Editorial
Bogdan Onac

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Commons service at USF. I also want to thank the Editorial Board members, many of whom have served for many years. They deserve our gratitude for the time and effort they have invested in the journal, which allowed it to grow and become the top karst science journal. The quality of the Associate Editors and the Editorial Board is very important for the journal’s scientific “health,” and my selection of whom to invite to join in the future will reflect this belief. I believe that young, energetic, and promising scientists can do an excellent job, even if they do not yet have dozens of publications. As you will see on the inside cover, more people will be added in the coming years to the Editorial Board, while others who have served on the journal’s board for many years will be replaced.

In this editorial, I want to share with you some of my plans for the journal. An important part of IJS continuing to be a high-impact journal is making it more attractive to authors, and to do so, I plan to put a serious emphasis on keeping the review process quick. In particular, I strive to have the first editorial decision within 40 days of the submission date. It is my goal that the time from submission to online publication will be reduced further during my tenure, so that within six months of first submission, the authors will see the paper published. The success of this endeavor will largely depend on the cooperation, efficiency, and timeliness of our referees and, of course, on authors submitting their best research to the journal. Finding the appropriate referee for a particular paper is not always easy, and this can delay the review process significantly. Reviewing a manuscript takes time, and people in the academic community are often overwhelmed with teaching, research, and administrative duties, making the incentives to review smaller than the incentives to write our own papers. Nevertheless, the peer review system depends on referees; therefore, I expect authors who contribute to IJS to be willing to participate in the review process when invited. Such cooperation will improve the journal significantly.

Another strategy aimed at increasing the quality of the journal will be to ensure a fair balance among the submitted papers, reflecting the large diversity of karst and cave disciplines. In addition, I plan to support the geographic diversity of papers we publish, even if the IJS Editorial Group may have to be more actively involved in editing some papers (e.g., from developing countries in Africa, Asia, and South America) with authors to make them more readable and of higher impact to our community.

It is important for IJS to continue attracting high-quality articles that garner interest and excitement in a growing readership within the karst/cave community and beyond. However, this depends on you, our authors and readers. Therefore, please consider this editorial an open invitation to support the growth of IJS by submitting your research results so that we can continue to publish high-quality manuscripts.

Bogdan P. Onac

Editor, IJS
Layer-bounding surfaces in stalagmites as keys to better paleoclimatological histories and chronologies

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Abstract: Petrographic recognition of layer-bounding surfaces in stalagmites offers an important tool in constructing paleoclimatic records. Previous petrographic efforts have examined thickness of layers (a possible proxy for annual rainfall) and alternation of layers in couplets (a possible indicator of seasonality). Layer-bounding surfaces, in contrast, delimit series of layers and represent periods of non-deposition, either because of exceptionally wet or exceptionally dry conditions. Two types of layer-bounding surfaces can be recognized according to explicitly defined petrographic criteria. Type E layer-bounding surfaces are surfaces at which layers have been truncated or eroded at the crest of a stalagmite. Keys to their recognition include irregular termination of layers otherwise present on the stalagmite's flank, dissolutional cavities, and coatings of non-carbonate detrital materials. Type E surfaces are interpreted to represent wet periods during which drip water became so undersaturated as to dissolve pre-existing stalagmite layers, and thus they necessarily represent hiatuses in the stalagmite record. Type L layer-bounding surfaces are surfaces below which layers become thinner upward and/or layers have lesser lateral extent upward, so that the stalagmite's layer-specific width decreases. They are thus surfaces of lessened deposition and are interpreted to represent drier conditions in which drip rate slowed so much that little deposition occurred. A Type L surface may, but does not necessarily, represent a hiatus in deposition. However, radiometric age data show that Type L surfaces commonly represent significant hiatuses. These surfaces are significant to paleoclimate research both for their implications regarding climate change (exceptionally wet or dry conditions) and in construction of chronologies in which other data, such as stable isotope ratios, are placed. With regard to climate change, recognition of these surfaces provides paleoclimatological information that can complement or even substitute for geochemical proxies. With regard to chronologies, recognition of layer-bounding surfaces allows correct placement of hiatuses in chronologies and thus correct placement of geochemical data in time series. Attention to changing thickness of annual layers and thus to accumulation rate can also refine a chronology. A chronology constructed with attention to layer-bounding surfaces and to changing layer thickness is much more accurate than a chronology in which hiatuses are not recognized at such surfaces.

Keywords: stalagmites; paleoclimate; petrography; age models; chronologies

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INTRODUCTION

In the last twenty years, the application of stalagmites to the study of past climate has expanded explosively: a bibliographic survey indicates a fifteen-fold increase in such papers from 1990 to 2010. By far the data most commonly collected and applied from stalagmites have been stable isotope ratios, and these are largely δ18O data. Combination of these data, which are now well understood (McDermott, 2004; Lachniet, 2009; Fairchild & Baker, 2012), with U-Th dating via inductively-coupled plasma mass spectrometry (Shen et al., 2002) and other methods (Drysdale et al., 2012) has allowed development of detailed and seemingly continuous records of climate change.

In the application of stalagmites to paleoclimateology, the petrography of stalagmites (Railsback et al., 1994; Turgeon & Lundberg, 2001; Frisia et al., 2002) has not been used widely. One occasional application of petrography has been the counting of apparently annual layers and measurement of their thickness (e.g., Baker et al., 1993; Brook et al., 1999; Polyak &
Asmerom, 2001). However, exact counting of layers is subject to interpretation (e.g., Railsback et al., 1994), and the complexities of the controls on layer thickness are only now becoming known (Tan et al., 2006; Mariethoz et al., 2012).

