g., Mizutani et al., 1992; Bird et al., 2007; Wurster et al., 2007; Onac et al., 2015). Many bats species are mostly insectivorous and the isotopic composition of their fluids and feces roughly represent that of the insects in their diets. In turn, the δ¹⁵N and δ¹⁵N of insect tissues are intrinsically linked to the type of vegetation (C3, C4, or CAM) on which they feed. Regarding δ¹³C in vegetation, for C3-type plants, the fixation of carbon dioxide through Calvin metabolic cycle -representing over 98% of vegetal species- produces tissues with typical isotopic values around -26‰. In contrast, the C4-type plants group, mainly composed by grasses and steppe species, is characterized by fixation of carbon dioxide through the Hatch- Slack metabolic cycle with less discrimination for ¹³C, giving rise to biomass isotopically enriched with a typical value around -12.5‰ for ¹³C (O’Leary, 1981; Vogel et al., 1986). This means that food chains based on C4 plants, typical of steppes and other areas with lower water availability, generally show higher δ¹³C values compared with C3 plant-based ecological systems.

The δ¹³C value of the guano sample from Kahf Kharrat Najem is -18.6‰. This strongly suggests a contribution of C4-type plants to the food chain. The yellow stalactite sample (RAK 3) shows a slightly
more enriched δ^{13}C value (-16‰). CO₂ degassing and diagenetic processes could produce this enrichment, especially during the dehydration of bat wastes that leads to the precipitation of urea-derived minerals.

The δ^{15}N in the guano sample was +7.5‰ and roughly agrees with values found in some bat guano deposits (Mizutani et al., 1992; Wurster et al., 2007), although it is considerably lower than values in the order of +10 to +20‰ found in other studies (Bird, 2007; Wurster et al., 2008). In general terms, and regardless to the nitrogen source, δ^{15}N has been found to increase with volatilization of ammonia from waste and feces (Wolterink et al., 1979; Geih, 2000). Ammonia displaying lighter nitrogen isotopes passes easily to the gaseous phase. This produces δ^{15}N-enriched guano. Nevertheless, Mizutani et al. (1992) suggested that this fractionation due to ammonia degassing is considerably reduced under conditions of low RH and rapid water loss from guano. The extremely dry environmental conditions in Kahf Kharrat Najem suit this scenario of fast “mummification” of guano and preservation of the original δ^{15}N. A similar interpretation of elevated δ^{15}N can arise from urea desiccation and crystallization in the yellow stalactites, which show the same δ^{15}N (within analytical uncertainties) as the guano deposits.

The presence of modern bat colonies in the cave suggests that both the yellow stalactites and the guano deposits could be relatively recent. This recent age is also supported by their soft (although dry) consistence and poor mineralization. In addition, the relatively thinness of the guano layers may indicate limited period of residence of the bats colony in this cave. Thus, relatively rapid growth rate is proposed for the yellowish urea stalactites.

**Urea stalactites, an indicator of bat ethology?**

The distribution of the yellowish urea stalactites is not related to any fissure or relief in the ceiling (Fig. 6). Therefore, it suggests a close relationship between these speleothems and the permanence of bat roosting spots. A similar observation was made in Hitchcock Cave (Israel) where Lesser mouse-tailed bats are clearly located in a recurrent place marked by small urea stalactites (Fig. 10). Likewise, in Cabrespine Cave (Southern France), individual horseshoe bats roosting at the tip of stalactites were responsible of deep biogenic corrosion pots filled with guano and phosphates, which could only be related to a single individual (obs. PA). Biogenic corrosion pots have not been found in Kahf Kharrat Najem Cave, where the cave atmosphere is too dry to allow for their development; however, we propose that the investigated yellowish urea stalactites could possibly be also interpreted as individual roosting spots.

These peculiar stalactites constitute a strong indicator of occupation of a cave by certain bats species. They may be an interesting indicator for the assessment of the regional distribution of species during time and of the evolution of their biotopes (Davis, 2007). The confirmation of the relationship between urea stalactites, biogenic corrosion pots, and certain bat species which do not live in dense colonies, also could help in understanding the bats behavior (ethology). In particular, this could shed light on understanding the attachment of some species to some precise roosting spots, and possibly also the ethology of lesser mouse-tailed bat that differently segregate on cave walls.

**CONCLUSIONS**

The small yellow stalactites from Kahf Kharrat Najem Cave, UAE are composed of urea and allantoin as an accessory byproduct. This cave represents an additional site of urea, a mineral that is seldom present in caves, and our report contains the first-ever mention of allantoin in cave as a urea byproduct. Kahf Kharrat Najem also contains rare sulfate minerals (aphthitalite, alunite) and not well-defined phosphates that likely correspond to the archerite-biphosphammite series, making this cave an outstanding site for cave mineralogy in arid regions. The occurrence of rare bat-related minerals is due to extremely dry conditions in the cave, which favor preservation of guano deposits and allows for the crystallization of these very soluble minerals. The rapid mummification of the guano deposits have subdued the amount of ammonia and CO₂ degassing, so that the isotopic composition of guano in caves of this region could indicate the paleo-diet of bats and...
provide palaeoenvironmental information more than guano deposits in other regions. This opens a field for future paleoclimate studies from guano deposits in caves similar to those of the UAE.

ACKNOWLEDGEMENTS

We are grateful to Mohammed Al-Hafytti who showed the cave, and to the Emirates Geographical Society, who provided the invitation to PA and DC to visit the cave and allow sampling in the field. To D. Borschneck who performed the XRD analysis in CEREGE, France. We are thankful to Prof. David Hodell for offering the facilities to perform the isotopic analyses, and to James Rolfe for technical support. The personnel from the GLI CAS Praha (Czech Republic) were very helpful, especially Mr. Pavel Lisý, who performed specimen macrophotography. We thank R. Ruggieri for photos and for additional sample transfer. Also, G. Pucej is warmly acknowledged for providing cave photos. Finally, the authors appreciate the corrections made by Professors Carol A. Hill and Bogdan P. Onac and a third anonymous reviewer.

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During the 2011 speleological expedition to the Puerto Princesa Underground River (Palawan, Philippines) a drapery characterized by several close-to-horizontal ribs has been noticed. Even without sampling and analyzing its internal growth layers, a detailed morphological study allowed to present a possible genetic model. The presented model helps to explain its evolution, which is mainly controlled by variation in water flow as a consequence of the Palawan climate. When validated by further analyses, the same genetic mechanism could define also the evolution of the very common but still unexplained complex flowstones, which exhibit several close-to-horizontal steps, widenings and narrowings along their growth axis.

INTRODUCTION

The morphological characteristics of speleothems are mainly controlled by the type of water flow feeding them (Hill & Forti, 1997). This characteristic allows for the creation of theoretical models that define the shape of some of the most common speleothems such as stalagmites (Franke, 1965; Curl, 1973; Dreybrodt, 1999; Kaufmann, 2003; Romanov et al., 2008), stalactites (Kaufmann, 2003; Short et al., 2005a, b), and rimstone dams (Wooding, 1991). These models are mainly based on the in-time evolution of supersaturation during the flow of the feeding water over the speleothem.