With the previously undeveloped state of stalagmite petrography in mind, this paper proposes that a more useful approach to understanding the history of a stalagmite’s deposition is not the study of just the layers themselves but instead the study of layer-bounding surfaces (Fig. 1). A layer-bounding surface is a surface at which underlying layers are terminated. Such surfaces can be divided into two types that can be identified by objective criteria and that have very different origins. One kind, Type E, results from dissolutional erosion, whereas the other, Type L, results from lessened growth (Table 1). This focus on layer-bounding surfaces, rather than layers themselves, is akin to the wildly successful application of sequence stratigraphy to sedimentary strata, where rock layers themselves are less critical than layer-bounding erosional surfaces (unconformities recognized as sequence boundaries) and condensation zones representing slower deposition (and commonly recognized as maximum-flooding surfaces) (Vail et al., 1977).

The recognition of layer-bounding surfaces is significant to paleoclimate studies for two reasons. First, both dissolutional erosion (suggested by Type E surfaces) and lesser deposition (suggested by Type L surfaces) imply significant changes toward wetter and drier climate regimes, respectively. Recognition of these surfaces thus provides evidence of climate change. Secondly, Type E surfaces necessarily represent hiatuses, and Type L surfaces likely represent hiatuses. Recognition of these surfaces thus changes the construction of chronologies and suggests that the “seemingly continuous records of climate change” mentioned above may not be as continuous as commonly thought.

### Table 1. Characteristics of, and inferences from, layer-bounding surfaces in stalagmites.

<table>
<thead>
<tr>
<th></th>
<th>Type E (for “Erosional”)</th>
<th>Type L (for “Lessened”)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defining morphology</td>
<td>Layer or layers truncated, commonly near crest of stalagmite</td>
<td>Layer, or more commonly layers, thin toward flanks of stalagmite</td>
</tr>
<tr>
<td>Key/diagnostic features</td>
<td>Micro-topographic features: mesas, escarpments, caves</td>
<td>Upwards-thinning, laterally less extensive layers</td>
</tr>
<tr>
<td>Relationship to width of stalagmite</td>
<td>Typically none</td>
<td>Stalagmite may narrow</td>
</tr>
<tr>
<td>Characteristics of sequence of layers below surface</td>
<td>Commonly no distinctive sequence; layers below may thicken upwards or contain detrital grains</td>
<td>Commonly thinning upwards to surface, so that interval below surface is a zone of transition</td>
</tr>
<tr>
<td>Mineralogical trends</td>
<td>Either aragonite or calcite below surface; commonly calcite above</td>
<td>Aragonite commonly below surface, especially in warmer settings</td>
</tr>
<tr>
<td>Non-carbonate detrital material</td>
<td>Commonly abundant; can contain coarse (silt to sand) grains</td>
<td>Commonly scarce; may be a thin horizon of very fine material (dust)</td>
</tr>
<tr>
<td>Stable isotope relationships</td>
<td>Commonly none; discontinuity across surface possible</td>
<td>Typically increase δ13C, and commonly increase in δ18O, of spelean CaCO3 below surface</td>
</tr>
<tr>
<td>Inferred processes</td>
<td>Dissolutional erosion by abundant undersaturated dripwater</td>
<td>Lessened deposition by diminished dripwater</td>
</tr>
<tr>
<td>Paleoecological implications</td>
<td>Increased atmospheric precipitation</td>
<td>Decreased atmospheric precipitation</td>
</tr>
<tr>
<td>Chronological implications</td>
<td>Non-representation (hiatus) of time in which dissolved layers were deposited</td>
<td>Likely non-representation (hiatus) of time after last diminished layers</td>
</tr>
</tbody>
</table>

### MATERIALS

This paper presents images of fourteen stalagmites from five continents and from the island of Madagascar (Table 2). The stalagmites are from locations with present average annual precipitation ranging from ≤ 400 to ≥ 2400 mm, and they range in age from 170 ka (Stalagmite GG) to 14 years BP (Stalagmite DP1), where “BP” indicates time before 1950.

### Table 2. Stalagmites illustrated in this paper.

<table>
<thead>
<tr>
<th>Stalagmite</th>
<th>Cave</th>
<th>Location</th>
<th>Annual atmospheric precipitation (mm)</th>
<th>Relevant literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANJ94-5</td>
<td>Anjohibe Cave</td>
<td>NW Madagascar</td>
<td>1500</td>
<td>Work in progress</td>
</tr>
<tr>
<td>BC97-14</td>
<td>Bone Cave</td>
<td>NW Botswana</td>
<td>450</td>
<td>Work in progress</td>
</tr>
<tr>
<td>BRU11</td>
<td>Las Brujas Cave</td>
<td>W Argentina</td>
<td>400</td>
<td>Work in progress</td>
</tr>
<tr>
<td>BZBT1</td>
<td>Box Tunich Cave</td>
<td>W Belize</td>
<td>2360</td>
<td>Akers (2011)</td>
</tr>
<tr>
<td>CH1</td>
<td>Yangshipo Cave</td>
<td>SE China</td>
<td>1140</td>
<td>Xiao (2003)</td>
</tr>
<tr>
<td>DP1</td>
<td>Dante Cave</td>
<td>NE Namibia</td>
<td>532</td>
<td>Stilten et al. (2013)</td>
</tr>
<tr>
<td>EG1</td>
<td>La Gruta de San Isidro, Apazco</td>
<td>SW Mexico</td>
<td>674</td>
<td>Work in progress</td>
</tr>
<tr>
<td>ESP03</td>
<td>Cova da Arcola</td>
<td>NW Spain</td>
<td>2500</td>
<td>Railsback et al. (2011)</td>
</tr>
<tr>
<td>ESP04</td>
<td>Cova da Arcola</td>
<td>NW Spain</td>
<td>2500</td>
<td>Work in progress</td>
</tr>
<tr>
<td>GG</td>
<td>Carlsbad Cavern</td>
<td>SW United States</td>
<td>379</td>
<td>Brook et al. (2006)</td>
</tr>
<tr>
<td>MA3</td>
<td>Anjohibe Cave</td>
<td>NW Madagascar</td>
<td>1500</td>
<td>Work in progress</td>
</tr>
<tr>
<td>MC</td>
<td>Macal Chasm</td>
<td>W Belize</td>
<td>1500</td>
<td>Webster et al. (2007)</td>
</tr>
<tr>
<td>WGI</td>
<td>Wow Gdom Cave</td>
<td>NW Namibia</td>
<td>300</td>
<td>Work in progress</td>
</tr>
<tr>
<td>Wudu</td>
<td>Wuxiang Cave</td>
<td>N China</td>
<td>480</td>
<td>Xiao (2003)</td>
</tr>
</tbody>
</table>