Detailed geochemical (stable isotope) studies and petrography on speleothems can reveal the processes that caused the feeding water to precipitate carbonate (mainly calcite and aragonite), and thus the evolution of waters in underground environments due to a variety of processes active in these environments (i.e., differential CO₂ and/or H₂O loss from a water film, differential aerosol deposition, deposition in subaqueous environments) (Caddeo et al., 2015). Recent studies focused on the effect of the steady flow hydrodynamics in developing “crenulations” (ripple-like structures characterized by a wavelength close to 1 cm) over stalactites, stalagmites and flowstones (Camporeale & Ridolfi, 2012; Vesipa et al., 2015). In this case the variation of the supersaturation was induced by changes in the thickness of the water film and/or the development of micro-bubbles of gas within the water, inducing enhanced diffusion of CO₂ to the cave atmosphere. All these models are based on the assumption of stationary homogeneous flow conditions. In reality, most speleothems display complex morphological patterns that develop in unsteady or heterogeneous flow conditions. Those conditions still require explanation from a genetic point of view.

This is the case of an astonishing “ribbed” drapery observed in the Puerto Princesa Underground River (PPUR) (Palawan, Philippines) during the 2011 La Venta expedition (Piccini & Iandelli, 2011; Coombes et al., 2015) (Fig. 1A). Until present, a drapery of this kind was never described in other caves in the world and this exploration offered the unique possibility to try and understand the special conditions that controlled the evolution of this speleothem. Because the PPUR is a protected area, and the speleothem is unique, sampling was not carried out. Nevertheless, modeling results from previous authors and basic scientific principles make it possible to propose a genetic mechanism for the formation of this ribbed drapery.
MORPHOLOGY OF THE PPUR DRAPIERY

The ribbed drapery, located approximately 2 km from the cave entrance (Fig. 1A), is developed along the God’s Highway, a strait 600 m long by 10-20 m wide by 15 m high SW-NE oriented passage through which flows the PPUR river. This drapery, over 10 m long, is characterized by the presence, along its lateral sides, of several close-to-horizontal symmetric ribs (Fig. 1B). The distance between the ribs ranges from a few decimeters to over 1 m, while their size is from a few to 20-30 cm wide. The increase in rib size corresponds to a larger deviation from the horizontality: in fact the largest ones are clearly bent upward with possible interference with the upper ribs, which may eventually be incorporated. Finally, at least in the area close to the contact with the cave wall (1 in Fig. 1B) it is evident that, at the beginning, the process was characterized by a higher number of smaller ribs, which progressively coalesced forming some larger ribs. The vertical section of the drapery is characterized by close-to-horizontal zones of sudden enlargement, followed by a downward, fast progressive reduction until its “normal” thickness is re-established. The enlargements are always symmetrical, being of the same size and at the same level on both sides of the drapery.

CAVE CLIMATE AND WATER REGIME

Palawan is located in the Intertropical Convergence Zone and therefore its average temperature is relatively high (~27°C) with daily, monthly and yearly excursion rarely exceeding ±5°C. The annual average relative humidity is high and almost constant staying around 80-85%.

The climate is “tropical wet and dry” (Aw in the Köppen climate classification): the wettest month is September while the driest month is February. The average rainfall is relatively high (close to 2,000 mm/yr), and 95% of it falls during the wet (monsoon) period (from May to November) and it is concentrated in few, short but heavy rainstorms.

Consequently the PPUR climate is extremely stable (Badino, 2010, 2013), controlled by the general island climate and by the ocean, which, during the dry season, floods every 12 hours a large part of the cave with more than 100,000 m³ of sea water (Forti, 2014). The flow regime within the PPUR dramatically changes from dry to wet period (the outflow discharge rises from less than 200 l/s to much more than 10 m³/s in only a few couples of hours): thus most of the hosted speleothems are active only during the floods but dry all the rest of the time.

Fig. 1. A) Location map of the PPUR; B) The ribbed drapery (photo by Natalino Russo, La Venta Esplorazioni Geografiche); 1) enlargement to highlight the mechanism of progressive coalescence of the small ribs starting with the closest ones giving rise to a few larger ribs.
The very high external relative humidity together with the extremely narrow temperature fluctuation and the presence within the cave of an enormous free water surface, greatly inhibit evaporation processes that are subdued in the presence of relatively high air currents (up to 1.5 m/s displacing over 100 m²/s, Badino, unpublished data). However, in some places of the cave system it is sometimes possible to see active condensation processes with the development of large clouds, the genesis of which is induced by cold air currents coming from the upper cave levels. This phenomenon has been observed when rainstorms that occur in the hot season cause a local lowering of cave temperatures. This occurs when cooler percolation water originating from the top of the mountain rapidly reaches the main cave passages. This phenomenon may also explain the fact that in the dry season of the cave the average temperature is almost 2°C lower than the external one.

GENESIS OF THE RIBBED DRAPERY

Normally the genesis, and most of the evolution, of a drapery is induced by water flowing exclusively along its external (lower-outer) edge, while the possible laminar flow on both sides of the speleothem can only enlarge its thickness without changing its shape and/or structure. The morphology of the PPUR drapery suggests that this conditions of enhanced deposition exclusively occur in the widened zones, where enlargement takes place. It is also clear that many of these initially favorable deposition zones enlarged themselves progressively merging with neighboring ribs giving rise to the evolution of the larger ribs (1 in Fig. 1B).

It is evident that the flow along the lower/outer edge of the drapery, which allows deposition of water, can only enlarge its thickness without changing its shape. These enlargements have to be caused by water flowing along the lateral sides of the drapery. In the PPUR cave system, the lateral sides of the draperies are totally dry most of the time, subjected to active flow only during rainstorms characterizing the wet season of Palawan. During these rare and relatively short (from few hours to a couple of days) events the water flow may also become very important and fast. We believe that ribs can grow only during such rainy periods, when wet conditions on the lateral sides of the drapery are easily achieved.

Evaporation processes are not considered important factors for inducing the sudden increase in supersaturation because, when the water flow is present, evaporation is very low or non-existent. There are several reasons for this: first because active dripping water occurs mostly during rainstorms, when, even in the hot seasons, the percolating waters are cooler than the cavern environment, which in turn is already saturated or even oversaturated with respect to H₂O (Badino, 2013). It would also be extremely complex to justify the perfectly symmetric development of the ribs on both drapery sides by evaporation, since this would mean that air currents would have to flow preferentially where the ribs are growing. Furthermore, the Palawan climate, characterized by stable temperatures and low diurnal and annual thermal excursion, along with the oceanic effect of a large entrance and underground river connection to the ocean, and the influence of tides on cave atmosphere, do not allow symmetrically distributed air flow during rainstorms.