FIG. 1. Schematic cross-section of a stalagmite showing idealized Type E and Type L layer-bounding surfaces.
TYPE E SURFACES

Petrography

Type E layer-bounding surfaces are surfaces at which layers evident on the flanks of a stalagmite have been truncated or eroded at the stalagmite’s crest (Fig. 1). The “E” is thus a reminder of erosion. This erosion commonly generates micro-topographic features analogous to landscape features in regions of eroded sedimentary strata. For example, erosion of most, but not all, of the lateral extent of a layer can leave an isolated remnant analogous to a landscape’s mesa (Figs. 2A and 2C). Truncation of layers generates stair-step-like surfaces analogous to erosional escarpments of landscapes (Figs. 2B, 2D, 2E, and 3A), and localized removal of layers generates valley-like features (Fig. 3B). Dissolution below the surface of the stalagmite can leave holes analogous to the caves of a karst landscape (Fig. 4). The latter features commonly develop where clays have coated most of the surface of stalagmite, precluding dissolution where that coating is present, but where gaps in the coating allow penetration of water that dissolves underlying CaCO₃ to create cavities analogous to caves. At least two examples show the transition in one layer from cavity-ridden microkarst to complete removal of that layer at the crest of the stalagmite (Figs. 4A and 4B).

Other features are commonly associated with Type E surfaces. Type E surfaces commonly have coatings...
The truncation of layers at Type E surfaces suggests that the surfaces result from some form of erosion. Removal of material within (rather than on) stalagmites and development of cavities analogous to microscopic caves demonstrates that this erosion is chemical rather than physical, and is the result of dissolution of spelean carbonate. This requires that the water falling on the stalagmite be undersaturated with respect to the stalagmite's calcite or aragonite. This conclusion, combined with the evidence from larger detrital material for gushing cave waters, suggests that Type E surfaces form in times of rainfall greater than normal. For example, undersaturation could occur either when water passes through the rock above the cave quickly enough to not reach chemical equilibrium with the CaCO₃ of the overlying limestone, or when that water enters the cave and reaches the stalagmite so quickly that it has no time to degas and go from undersaturation to supersaturation (Fig. 5).

The Type E surfaces described above are evident at scales from 0.1 mm to 1 cm, and they typically cut across multiple crystals. Features at smaller scales of 0.02 to 0.5 mm that incise single crystals also exist and have been referred to as "micro-corrosion" (Fig. 6A). For example, Spötl et al. (2008) demonstrated microscopic evidence for micro-corrosion (their Fig. 3D) in Stalagmite SPA121 from Spannagel Cave in the Austrian Alps, and Driese et al. (2012) observed similar features in a stalagmite from Raccoon Mountain Cave in eastern Tennessee, USA. Luetscher et al. (2011) reported micro-corrosion from Milchbach Cave in the Swiss Alps on a stalagmite surface at which dissolution had followed crystallographic planes (their Fig. 5C). These micro-corrosion surfaces are small-scale Type E surfaces, and in fact crystallographically controlled micro-corrosion can be seen on surfaces that, at larger scale, meet the criteria for Type E surfaces discussed above (Figs. 6B and 6C).

Fig. 4. Cave-like dissolutional cavities at Type E layer-bounding surfaces. On images, “A” indicates aragonite and “C” indicates calcite. A. Type E surface (two shorter arrows) with an escarpment in which cavities (V) have been opened by dissolution of aragonite. Longer arrow points to a remnant of the aragonite layer seen at right. Stalagmite ESP03 from northwestern Spain. B. Type E surface (arrows) with an escarpment in which cavities (V) have been opened by dissolution of aragonite in Stalagmite DP1 from northeastern Namibia. C. Cavities (V) opened by dissolution of aragonite in Stalagmite DP1 from northeastern Namibia. In both B and C, note dark coatings of detrital material, mostly clay, inhibiting dissolution of aragonite surface; cavities presumably develop where coating is penetrated by corrosive drip water.

of non-carbonate clay (or, more generally, "mud"), although aggregates of clay as large as silt and fine-grain sand can commonly be recognized within these coatings (Figs. 2A, 3C, 3D, 3E, 4B, and 4C). Larger detrital grains, as large as medium-grained sand and consisting of carbonates, phosphates, and silicates, are also sometimes found near Type E surfaces. One might infer that the fine-grained (clay-size) material could have been deposited either as wind-blown dust or in drip water, but the coarser detrital grains and aggregates are too large to have been carried by air in the interiors of caves and instead suggest deposition from gushing drip water.

The Type E surfaces described above are evident at scales from 0.1 mm to 1 cm, and they typically cut across multiple crystals. Features at smaller scales of 0.02 to 0.5 mm that incise single crystals also exist and have been referred to as “micro-corrosion" (Fig. 6A). For example, Spötl et al. (2008) demonstrated microscopic evidence for micro-corrosion (their Fig. 3D) in Stalagmite SPA121 from Spannagel Cave in the Austrian Alps, and Driese et al. (2012) observed similar features in a stalagmite from Raccoon Mountain Cave in eastern Tennessee, USA. Luetscher et al. (2011) reported micro-corrosion from Milchbach Cave in the Swiss Alps on a stalagmite surface at which dissolution had followed crystallographic planes (their Fig. 5C). These micro-corrosion surfaces are small-scale Type E surfaces, and in fact crystallographically controlled micro-corrosion can be seen on surfaces that, at larger scale, meet the criteria for Type E surfaces discussed above (Figs. 6B and 6C).

Process

The truncation of layers at Type E surfaces suggests that the surfaces result from some form of erosion. Removal of material within (rather than on) stalagmites and development of cavities analogous to microscopic caves demonstrates that this erosion is chemical rather than physical, and is the result of dissolution of spelean carbonate. This requires that the water falling on the stalagmite be undersaturated with respect to the stalagmite’s calcite or aragonite. This conclusion, combined with the evidence from larger detrital material for gushing cave waters, suggests that Type E surfaces form in times of rainfall greater than normal. For example, undersaturation could occur either when water passes through the rock above the cave quickly enough to not reach chemical equilibrium with the CaCO₃ of the overlying limestone, or when that water enters the cave and reaches the stalagmite so quickly that it has no time to degas and go from undersaturation to supersaturation (Fig. 5).

The common deposition of calcite over Type E surfaces, even in stalagmites dominantly of aragonite (as in Figs. 3C, 3E, 4B, and 4C), further suggests the association of Type E surfaces with wetter conditions (Murray, 1954; Pobeguin, 1965; Siegel, 1965; Siegel & Dort, 1966; Thrailkill, 1971; Cabrol & Coudray, 1982).

Stalagmites can also be truncated by breakage, most commonly by seismic disturbance (e.g., Forti, 2001; Sebela, 2008; Fairchild & Baker, 2012). However, in contrast to Type E surfaces, such breakage commonly does not follow layers (e.g., Photo 3 of Cadorn et al., 2001), and even if it followed layers it would not be localized at the crest of a stalagmite (cf. Fig. 1). Furthermore, breakage cannot remove material from
Layer-bounding surfaces in stalagmites

Small or insignificant hiatuses may especially be characteristic of Type E surfaces with little vertical expression. For example, the chronology of Spötl et al. (2008) provided little evidence of a significant hiatus at their reported micro-corrosion surface, and similarly Luetscher et al. (2011) reported a surface of micro-corrosion but their chronology did not show a hiatus in that time interval. On the other hand, Driese et al. (2012) inferred hiatuses of at least centuries at the micro-scale corrosion surfaces in their Holocene stalagmite.

Duration of hiatus

The apparent removal of CaCO$_3$ at Type E surfaces suggests that any such surface represents a hiatus in the stalagmite’s record of time. This relationship has been confirmed in Stalagmite ESP03 from Cova da Arcoia in northwestern Spain, where one Type E surface represents a hiatus of about 1000 years in a 9600-year record, and several others represent hiatuses of multiple centuries (Fig. 6 of Railsback et al., 2011). A Type E surface in Stalagmite BZBT1 from Belize likewise may represent a hiatus of 200 years in a record of at most 2000 years (Akers, 2011). On the other hand, the Type E surfaces in Stalagmite DP1 from northeastern Namibia represent such small hiatuses that they are insignificant in the construction of a chronology (Sletten et al., 2013), and the same is true of Type E surfaces in the Wudu stalagmite from north-central China studied by Xiao (2003). Where Type E surfaces represent a hiatus of significant duration, they can introduce abrupt changes in proxy records, as discussed further below.

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TYPE L SURFACES

Petrography and process

Type L layer-bounding surfaces are surfaces below which layers have lesser lateral extent upward and/or become thinner upward (Figs. 1, 7, and 8). They thus represent lessened deposition, and hence the “L”. The lesser lateral extent of layers below Type L surfaces means that stalagmites are commonly narrower beneath such surfaces (e.g., Figs. 8A and 8D). Layers beneath Type L surfaces are commonly aragonite rather than calcite (e.g., Figs. 7A and 7B). Stable isotope ratios, and especially δ$^{13}$C, commonly increase beneath Type L surfaces, as in Stalagmite...
Fig. 6. Type E layer-bounding surfaces (A, C, D, and H to I) at which there is microscopic expression of corrosion (B, C, F, and G). A. Two Type E surfaces (arrows) in Stalagmite EG in southern Mexico. Note coating of detrital material on most of the two surfaces. Rectangle encloses non-coated area shown in Part B. B. Enlargement of non-coated area in rectangle in Part A. Corroded surface of calcite (arrows) in area enclosed by rectangle in Part A. C. Dissolutional pits (arrows) in calcite. D. Type E surface with underlying layer increasingly truncated to left. White rectangle at right encloses area shown in Part E. E. Corroded terminations of calcite crystals in area enclosed in white rectangle in Part D. Examples shown in Parts C, D, and E are from Stalagmite GG from New Mexico in the southwestern United States. F and G. Corroded surface of calcite (arrows) in rectangles along Type E surface that is shown in Parts H and I. H and I. Type E surface (arrows) in Stalagmite MA3 from Madagascar. H is left half and I is right half of one view along one Type E surface; white circle highlights on reference point visible in both images. Quadrilaterals in H outline areas shown in Parts F and G. Note extreme topography developed to right in Part I.
Layer-bounding surfaces in stalagmites

One might argue that Type L surfaces could form not only as the result of drier climate but also as the result of hydrogeological changes, such as blockage of dripwater pathways or lateral drift of drip points. However, these changes would have to be reversible, because the Type L layer-bounding surfaces considered here both overlie previous deposits and, more critically, underlie later deposits that require resumption of their drips. In addition, lateral drift of a drip point should be evident in migration of the crest of a stalagmite’s layers below the Type L surface. Minor migration of the crest is evident at some Type L surfaces, as in Figure 8a, but the migration is not sufficient to indicate drift of the drip off the stalagmite.

Esp03 from northwestern Spain (Railsback et al., 2011), in the Wudu stalagmite from north-central China (Xiao, 2003), and in Stalagmite BZBT1 from Belize (Akers, 2011). In contrast to Type E surfaces, fine-grained detrital sediment is not notably associated with Type L surfaces, and coarser silt-to-sand-sized detrital grains are not associated with Type L surfaces at all.