Discarding evaporation as a possible cause, the water flow over the drapery sides is the only possible factor able to induce the sudden stationary supersaturation at the position where the ribs develop. To understand the development of the ribs, it is fundamental to define how the first symmetric discontinuities, which later induced the evolution of the ribs, were formed.

The fact that all the “embryonic” ribs are close-to-horizontal and always at the same level on both sides strongly support the hypothesis that a single simultaneous process is responsible for their genesis. The increase in flow rate and velocity (with possible transition from laminar steady flow to subcritical or critical flow) (Chanson, 2009, 2012) affects the shape and the thickness of the water layer with consequent local narrowings or enlargements. This may cause the splitting-up of the water film due to insufficient surface tension (Eggers, 1997). Such events are greatly enhanced by the irregularities of the rock over which the drapery starts to develop.

All these phenomena, locally lowering the internal pressure of the water film, not only cause an enhanced CO₂ diffusion into the cave atmosphere, but also induce the development of micro-bubbles of CO₂ within the solution (Brennen, 1995). The presence of these bubbles further increases the supersaturation, such that they will not be so easily reabsorbed when the original condition of laminar flow is restored.

In this manner any further even minimal decreases of the fluid’s internal pressure enhances bubble development, thus inducing a relatively high increase in supersaturation downstream of where bubbles are formed. Finally, these instabilities in the water regime are responsible for a local decrease of the flow rate. These processes increase the deposition of CaCO₃ due to enhanced supersaturation and an increase in time of water-drapery surface contact over a short distance. This also causes a decrease of the steepness of the drapery edge and/or an increase in roughness. Being induced by local factors, these discontinuities develop in places that may result at regular intervals.

When at the end of a pulse the water flow ends, evaporation will become active for just a short time lapse: as a result of capillary uplift, this process will be efficient mainly on top of the small protrusions. In this manner the protrusions, generated by the critical flow during the rainstorms, undergo a slight lateral enlargement exactly at the same level on both sides of the drapery. The subsequent flows and dry periods are obviously forced to induce the same effects exactly on the same protrusions. The final result will be the development of embryonic close-to-horizontal ribs (Fig. 2A1). In conclusion, the variation of the water flow over the drapery edge is the only possible parameter responsible for the development of the embryonic ribs.
It is evident that the water flow along the lower/outer edge cannot induce the deposition of calcium carbonate along the lateral sides of the drapery, therefore other processes must control the progressive enlargement of the ribs. The Palawan climate influences the water regime over the lateral sides even more than on the drapery edge: consequently the flow regimen variations and induced supersaturation discussed earlier are also valid here. The only difference is that close-to-horizontal embryonic ribs already exist on the lateral sides and therefore flow perturbations are forced to occur exactly on these protrusions: so the consequent enhanced deposition will progressively enlarge them.

The rib enlargement will be a rapid process because just below the point of abrupt decrease of steepness, the convex shape of the rib will activate the enhanced CO₂ diffusion phenomenon (2 in Fig. 2A): practically this is the same well-known process allowing the selective deposition just on top of the rimstone dams (Wooding, 1991; Hill & Forti, 1997). The protrusions, i.e., the ribs, are the favored place for capillary uplift and evaporation (Badino et al., 2011), because they are slightly enlarged by selective deposition. This process occurs when the water flow over the lateral sides ends and the water layer completely dries up.

The growth of all the embryonic ribs continues until their dimensions interfere with the next rib causing a progressive coalescence of some of them into a larger one. This explains the existence of ribs of very different size but of the same age (1 in Fig. 1B).

The feature yet to be explained is the progressive upward deflection of the largest ribs towards the drapery edge (Fig. 1B). As progressive enlargement occurs along all of the ribs, it also occurs along the drapery edge. As a result the edge thickens and the shape progressively increases. But the portion of the rib over its lower edge will continue growing not only during the short period of water flow along the drapery sides, but also during the longer ones in which drip flow is active just over the lower (outer) edge. As a consequence the deposition on the lower edge will necessarily be slightly greater than that on both drapery sides, inducing a progressive upward growth of the rib towards the edge (Fig. 2B).

In particular during high water flows, the greater supersaturation occurs just above the point where turbulent flow begins and will rapidly decrease downstream due to the CaCO₃ deposition, which will completely stop far before the end of the convex shape of the rib (1 in Fig. 2B). At the end of the water pulse, when for a short span of time the dominant processes are capillary movement and evaporation, the deposition will migrate toward the most prominent sites, specifically to the top of the small domes protruding from the drapery edge (2 in Fig. 2B), which will slowly expand.

Due to the presence of larger close-to-horizontal (or at least less sloping) surfaces, the efficiency of the just outlined process is proportional to the size of the ribs and therefore the upward deflection is directly controlled by the rib size.

In conclusion, the fundamental factor ruling the development of the ribbed drapery of the PPUR is the unique climate of Palawan Island, that is characterized by long dry periods interrupted by heavy rainstorms. In fact, it is the great variability of the feeding water which allows not only the genesis of the close-to-horizontal symmetric embryonic ribs induced by the perturbation of the flow along the external edge of the drapery, but also their progressive evolution due to the water flow along both lateral sides. The alternation of dry and wet periods also justifies the possible coalescence of two or more ribs to form a larger one and it is also responsible for the slight upward deflection, more evident in the larger ribs.

![Diagram of the drapery showing the shape of the ribs.](image)
The processes ruling the genesis and the development of this unique drapery are of the “positive feedback” type. In fact, when in a given point the condition of preferential deposition is attained, this process progressively increases the further deposition exactly in the same point. This happens because the presence of a discontinuity enhances both changes in the flow regimen during water pulses and evaporation at the beginning of the dry periods.

CONCLUSIONS

The morphological analysis of the Puerto Princesa Underground River (PPUR) ribbed drapery suggest that its evolution is totally controlled by the flow regime over both its lateral sides during the short but strong rainstorms, while the consequent transition from subcritical to critical flow velocity and/or by the variation in the water layer thickness are the parameters ruling the development of the drapery ribs. The UNESCO World Heritage Site status that is afforded to PPUR does not allow destructive analyses of any of its hosted speleothems so it is not possible to experimentally confirm the proposed genetic mechanism.

Stepped flowstones and speleothems similar to the PPUR drapery ribs are present in other caves of the world so it is possible in the future to be able to conduct the required analyses to validate our model.

Once confirmed, the genetic-evolutionary mechanisms ruling the formation of this unique drapery may be useful to better define those controlling the development of other speleothems, which were never analyzed from the genetic point of view until present, despite their widespread occurrence in caves all over the World: the “stepped” flowstones.

ACKNOWLEDGEMENTS

We thank P. Kambesis for carefully reviewing our manuscript and providing suggestions that helped improve the paper.