The lesser lateral extent of layers below Type L surfaces suggests that they form during diminishing flow of water onto the stalagmite (Dreybrodt, 1999), so that little drip water reaches the stalagmite and, in caves where evaporation occurs, even less water reaches its flanks. Greater values of δ¹³C associated with Type L surfaces likewise suggest drier conditions, in that less extensive soil respiration and/or the presence of C4 vegetation can lead to greater δ¹³C of speleal CaCO₃. The frequency of aragonite beneath Type L surfaces further suggests that such surfaces form in drier conditions (Murray, 1954; Pobeguin, 1965; Siegel, 1965; Siegel & Dort, 1966; Thrailkill, 1971; Cabrol & Coudray, 1982). Finally, the scarcity of silt-to-sand-sized detrital grains near Type L surfaces is at least compatible with, if not suggestive of, development of those surfaces in drier conditions because less intense dripwater flow would lessen water-borne transport of coarser detrital grains. On the other hand, Dredge et al. (2013) have proposed that aerosols might allow deposition of some very fine-grained material during cessation of drips, and both Bertaux et al. (2002) and Webster et al. (2007) inferred deposition of dust on surfaces at which precipitation of CaCO₃ ceased (Fig. 7c).

One might argue that Type L surfaces could form not only as the result of drier climate but also as the result of hydrogeological changes, such as blockage of dripwater pathways or lateral drift of drip points. However, these changes would have to be reversible, because the Type L layer-bounding surfaces considered here both overlie previous deposits and, more critically, underlie later deposits that require resumption of their drips. In addition, lateral drift of a drip point should be evident in migration of the crest of a stalagmite’s layers below the Type L surface. Minor migration of the crest is evident at some Type L surfaces, as in Figure 8a, but the migration is not sufficient to indicate drift of the drip off the stalagmite.

Fig. 7. Small-scale examples of Type L layer-bounding surfaces. A. Type L surface below which an interval of clear layers thins to flank of stalagmite at left. B. Type L surface below which layers in bracketed interval at right thin to flank of stalagmite at left. A and B are from Stalagmite ESP03 from northwestern Spain. C. Type L surface in Stalagmite MC1 from Belize. Note extent to which layers in bracketed interval at right thin toward flank of stalagmite at left. Dark color is result of fine-grained non-carbonate material that may be dust accumulated as aerosol during dry period, as proposed by Webster et al. (2007). D and E. Type E surfaces (arrows) at which bracketed intervals at right thin toward flank of stalagmite at left in Wudu stalagmite from north-central China.
Railsback et al. do not represent hiatuses recognizable at the scale of the dating available for that stalagmite (Railsback et al., 2011). An even more striking example comes from a stalagmite from Obir Cave in Austria, where Fairchild et al. (2010) in their Figure 4b documented a Type L layer-bounding surface that they inferred to result from lessened drip rate and that represented a hiatus of just one year.

**SIGNIFICANCE OF LAYER-BOUNDING SURFACES TO PALEOClimATE STUDIES**

**Interpretation of changing climate**

The association of Type E and Type L surfaces with wetter and drier conditions, respectively, has obvious implications for the development of histories of climate change. Most paleoclimate research has depended on oxygen and carbon stable isotope data for indications of climate change, but the interplay of atmospheric temperature, amount effects, and cave temperature make the interpretation of δ¹⁸O data complex if not uncertain (McDermott, 2004), and uncertainties in interpreting δ¹³C data lead many authors to not report their C isotope data at all. Recognition of Type E and Type L surfaces allows inferences of wetter and drier conditions that can support or discredit hypothesized interpretations of a set of stable isotope data, and thus it can improve the ultimate interpretation of those data.

Petrographic recognition of layer-bounding surfaces can additionally inform climate histories from stalagmites in which recrystallization has made isotope data suspect. For example, where chronologies can be based on non-carbonate material, as by radiocarbon dating of organic matter (Akers, 2011) or OSL dating of detrital material (Chirienco et al., 2010), recognition of layer-bounding surfaces allows development of a paleoclimate history not reliant on a stalagmite’s carbonate geochemistry.

**Chronologies and interpretation of time-series paleoclimate data**

One important component in the application of a stalagmite to paleoclimatology is construction of an age model or chronology, where these terms refer to a graphic and/or mathematical relationship assigning an age to every positional increment along the stalagmite’s growth axis. This effort commonly results in a published plot of age and position in which the chronology is represented by a line, series of line segments, or curve that relates position to inferred age (e.g., Fig. 4 of Spötl et al., 2008; Fig. 3 of Holzkämper et al., 2009; Fig. 2 of Dominguez-Villar et al., 2009; Fig. 5 of Brook et al., 2010; Fig. 6 of Railsback et al., 2011; Fig. 3 of Luetscher et al., 2011; Fig. 9 of this paper). The construction of such age models or chronologies is critical to the development of an environmental history (Telford et al., 2004; Blaauw, 2010; Scholz et al., 2012).

Recognition of layer-bounding surfaces can profoundly affect the construction of the age model or chronology from a stalagmite. That is because such surfaces indicate the existence of a hiatus (where

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**Duration of hiatus**

A Type L surface may, but does not necessarily, represent a hiatus in deposition. In the Wudu stalagmite from north-central China, Type L surfaces represent hiatuses of as much as 3000 years (Xiao, 2003), and the Type L surface in Stalagmite ANJ94-5 from Madagascar represents a hiatus of about 900 years. Type L surfaces in Stalagmite DP1 from northeastern Namibia do not represent demonstrable hiatuses but are in a zone of extremely slow growth (Sletten et al., 2013). On the other hand, Type L surfaces in Stalagmite ESP03 from northwestern Spain

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**Fig. 8. Large-scale examples of Type L layer-bounding surfaces. A. Type L surface in Stalagmite ANJ94-5 from Madagascar. Note lesser lateral extent of layers in 1 to 2 cm below surface. B and C. Type L surfaces in Stalagmite WG1 from Namibia. Note lesser lateral extent of white intervals below surfaces. D. Type L surface in Stalagmite BRU 11 from Argentina. Note lesser lateral extent of dark interval just below surface.**