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Bioaccumulation of metallic trace elements and antioxidant enzyme activities in *Apfelbeckia insculpta* (L. Koch, 1867) (Diplopoda: Callipodida) from the cave Hadži-Proданова Pećina (Serbia)

Jelena Vranković1, Slavica Borković-Mitić1, Bojan Ilić2, Milanka Radulović3, Slaviša Milošević4, Slobodan Makarov2, and Bojan Mitić2

Abstract: The concentration of 10 metallic trace elements or MTE (Cu, Fe, Zn, Mn, As, Hg, Pb, Cd, Ni, and Cr) was measured in specimens of the troglophilic millipede *Apfelbeckia insculpta* (L. Koch, 1867) and sediment of the cave Hadži-Prodanova Pećina in western Serbia. Some MTE, like Fe and Mn, displayed much higher concentrations compared to other elements, both in the sediment and in the body of *A. insculpta*. On the other hand, estimation of the bioaccumulation factor (BAF) in both males and females of *A. insculpta* showed values greater than 1 for xenobiotic elements compared to those that are essential. In addition to chemical analyses, we examined the activities of antioxidant enzymes (SOD, CAT, GPX, and GR) and the phase II biotransformation enzyme GST, as well as the content of –SH groups, in the body of *A. insculpta*. Activities of two (GR and GST) out of the five tested enzymes showed significant differences between the sexes. These results represent the first comprehensive report of antioxidant enzymes in myriapods. The noted differences in the investigated MTE and enzyme activities between the sexes of *A. insculpta* most likely reflect different metabolic activities and responses to environmental conditions in males and females.

Keywords: *Apfelbeckia insculpta*, cave, MTE concentration, BAF, antioxidant enzyme


INTRODUCTION

Trace elements, especially metallic trace elements (MTE), are considered to be one of the main pollutants in the environment since they can reside in soils for long periods and consequently have the potential to damage living organisms. Natural forms of trace elements are usually present in relatively low concentrations, but in recent years a number of anthropogenic sources, such as industrial plants, motor vehicles, thermal power stations, mines, quarries and various waste-producing commercial enterprises, have made notable contributions to a discernible increase of environmental metal concentrations (Marjanović et al., 2009).

Due to the difficulty of accessing and studying many of their inhabitants, cave ecosystems as part of the larger karst ecosystem are one of the least known ecosystems in the world. In cave systems, the range of variation of environmental parameters, especially temperature, is narrow and light is totally absent, circumstances which exert intense evolutionary pressure on existing species (Culver & Pipan, 2009). The cave environment depends on trophic contributions from the outside since photosynthesis is impossible in the absence of light (Paoletti et al., 2009). Food gets into a cave through natural meteorological events like floods or is brought in by trogloxenes and troglophiles who live next to the caves.

The cave Hadži-Prodanova Pećina (Figs. 1A-C) is located near a quarry on one side and next to the Ivanjica-Guča regional road near the urban settlement of Ivanjica on the other. This road passes...
about 10 feet in front of the cave entrance, making it possible for engine exhaust gases to reach inside the cave. Like every mining operation, quarrying is a destructive activity with detrimental effects on natural ecosystems. The processes involved release particulate matter and dust of different metallic constituents from the machinery and blasting and crushing of rocks (Ayodele et al., 2014), which can freely pass through the entrance of the cave Hadži-Prodanova Pećina and penetrate its interior, where it is deposited in the damp walls.

Certain metals [mainly those considered as “heavy metals”, like mercury (Hg), lead (Pb), cadmium (Cd) and arsenic (As)] are among the most abundant, toxic and persistent inorganic environmental pollutants. Metals essential for animal life include sodium, potassium, calcium, magnesium, iron (Fe), copper (Cu), cobalt, zinc (Zn), nickel (Ni) and molybdenum, which are also poisonous to animals if excessive amounts are ingested (Bulog et al., 2002). Metallic trace elements present in excessive concentrations have toxic effects on organisms and can, directly or indirectly, produce reactive oxygen species (ROS), causing adverse alterations in proteins, nucleic acids and lipids, finally leading to cell death by necrosis or apoptosis (Pulido & Parrish, 2003). The excessive formation of ROS and their toxicity to the main biological components is counteracted by the activities of many antioxidant cell defence mechanisms (Stohs et al., 2000). As a part of these defence mechanisms, antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and the phase II biotransformation enzyme glutathione-S-transferase (GST).

Millipedes are slow-moving detritus feeders that generally spend their time burrowing through soil and litter, consuming plant remains and converting vegetable matter into humus (Brusca et al., 2016). Many species colonized caves and other subterranean habitats in which diurnal and seasonal variations in temperature and humidity are much reduced. Millipedes are often numerically dominant in these environments, which are inhabited by both surface-dwelling species that enter caves sporadically and seasonally (troglophiles) and obligate cave dwellers (troglobionts) (Culver & Pipan, 2009). The callipodidan genus Apfelbeckia Verhoef, 1896 is an endemic taxon which usually inhabits caves and other underground habitats of the Dinarides and Greece. Among members of the genus, Apfelbeckia insculpta (L. Koch, 1867) has the largest distributional range and can be found in Serbia, Montenegro, Bosnia and Herzegovina, Croatia and Albania (Stoev & Enghoff, 2008; Ilić et al., 2016). In contrast to most millipedes, A. insculpta feeds on earthworms, flies, spiders, and centipedes (Hoffman & Payne, 1969; Stoev & Enghoff, 2008) or on decomposing animal materials (B. Ilić, pers. observ.).

Previous studies have reported on human disturbance as detrimental to the biota of caves (Bulog et al., 2002; Mikac et al., 2011), but the very limited number of them that deal with the effects of pollutants on cave organisms at the biochemical and molecular levels prompted us to undertake the present work. We here investigate the concentrations of MTE (Cu, Fe, Zn, Mn, As, Hg, Pb, Cd, Ni, and Cr) in the cave’s sediment and establish their bioavailability to A. insculpta. In addition, the present study was conducted to integrate chemical analyses of selected MTE in this endemic macroinvertebrate species with its antioxidant enzyme and GST responses.

MATERIALS AND METHODS

Site description and sampling

The cave Hadži-Prodanova Pećina is situated in the valley of the river Raščićka Reka in the village of Raščići 7 km from the city of Ivanićica (western Serbia). The entrance to the cave lies at 630 m a.s.l., about 25 m above the valley floor. Although it is not set up for mass tourism, the cave Hadži-Prodanova Pećina is a local tourist attraction and visitors emerge as an additional factor violating the constant conditions. The cave (43°35′47″N lat., 20°13′19″E long.) has a complex morphology with a number of lateral channels at different levels, a situation which corresponds to the type of a complex branched cave (Figs. 1A-C). Total length of the galleries is 420 m. The gallery floor is covered with a film of water which slowly flows to the lower chambers, where it sinks or forms lakes. Generally, the explored galleries of the cave Hadži-Prodanova Pećina are dry, without a cave stream (Đurović, 1998).

Several cave-dwelling arthropod species inhabit this locality, among them our model organism (Fig. 1D). The population of A. insculpta in the cave Hadži-Prodanova Pećina can tolerate periodic sampling for biomonitoring because of its great abundance. It is also suitable for sampling due to easiness of collection.