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Fig. 9. Five possible chronologies, or age models, for the hypothetical stalagmite shown in Figure 10. For each, horizontal axis is time during deposition, and vertical axis is position in stalagmite. Series of filled circles to the left of each plot indicate positions of geochemical samples; measured results are greater from left to right. “CDS” indicates a “chronologically disjunct sample” of speleant carbonate from both below and above a layer-bounding surface, and thus from both before and after a hiatus. Figure 11 shows plots of the geochemical data generated assuming the different chronologies shown here. Roman numbers I and VIII indicate two of eight radiometric dates whose positions are shown in Figure 10. A, B, and C are examples of poorly constructed chronologies, as discussed in text. (Fig. 9 continued on following page.)
a hiatus is otherwise not recognized) or the correct placement of a hiatus (where a hiatus is suspected but its position is otherwise unknown). To illustrate this, Figure 9 shows five different chronologies constructed from one hypothetical set of radiometric dates from one hypothetical stalagmite, which is shown in Figure 10. Figure 11 correspondingly shows five time series generated from one hypothetical set of isotopic data plotted according to the five chronologies in Figure 9. The dates come from a hypothetical stalagmite in which there are two Type L surfaces and one Type E surface (Fig. 10).

Of the five chronologies, Chronologies A, B, and C are constructed with no awareness of the layer-bounding surfaces. Chronology A assumes uninterrupted deposition and a constant rate of deposition for the entire stalagmite, and thus it consists of one straight line. In modern research, Chronology A would likely be questioned because it assigns ages outside the range of uncertainty for many of the dates. However, some papers published recently each include at least a single case of such nonconformity to radiometric dates and their uncertainties. Examples include the date 6 cm from the top of Stalagmite W5 studied by Holzkämper et al. (2009) and the date 18.5 mm from the top of Stalagmite ESP03 by Railsback et al. (2011). Application of Chronology A to a set of stable isotope or other data from the stalagmite in Figure 10 would yield the time-series of data in Figure 11A. Inattention to layer-bounding surfaces would lead to some data from diachronous samples taken across the unrecognized layer-bounding surfaces; these chronologically disjunct samples (“CDS” in Figs. 9 and 11) falsely smooth abrupt transitions in the data. In addition, some transitions in the time series would seem exceptionally abrupt because the large times that they span would not be recognized without recognition of the layer-bounding surfaces.

Chronology B assumes uninterrupted deposition but changing rates of deposition, and it thus consists of a series of joined line segments. It resembles many chronologies published in recent years, including
layer-bounding surfaces in stalagmites

those for Stalagmite SPA121 of Spötl et al. (2008) and Stalagmites MB3 and MB5 of Luetscher et al. (2011), and for large intervals of the chronologies for Stalagmite LV5 of Domínguez-Villar et al. (2009) and Stalagmite W5 of Holzkämper et al. (2009). It would, however, indicate incorrect ages for the intervals between Dates II and III, V and VI, and VII and VIII, because it does not take into account the three hiatuses at the three layer-bounding surfaces. As with Chronology A, stable isotope and other data taken using Chronology B could include diachronous samples across the unrecognized layer-bounding surfaces, and some transitions in the time series would be exceptionally abrupt and thus suggest improbably rapid ecological transitions. Chronology B would also incorrectly suggest an uneven spacing of samples in the time domain (Fig. 11B). Diligent researchers would take additional samples in time intervals that were seemingly sparsely sampled, which would only help to conceal the displacement of data in time and would likely lead to recovery of more diachronous samples across the unrecognized layer-bounding surfaces.

Chronology C assumes discontinuous deposition, with breaks in deposition where radiometric dates suggest either very slow growth or hiatuses. Those breaks must fall between two noticeably different ages, but the radiometric data provide no other criteria to dictate the exact positions of those breaks. As a result, the specific example shown as Chronology C in Figures 9 and 11, with its specific positioning of hiatuses, is just one of a large number of chronologies that could be generated using the same principles but with differing arbitrary positioning of the inferred hiatuses. Chronology C resembles many chronologies published in recent years, including that of Brook et al. (2010) where a hiatus inferred at 46 cm could seemingly be anywhere between 42 and 48 cm of a sequence 55 cm long. It also resembles the chronology derived from a hypothetical set of data by Scholz & Hoffman (2011), where radiometric data only constrain a hiatus to be anywhere between 380 and 400 mm from the top of a stalagmite 450 mm long. Differing choices for the positions of hiatuses can lead to very different times assigned to geochemical samples (Fig. 11C). In the real-world chronology by Brook et al. (2010), differing placement of the hiatus inferred at 46 cm would change the age of some samples by 9,000 to 10,000 years, and in the hypothetical example by Scholz & Hoffman (2011) differing arbitrary placement of the hiatus would change the age of some samples by about 8,000 years.

Chronology D assumes discontinuous deposition, with breaks in deposition at layer-bounding surfaces. Its breaks in deposition thus coincide with the best available evidence for timing of the hiatus. In this respect, it is superior to Chronology C, where positions of the inferred hiatuses are not supported by petrographic evidence, and far superior to Chronologies A and B, which have no hiatuses at all. Chronology D assumes constant rates of deposition within a single continuous growth interval, but rates that may differ from one interval to the next. It thus consists of a series of isolated line segments. Chronology D is an analog of the chronology of Stalagmite ESP03 from Cova da Arcoia in northwestern Spain by Railsback et al. (2011) and of Stalagmite CBD-2 from southwestern Mexico by Bernal et al. (2011), who included photomicrographs of what we would categorize as Type E surfaces.