In November of 2012, millipede samples were collected from the cave walls and sediment in the main passage of the cave (Fig. 1A). Samples of sediment were collected using a plastic gardening trowel and put in labeled plastic bags. We used latex gloves to reduce the possibility of contaminating the samples during collection. Between samples, the trowel was sanitized using doubly deionized water and sterile wipes. Animals were collected by hand using latex gloves and put in labeled plastic bags alive. During their collection, they were housed for a short time in boxes with sediment in order to minimize stress. Afterwards the animals were frozen in liquid nitrogen and then transported to the laboratory. Sex of the animals was determined, after which they were divided into same-size groups of males (n = 20) and females (n = 20) and subsequently stored at -80°C until further analysis.

The site of sampling of sediment and animals was located in an area about 20 to 50 m away from the main entrance to the cave. Since the cave entrance is narrow, the sampling site was in complete darkness. Air temperature in the cave was 9°C, while outside it was 11°C.

Sediment analysis

Composition of the sediment was determined by element analysis. Values of moisture and element composition of the sediment are given in Table 1.
Metal bioaccumulation in Apfelbeckia insculpta

Table 1. Element composition (%) of the cave Hadžić-Prodanova Pećina and N content (%) determined by the Kjeldahl method (Bremner, 1960) in sediment and organic matter content in sediment of the cave Hadžić-Prodanova Pećina with its element analysis. The percentage of moisture was obtained by subtracting the weight before and after the drying process (100°C for 48 h) from a fraction of the collected sediment samples. All results are expressed in % of the dry mass.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Moisture</th>
<th>C (%)</th>
<th>H (%)</th>
<th>N (%)</th>
<th>S (%)</th>
<th>Organic matter (%) in the sediment</th>
<th>Element analysis of organic matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment</td>
<td>23.78</td>
<td>46.65</td>
<td>5.22</td>
<td>3.40</td>
<td>1.23</td>
<td>2.30</td>
<td>%C</td>
</tr>
</tbody>
</table>

The results of element analysis of the sediment made it possible to estimate the content of organic matter and its element composition. The content of organic matter in the investigated sediment samples was determined by element microanalysis (using a Varian El III CHNS/O element analyzer from Hanau Instruments GmbH) and is also shown in Table 1.

Analysis of metals in sediment and animals

Metal concentrations in the body of adult animals (20 males and 20 females) were determined after purging of their gut to ensure that the actual concentrations in their tissues are being measured. The whole animal (with approximate body length of 89.5 ± 0.3 mm and weight of ~1 g) was dissected (after removal of the gut) and equal 0.5-g batches were then separated from the macerated mass for measuring of enzyme activity and the concentration of metals.

Metal content was determined in sediment and specimens of A. insculpta according to Beck & Sneddon (2000) by means of flame atomic absorption spectroscopy (using a Varian Spectra A55 spectrophotometer). To check the accuracy and precision of the instruments, the TORT-2 certified reference material (lobster hepatopancreas reference material for trace metals from NRC Canada) was employed. The samples were oven-dried at 105°C to a constant weight. Digestion was performed according to a program that included warm-up to 200°C for 10 min and heating for 15 min. After a cooling-down period, the samples were quantitatively transferred to a volumetric flask (25 mL) and diluted with distilled water. The metal content of samples was determined after digestion using a known analytical procedure (70 cm$^3$ 2% HClO$_4$, 10 cm$^3$ conc. HNO$_3$, 5 cm$^3$ 20% H$_2$O$_2$) (Batley, 1989). Values of ash and metal content of all samples are summarized in Table 2.

The bioaccumulation factor (BAF) was estimated for each MTE-tissue combination in order to detect the effects of elements. This was calculated as the...
Table 2. Ash content (%) and metal trace element (MTE) content (mg kg\(^{-1}\)) in the sediment and in the body (males and females) of *Apfelbeckia insculpta* collected from the cave Hadži-Prodanova Pećina. MAC(s) - maximum allowed concentrations of potentially toxic elements in the soil as prescribed by Serbian legal regulations, Official Gazette of FRY, No.23/94. MTE concentrations in 20 males and 20 females are presented as mean ± SE. Significant differences in MTE concentrations between males and females were calculated by one-way ANOVA. The level of statistical significance of differences between the sexes was defined as \( p < 0.05 \). Statistical significance is marked with an asterisk (*).

<table>
<thead>
<tr>
<th>Ash content (%)</th>
<th>MTE concentrations (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cu</td>
</tr>
<tr>
<td>Sediment</td>
<td>11.20</td>
</tr>
<tr>
<td>Males</td>
<td>7.43</td>
</tr>
<tr>
<td>Females</td>
<td>6.36</td>
</tr>
<tr>
<td>MAC(s)</td>
<td>/</td>
</tr>
</tbody>
</table>

quotient of the MTE concentration in each individual divided by MTE content in the sediment as described by Dabrowska et al. (1996).

### Tissue processing

The rest of the macerated tissues (of 20 males and 20 females) was minced and homogenized in 5 volumes of 25 mmol/L sucrose containing 10 mmol/l Tris-HCl, pH 7.5, at 4°C (Lionetto et al., 2003) using an IKA-Werk Ultra-Turrax homogenizer (from Janke and Kunkel, Staufen, Germany) (Rossi et al., 1983). The homogenates were sonicated for 30 s at 10 kHz on ice to release enzymes (Takada et al., 1982), and then centrifuged in a Beckman ultracentrifuge at 85,000 x g for 90 min at 4°C. The resulting supernatants were used for biochemical analyses.

### Biochemical analyses

The activities of antioxidant enzymes were measured simultaneously in triplicate for each sample using a Shimadzu UV-160 spectrophotometer with a temperature-controlled cuvette holder.

#### Superoxide dismutase

The activity of SOD was assayed by the epinephrine method (Misra & Fridovich, 1972). One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the autoxidation of adrenaline at 26°C and was expressed as specific activity (U/mg protein).

#### Catalase

Catalase activity was evaluated from the rate of hydrogen peroxide (H\(_2\)O\(_2\)) decomposition (Clairole, 1984) and expressed as μmol H\(_2\)O\(_2\)/min/mg protein.

#### Glutathione peroxidase

The activity of GPX was determined by following the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) as a substrate with t-butyl hydroperoxide (Tamura et al., 1982) and expressed in nmol NADPH/min/mg protein.

#### Glutathione reductase

The activity of GR was measured using the method of Glatzle et al. (1974), which is based on the ability of GR to catalyze the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH as a substrate in a phosphate buffer (pH 7.4). Activity of this enzyme was expressed as nmol NADPH/min/mg protein.

Glutathione S-transferase

The activity of GST towards 1-chloro-2,4-dinitrobenzene (CDNB) was determined by the method of Habig et al. (1974) and expressed as nmol GSH/min/mg protein. The given method is based on the reaction of CDNB with the SH group of GSH, which is catalyzed by GST contained in the samples. 

- **SH groups**

  The concentration of sulphhydryl (-SH) groups was determined using 5,5’-dithio-bis-(2-nitrobenzoic acid) (DTNB) according to the Ellman (1959) method and expressed in nmol/g wet mass.

#### Protein quantification for enzymes

Protein concentration in the supernatants was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

### Statistical analyses

Gender variation in MTE bioaccumulation and biochemical parameters was tested by using one-way ANOVA, followed by Tukey’s post-hoc analysis. Correlation analyses were performed between biochemical data using the Pearson correlation coefficient. Differences were considered significant at \( p < 0.05 \). All analyses were performed using SAS 9.1.3 software (SAS Institute, Cary, NC, USA).

### RESULTS

The quantity of mineral components of the studied sediment was expressed in the form of ash content (Table 2). Table 2 also contains data on MTE concentration in cave sediment and in *A. insculpta* specimens. It can be seen that the tendency of MTE presence in sediment of the cave Hadži-Prodanova Pećina was as follows: Fe > Mn > Cr > Cu > Ni > Zn > As > Pb > Cd > Hg. The level of MTE in the cave sediment was relatively high, but lower than the maximum set forth in Serbian legal regulations (Table 2). Patterns of bioaccumulation of the 10 MTE in *A. insculpta* differed depending on the gender. The concentrations of MTE were in the following descending order: Fe > Mn > Cr > Ni > Zn > Pb > Cu = As (for males) and Cu > Pb > As (for females) > Cd > Hg (Table 2). In all specimens,
In female specimens, the concentration of Pb was 1.88 times higher than in the sediment, while male individuals had a Pb concentration which was double the one in the sediment. Body concentrations of other metals were either lower than (Cu, Zn, Mn, and As) or the same as (Fe, Hg, and Cd) compared to those in the cave sediment. The concentrations of Ni and Cr in the body of the animals were slightly higher than their concentrations in the sediment (1.07 times higher in males and 1.38 times higher in females for Ni; 1.13 times higher in males and 1.05 times higher in females for Cr). In addition, measured concentrations of Cu, Fe, and Ni were significantly higher in female individuals compared to males, whereas the concentration of Mn was significantly higher in males than in females (Table 2).

Expressed as the concentration of metals in the body of *A. insculpta* divided by their concentration in the sediment, the bioaccumulation factor (BAF) can serve as an indicator of metal accumulation by the animals. In the present study, the BAF values of measured metals were estimated and compared (Table 3) in both genders of this callipodidan species. The tendency of BAF values determined in both genders of *A. insculpta* was as follows: Pb > Ni > Hg, Cr > Fe > Cd > Mn > Zn > As > Cu.

If the BAF is lower than one (< 1), it indicates that accumulation in the organism is lower than that of the medium from which the xenobiotic was taken, and the organism can be considered an excluder. It can be seen from Table 3 that four of the 10 elements have the highest BAF values: Pb > Ni > Hg = Cr.

Table 4 shows inter-individual correlations between enzyme activities and –SH group concentrations measured in females (n = 20) and males (n = 20) of *Apfelbeckia insculpta* from the cave Hadži-Prodanova Pećina. Activities of GR and GST (Figs. 2A and B) were significantly lower (p < 0.05) in males of *A. insculpta* compared to female specimens. All other enzymes also showed lower activities in male specimens, but with a lack of statistical significance (Fig. 2). In addition, the concentration of -SH groups was lower in males compared to females without any statistically significant difference (Fig. 2C).

**DISCUSSION**

Mining and industrial processes lead to increased release of MTE into the surrounding environment. Accumulation of MTE is a great threat to the entire biota directly or through the food chain. Excessive amounts of various chemicals interfere with complex processes occurring in soils, modifying soil properties and assimilation of some elements by terrestrial organisms (Kania & Lechowski, 2014).

The cave Hadži-Prodanova Pećina is located close to a granite-quarrying and stone-crushing facility, making it constantly exposed to MTE pollution. Also, this cave is home to a large population of insectivorous bats and hence has bat guano accumulations.

The most important parameters affecting MTE bioavailability are soil/sediment pH and organic content (Yu et al., 2001; Takač et al., 2009). In caves, guano deposits influence sediment pH and organic content (Wurster et al., 2015) via the presence of phosphate and sulphate minerals characteristic of the decomposition of bat guano with the passage of time (Onac & Forti, 2011). In addition, strongly acidic conditions are usual in guano deposits owing to the degradation of organic matter, as it is known that waste products of bacterial degradation are mostly acids (Shahack-Gross et al., 2004).

### Table 3. Values of the bioaccumulation factor (BAF) for metals in males (n = 20) and females (n = 20) of *Apfelbeckia insculpta* collected from the cave Hadži-Prodanova Pećina (BAF values greater than 1 are presented in bold).

<table>
<thead>
<tr>
<th>Gender</th>
<th>BAF</th>
<th>Cu</th>
<th>Fe</th>
<th>Zn</th>
<th>Mn</th>
<th>As</th>
<th>Hg</th>
<th>Pb</th>
<th>Cd</th>
<th>Ni</th>
<th>Cr</th>
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<tbody>
<tr>
<td>Male</td>
<td></td>
<td>0.19</td>
<td>0.95</td>
<td>0.80</td>
<td>0.84</td>
<td>0.59</td>
<td>1.06</td>
<td>2.00</td>
<td>0.89</td>
<td>1.07</td>
<td>1.13</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>0.26</td>
<td>0.98</td>
<td>0.67</td>
<td>0.73</td>
<td>0.38</td>
<td>1.13</td>
<td>1.88</td>
<td>0.94</td>
<td>1.38</td>
<td>1.05</td>
</tr>
</tbody>
</table>

### Table 4. Overall Pearson correlations among enzyme activities and –SH group concentrations measured in females (n = 20) and males (n = 20) of *Apfelbeckia insculpta* from the cave Hadži-Prodanova Pećina (statistically significant values are presented in bold).

<table>
<thead>
<tr>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>CAT</td>
</tr>
<tr>
<td>SOD</td>
<td>-</td>
</tr>
<tr>
<td>CAT</td>
<td>0.08</td>
</tr>
<tr>
<td>GPX</td>
<td>0.03</td>
</tr>
<tr>
<td>GR</td>
<td>0.63</td>
</tr>
<tr>
<td>GST</td>
<td>0.64</td>
</tr>
<tr>
<td>-SH groups</td>
<td>-0.89</td>
</tr>
</tbody>
</table>
Metal solubility tends to increase at lower pH, and greater quantities of dissolved metals become potentially available for incorporation in biological processes as pH decreases (Salomons, 1995). In that context, our results showing BAF values for Pb, Ni, Hg and Cr, greater then 1 suggest that these metals are more bioavailable than others. Apart from these elements, BAF values for Fe, Zn, Mn, and Cd were nearly one, showing that accumulation in the organism is similar to that in the medium from which the MTE was taken. Some authors (Miko et al., 2002; Bird et al., 2007) have already suggested that cave guano is particularly enriched in transition metals (like Cr, Ni, Hg, Fe, Cd, Mn, and Zn) compared to local soils.