Chronology E, like Chronology D, assumes discontinuous deposition with breaks in deposition at layer-boundary surfaces, and thus like Chronology D it has hiatuses at positions supported by petrographic evidence. However, Chronology E assumes rates of deposition that vary as suggested by changing layer thickness. Thus, where layers thin beneath Type L surfaces and thereby suggest a lesser rate of deposition or growth rate, rate of deposition in the chronology diminishes. The exact extent of this change in rate cannot be determined precisely without an infinite number of radiometric dates spaced infinitely closely. However, Chronology E presents a more realistic and more probable relationship between position and age than does Chronology D, which is in turn much more realistic than Chronologies A, B, and C.

CONCLUSIONS

Two kinds of layer-boundary surfaces in stalagmites, Type E and Type L, can be recognized according to explicitly-defined criteria (Table 1; Fig. 1). Recognition of these surfaces provides paleoclimatological information that can complement or even substitute
for geochemical proxies. Recognition of these surfaces also greatly improves development of chronologies or age models, in that petrographically-constrained incorporation and placement of hiatuses yields more accurate relationships between position and age. Future paleoclimatological research using stalagmites would therefore be enhanced by petrographic, as well as geochemical, analysis of stalagmites. Further research may also recognize other kinds of layer-bounding surfaces and/or other origins of hiatuses as this area of speleothem research develops.

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**INTRODUCTION**

Chemolithoautotrophic microbial processes control metal and nutrient cycling in karst aquifer systems (e.g., Sarbu et al., 1996; Northup et al., 2003; Opsahl & Chanton, 2006; Gray & Engel, 2013) and support rich and biologically diverse ecosystems with complex trophic-level interactions and food webs in the absence of allochthonous input (e.g., Holmes et al., 2001; Farnleitner et al., 2005; Goldscheider et al., 2006; Macalady et al., 2008; Birdwell & Engel, 2009; Birdwell & Engel, 2010). These findings contradict a long-held tenet that nearly all ecosystems on Earth are dependent on oxidative photosynthesis for energy and organic carbon (e.g., Horner-Devine et al., 2003; Amend & Teske, 2005; Simon et al., 2007; Birdwell & Engel, 2009; Birdwell & Engel, 2010). These findings contradict a long-held tenet that nearly all ecosystems on Earth are dependent on oxidative photosynthesis for energy and organic carbon (e.g., Horner-Devine et al., 2003; Amend & Teske, 2005; Simon et al., 2007;
However, microbial food webs from subaerially exposed (i.e. terrestrial) mineral deposits in caves have received relatively limited attention with respect to microbial food web structure, despite their importance for troglobionts (cave-adapted, terrestrial invertebrates) and even the possibility that their association can alter climate records from speleothem deposits (e.g., Lacelle et al., 2004; Blyth et al., 2008).

Moonmilk -- a spongy to powdery assemblage of microcrystalline carbonate minerals that resembles toothpaste when hydrated -- is a type of secondary carbonate speleothem that occurs subaerially in caves, usually on cave walls (Hill & Forti, 1997). Microbial and fungal biomass is prevalent in moonmilk (e.g., Cañaveras et al., 2006; Rooney et al., 2010; Baskar et al., 2011; Braissant et al., 2012; Sanchez-Moral et al., 2012). Although some investigations suggest that microbes mediate moonmilk development (e.g., Mason-Williams, 1959; Northup et al., 2000; Cañaveras et al., 2006; Richter et al., 2008), microbes are not essential for its formation (Borsato et al., 2000). Recent studies of moonmilk examine biogeochemical and geomicrobiological interactions (e.g., Mulec et al., 2002; Cacchio et al., 2004; Van de Kamp, 2004; Portillo & Gonzalez, 2011; Sanchez-Moral et al., 2012), but there still has been scant effort to evaluate the microbial diversity of moonmilk using culture-independent methods or to consider the possibility that moonmilk, because of the abundance of microbial biomass, may serve as a potential food and energy source for troglobiotic invertebrates when allochthonous organic carbon input would be limited (Paoletti et al., 2011). Inclusion of moonmilk into terrestrial cave food web structures represents a significant paradigm shift.

Extensive moonmilk deposits in the Italian caves, Grotta della Foos and Bus della Genziana (Fig. 1A), are associated with the occurrence of Cansiliella, a group of troglobitic beetles. Cansiliella servadeii Paoletti 1972 (Coleoptera: Leptodirinae) browses the interface of percolating waters and moonmilk in Grotta della Foos, and Cansiliella tonielloi is found with moonmilk in Bus della Genziana (Beggio, 2007). Cansiliella spp., as well as other beetles from the genus Hadesia and possibly the recently described genera Radziella, Velebitodromus, Croatodirus, Nauticella, Tartariella, and Kircheria, have body characteristics distinct from the majority of other troglobitic Leptodirinae and have peculiar, semi-aquatic feeding behaviors attributed to inhabiting rocky surfaces, such as cave walls (Jeannel, 1924; Remy, 1940; Paoletti, 1973; 1980; Moldovan et al., 2003; Sket, 2004; Giachino & Vailati, 2006). Their feeding and mouthpart morphologies suggest a microphagous feeding habit associated with filtration of allochthonous organic material from the hygropetric, cave-wall habitat (Sket, 2004). Prior to our investigations (Paoletti et al., 2009; Paoletti et al., 2011; Paoletti et al., 2013), no previous studies directly evaluated the food source for Cansiliella spp.

Our observations of Cansiliella spp. led to the working hypothesis that microbes associated with the moonmilk serve as food for the beetles. Using stable isotope ratio systematics, we attempted to establish the food web position of C. servadeii and other cave animals (including Monolistra tavalensis, Niphargus sp., and Androniscus noduliger) with respect to moonmilk (Paoletti et al., 2011). The carbon isotope values of C. servadeii are consistent with moonmilk potentially serving as a food source. However, potential overgrazing of the moonmilk by the beetle to obtain sufficient nutrition, and unique and even unknown microbial activities in the moonmilk associated with nitrogen cycling, suggest that the trophic level position for C. servadeii is incompatible with moonmilk serving as a food source. Therefore, the goal of the current study is to enhance our understanding of moonmilk microbial diversity from Grotta della Foos and Bus della Genziana that could reveal the underpinnings of a moonmilk nitrogen cycle. This research identifies a common community structure for the moonmilk bacterial communities, and also offers an explanation for why some troglobitic invertebrates, such as C. servadeii, have evolved unique body morphologies and behaviors to take advantage of a cave hygropetric habitat like moonmilk.