The presence of high content of organic matter (89.25%) in sediment of the cave Hadži-Prodanova Pećina could indicate increased adsorabilities of heavy metals by the sediment and thus their lower bioavailability, as was demonstrated by Lin & Chen (1998), suggesting that organic substances are important scavengers of metals in river sediments.

Shahack-Gross et al. (2004) reported that sediments with higher organic content (as opposed to fresh guano) have lower pH values, reflecting the presence of still decomposing organic matter that is releasing acid. Keeping in mind the high organic content (Table 1) in sediment of the cave Hadži-Prodanova Pećina and the fact that lower pH increases bioavailability and higher organic content lowers bioavailability, we can assume the presence of still decomposing organic matter that is releasing acid. Apfelbeckia insculpta, a resident millipede species of the investigated cave, was chosen as the object of research due to its great abundance and easy availability. As the first analysis of MTE in sediment of the cave Hadži-Prodanova Pećina and the body of this European callipodidan species, our study represents a simple approach to assessment of site-specific metal bioavailability.

The elements Cu, Fe, Zn, Mn, and Ni are considered essential for living organisms. An essential metal for invertebrates which in several species can be regulated to a certain degree, Cu exhibits a concentration in animal bodies that is rather constant over a range of soil concentrations (Heikens et al., 2001). Copper concentrations measured in the present study were approximately four (in females) to five (in males) times lower than in the sediment (Table 2). The highest concentrations of all of the measured MTE were obtained for Fe, but these concentrations varied in a narrow range between both genders of A. insculpta and the sediment.

Synergistic action of Fe and Cu when they are present in excessive concentrations may result in generation of ROS by Fe/Cu ions via the Fenton or Haber-Weiss reactions. In addition, the Cu ion displays a high affinity for protein thiol and amino groups (Letelier et al., 2005). However, our results showed that regardless of the high concentration of Fe in the body A. insculpta, the BAF value for this metal is just below 1, while the BAF value for Cu is very low, around 0.2 (Table 3). Concentrations of other essential elements Zn, Mn, and Ni also varied in a narrow range between males, females and the sediment, with BAF values in the range of 0.8-0.95.

With respect to xenobiotic elements, As is a common constituent of most soils, whereas Hg and Cd are generally related to human activities. In spite of their low content in the sediment, Hg and Cd are toxic at much lower concentrations than the other elements analysed (US EPA 1999).

An additional mode of MTE entry into caves is via bat guano. Clark et al. (1986) reported that bats were exposed to Cd by ingesting insects from a polluted habitat. Cadmium-enriched guano was then voided by the bats and deposited in three caves of Florida. But to our knowledge, only a few investigations of trace metals in bat guano exist, making comparison of our results with other studies difficult. Arsenic, Pb and Cr are not essential elements and are also considered toxic. In the body of A. insculpta, the BAF value for Pb is above 1 in females and reaches a value of 2 in males (Table 3), suggesting a higher rate of entry into the body.
accumulation than elimination of this metal in males as compared to females.

Comparing our results with those obtained in other areas affected by metal pollution, we note that concentrations of MTE (except Mn) in the cave sediment in our study are several times lower than the mean concentration in the Ebro River basin, which is heavily impacted by industrial, urban and agricultural activities (Cid et al., 2010). On the other hand, the mean values of MTE content in sediment from the cave Hadžić-Pradanova Pčina obtained in the present work are very similar to those measured in soils near a granite quarry at Ikole-Ekiti in Nigeria (Ayodele et al., 2014), where assessed levels of MTE pollution indicated that some level of pollution exists, although it is low.

Soil invertebrates belonging to the same trophic level exposed to the same environmental concentrations of MTE often display different internal concentrations of elements and strategies for decontamination (van Straalen & van Wensem, 1986; Grodzinska et al., 1987; Laskowski & Maryanski, 1993). A number of them can retain metals in inactivated forms by initiating intracellular compartmentalization, which leads to bioaccumulation and some discharge of assimilated MTE into the environment. Animals which apply this strategy have the ability to concentrate elevated amounts of MTE and are considered as “macroconcentrators” (Dallinger, 1993). Employing a second strategy, “deconcentrators” are animals which possess low concentrations of MTE. On a trophic level including predators, “macroconcentrators” are represented by spiders, “deconcentrators” are represented by carabid beetles, while millipedes (the group to which A. insculpta belongs) are regarded as being intermediate between these two opposite strategies. It has been previously documented that diplodops themselves possess effective mechanisms to bind and detoxify MTE in tissues (Köhler et al., 1995). The first study carried out with millipedes as possible bioindicators was conducted by Hopkin et al. (1985) and involved assimilation of metals by the species Glomeris marginata (Villers, 1789). In this study with G. marginata, uptake of Cu, Zn and Cd by animals collected in contaminated soils was shown to be higher than their uptake by animals collected in uncontaminated environments. The same authors postulated that terrestrial arthropods which feed on decomposing materials (such as some representatives of Isopoda, Collembola and Diplopoda) are among the most appropriate organisms on which to evaluate the effects of accumulation of pollutants existing in the soil, due to their direct contact with contaminants present in it.

Invertebrates, when in direct contact with MTE, may absorb them through their exoskeletons or other body coverings (Heikens et al., 2001; Hobbelen et al., 2006). Conducted by Heikens et al. (2001), studies of Cd, Cu, Pb, and Zn accumulation in some invertebrate taxa from 1993 to 1998 showed that the content of all metals was highest in Isopoda, intermediate in Lumbricidae and low in Coleoptera. Copper and Zn in Diplopoda had concentrations similar to those in Isopoda, the concentration of Cd was high in Formicidae and Lumbricidae, whereas that of Cu was high in Diplopoda and Collembola. To judge from the BAF values obtained in this study, it seems more likely that certain elements accumulate to a greater extent than others. Gall et al. (2015) have suggested that due to their habitat, diets and physiological responses, certain invertebrate species may have higher accumulation rates for some MTE. Although the millipede in question feeds on animal material, it is not known to what extent its accumulated MTE concentrations originate from its prey. As for sexual differences in bioaccumulation of metals, our study showed different bioaccumulation rates for various MTE in males compared to females (Table 3). Many invertebrates (including ones that are food for A. insculpta) evolved metal tolerance or detoxification mechanisms like excretion of metals with their faeces, thus limiting MTE uptake and consequently reducing biomagnification onto higher trophic levels (Przybyłowicz et al., 2003).

Elevated levels of MTE are associated with increased generation of reactive oxygen species (ROS) such as superoxide free radicals (O$_{2}^•−$), hydroxyl free radicals (OH$^•−$) and non-free radical species (molecular forms) like singlet oxygen (O$_{2}^•$) and hydrogen peroxide (H$_2$O$_2$), which can induce oxidative stress and stimulate the involvement of antioxidant enzymes as cellular defence mechanisms for metal detoxification (Kim et al., 2014).

Superoxide dismutase represents the first line of defence in the process of ROS neutralization. This enzyme catalyzes the dismutation of superoxide anion radicals (O$_{2}^•−$) to H$_2$O$_2$, which is reduced to water and molecular oxygen by CAT or through the GSH-dependent pathway. In females, concentrations of –SH groups displayed negative correlations with the activities of SOD and GR (Table 4). Positive correlations were obtained between GST and SOD, as well as between GST and GR, in females (Table 4). In some beetle species from metal-polluted sites, SOD activity ranged from approximately 2 to 7 U mg$^{-1}$ protein (in our study it was around 15 U mg$^{-1}$ protein), with values being the highest in specimens from a site with moderate to low metal pollution, while CAT showed the highest activity in beetles from sites with lower metal pollution. The CAT activity in the present work was around 20 μmol H$_2$O$_2$/min/mg protein, like that measured in beetles collected from the most polluted sites (Migula et al., 2004). These values of SOD and CAT activities (Fig. 2) indicate that some factors caused increased levels of ROS in A. insculpta.

It has been previously reported that Pb is responsible for ROS generation (Bokara et al., 2008). Hansen et al. (2007) demonstrated that Pb exposure has an inhibitory effect on SOD; thus, it can cause alterations in the mitochondria and facilitate the release of O$_{2}^•−$, which can inhibit CAT activity. We can assume that the presence of a higher Pb concentration lowers SOD and CAT activities in males compared to females (Fig. 2).

In A. insculpta, As concentration is higher in males than in females by 57%, but all measured enzyme
activities have higher values in females compared to males, indicating that the mechanism governing the arsenic-induced physiological reaction could involve decline of SOD, CAT, GPX, GR, and GST activities. Despite the fact that a statistically significant difference between genders in favour of females was obtained only for GR and GST activities (Figs. 2A and B), the validity of our findings is supported by the results of previous authors, who reported decrease of SOD (Zaman et al., 1995; Wang et al., 2006; Altikat et al., 2015), CAT (Wang et al., 2006; Altikat et al., 2015) and GPX, GR, and GST (Wang et al., 2006) activities after exposure to As.

Glutathione peroxidase in a reaction coupled with GR reduces free H_2O_2 to water and lipid hydroperoxides to lipid alcohols. Inasmuch as lower GPX activity was measured in male individuals, it would appear that metabolism of H_2O_2 and/or organic hydroperoxides is less intensive in this gender. Glutathione reductase reduces glutathione disulphide (GSSG) to the sulphhydryl form GSH, which is also an important cellular antioxidant. Glutathione-S-transferase can remove free radicals, and its levels can reflect the antioxidant capacity of the organism. Migula et al. (2004) showed that GST activity was the highest from sites with lower metal concentrations, but with values not exceeding 200 nmol GSH/min/mg protein, while GR and GPX exhibited a varying trend in different beetle species depending on the gradient of metal pollution. In our study, GST activity was four and six orders of magnitude (in males and females, respectively) higher than the activity mentioned above. A positive correlation between GSH-related enzymatic activities (GR, GPX, and GST activities) in both sexes of A. insculpta could indicate coordinated enzymatic regulation of these enzymes aimed at restoring the GSH pool, in turn permitting an efficient antioxidant response.

In view of the fact that diplopods possess effective mechanisms enabling them to bind and detoxify potentially hazardous MTE in tissues, the existence of such mechanisms in A. insculpta may explain the absence of significant differences between MTE concentration in animals and sediment.

**CONCLUSION**

Results of the first study of A. insculpta specimens from the cave Hadži-Prodanova Pećina revealed differences of MTE distribution and antioxidant enzyme activities between the sexes. The obtained BAF values showed that the rate of MTE accumulation is greater for xenobiotic elements compared to essential ones. In general, females of A. insculpta have higher enzyme activity and –SH group concentration compared to males, suggesting that the measured biochemical parameters are more inducible in female individuals. Females may be more susceptible to fluctuations in enzyme levels as a consequence of reproductive physiology or other processes operating independently from responses to the presence of MTE. In concluding this work, we emphasize the need to use local specimens to infer the effects of pollution on caves and define specific environmental quality thresholds, which will certainly contribute to the protection of this specific type of ecosystem.

**ACKNOWLEDGEMENTS**

This work was supported by the Serbian Ministry of Education, Science and Technological Development under Grants Nos. 173025, 173038 and 173041. We wish to thank representatives of the Tourist Organization of Ivanića for their kindness and cooperation. Special thanks to three anonymous reviewers for their valuable comments that improved the manuscript.

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I "Travelling into the Underground" is more than a chronology of explorers and institutions. The book investigates the history of caving and speleology, with an emphasis on the regions of Austria and the Austro-Hungarian Empire, by means of the historical discourse analysis. It spans mainly the period from the Age of Enlightenment to the 1920s, but ancient and medieval times are also considered. The author searches for the links, relationships, and influences that cave exploring and other sciences, arts, philosophy and even theology and psychology had on each other. Nevertheless, the book has a clear structure, from antiquity through the 18th century and the Romantic period up to the Age of Nationalism and Imperialism. For each period, it investigates different perspectives and themes such as the perception of rooms and space, social aspects, science, technical approaches, and documentations. What were the motivations that drove travellers, scientists, and trained laymen into caves in these different periods? How were their perceptions formed by their view of the world, paradigms, and patterns of thought and vision? And vice versa: how did this influence their world-view?

Johannes Mattes is an expert on both caving and history. He is a professional historian at the University of Vienna and a dedicated caver.

Reading the book is like wandering along galleries of ages, following side passages of arts, philosophy, speleo-cartography, and others, and finding different perspectives lurking in the abyss of the psyches of travellers and explorers. The reader is terrified by dragons in the medieval times, searches for bizarre objects and fossils in the Baroque period, shrugs with a delightful horror in the Romantic era and strives for victory, honor, and a record of surveyed meters in the Age of Imperialism. As a local, he or she preserves cheese in an Alpine ice cave, or even worships, hidden with Protestants. Serendipitously, there are lots of fascinating details, such as early depth records and how the water supplies of Trieste and Vienna have been fed by karst waters since the 19th century. Details about amazing ancient caving equipment and technics, the more than 30 caving clubs of the Austro-Hungarian Monarchy, and the development of different theories concerning speleogenesis are just a few of the themes dealt with by the book.

Additionally, there are more than 100 pages of bibliography and footnotes, as well as a register of persons and locations. Perhaps the figures and images could have been larger or in color, as they have a major role in understanding the issues presented throughout the text.

"Travelling into the Underground" is pleasant to read, full of interesting and sometimes peculiar details. It uniquely shows new links between familiar theories, and opens the readers’ eyes and mind to a new approach to and perception of caves and caving.

Monika Schöner