Fig. 1. (A) General location map for Grotta della Foos, Italy (star labeled “F”) and Bus della Genziana (star labeled “G”) with (B) one of the moonmilk sampling sites in Grotta della Foos, modified from Paoletti et al. (2011). Scale bar = 0.5 m.
MATERIALS AND METHODS

Sample collection
Moonmilk was collected from Grotta della Foos in March 2008 and from Bus della Genziana in January 2009 (Beggio, 2007). Both caves formed in Cretaceous limestone, and Grotta della Foos is located at an elevation of 422 m and Bus della Genziana at 1020 m. The primary sample location in Grotta della Foos where C. servadeii is found is “Site B” (Fig. 1) (Paoletti et al., 2011). Further sampling location details are withheld because the Cansisiella spp. population sizes are not known and the caves are not well-protected, although Bus della Genziana is located in a natural speleological preserve (Paoletti et al., 2011).

Aqueous geochemistry and moonmilk characterization
Basic geochemical analyses of percolating water samples were done, including pH, temperature, and specific conductance. Water was filtered to 0.2 μm, and anions and cations were measured by single column ion chromatography. The geochemical program Phreeqc version 2.15.0 was used to determine the saturation indices (SI) of mineral phases according to the logarithm of the quotient of the ion activity product and solubility product constant (Parkhurst & Appelo, 1999). Organic carbon compositions were measured following the removal of inorganic carbonate with a solution of 1N HCl for 16 hr at room temperature while monitoring the reaction and pH (Midwood & Boutton, 1997). Samples for each were rinsed to about pH 5.5 in DI H2O, dried at 50 °C, and then ground to a fine powder. Because the acidification procedure could produce a loss of nitrogen-acid soluble organic matter and enhance ingassing of NH3, nitrogen content was done on non-acidified samples. Aliquots were flash combusted on an EA 1110 Carlo Erba elemental analyzer.

Moonmilk from Bus della Genziana was thinner and patchier, making collection for additional geochemical and biological characterization difficult. Therefore, examination of chlorophyll content and meiofaunal and microbial cell counts (below) was done only from Grotta della Foos. Chlorophyll and photosynthetic pigment content (Chl a) was measured spectrophotometrically to assess the potential presence of photosynthetically-derived (allochthonous) organic matter. For the water, the method of Innamorati (1990) was used. Briefly, 0.5 to 1 L of water was filtered onto a 1μm glass fiber filter, which was placed into 80% acetone. The filter-acetone mixture was ground, then incubated for 24 hr before centrifugation. For moonmilk, the following modifications were made from Innamorati (1990). Moonmilk was dried for 24 hr at 60 °C. The dried material was pulverized and 1 g was used to extract chlorophyll using 1:1 chloroform-methanol. The slurry was incubated for 24 hr, than centrifuged for 25 min at 4000 rpm. Absorbance was measured from the supernatant. Detailed calculations can be found in Beggio (2007).

Micro- and meiofaunal and microbial cell counts
Twenty glass slides were placed in the percolating water on the surface of moonmilk at Site B in Grotta della Foos (Fig. 1) for ~5 months to capture and enumerate micro- and meiofauna. Slides were transported to the lab in sterilized boxes containing the same percolating water and maintained at 4 °C until analysis. Slides were examined by stereomicroscope and transmission microscope for general identification. For microbial cell abundance, aliquots of the moonmilk with associated percolating waters were fixed in 4% formaldehyde. Briefly, 1 ml of the moonmilk slurry was resuspended in 5 ml of MQ water and vortexed for 5 min. An aliquot of 1ml of suspension or 1ml of water was vacuum filtered on a 0.2 μm pore size polycarbonate filter (Nuclepore) and stained with DAPI (α’, 6-diamino-2-phenylindole) at a final concentration of 0.2% wt/vol (Porter & Feig, 1980). Cell counts were done with a Zeiss Axioplan epifluorescence microscope, equipped with an HBO 100 W lamp, a Neofluor 100 x objective, 1.25x additional magnification, and filter sets for UV light excitation (Zeiss filter set G365, FT395, LP420). Microscopic fields for counting were grabbed with an image analysis system (Image ProPlus; Media Cybernetics, Silver Spring, MD, USA), equipped with a high sensitivity video camera (CoolSNAP Pro-cf, Media Cybernetics).

DNA extraction from moonmilk and percolating waters
Two separate samples of moonmilk from Grotta della Foos were collected from Site B (referred to as “Foos7B”) and “Foos8B”), and one site from Bus della Genziana (referred to as a “GMM”). Total environmental nucleic acids were extracted within 72 hr of collection. Various extraction methods were considered (e.g., Barton et al., 2006), but based on cell count estimates from DAPI, the following method was considered sufficient to retrieve high purity, representative DNA from the microbial community in the moonmilk. Specifically, 2 ml of moonmilk slurry were placed into 10 ml of sterile cell lysis buffer (10 mM Tris and 100 mM EDTA, pH 8.0), homogenized with a sterile tissue homogenizer for 2 min, or until large particles and clumps were thoroughly disaggregated to particles no bigger than grains of sand. The suspensions were incubated at 55°C on a shaker overnight at 250 rpm, following addition of 40 ml of proteinase K (20 mg/ml). Protein precipitation was accomplished by combining 1ml of cooled suspension with 1 ml 7.5 M ammonium acetate. The solutions were mixed thoroughly, and then incubated on ice for 15 min prior to centrifugation for 5 min at high speed. The supernatant was transferred to a new tube with an equal volume of 100% isopropanol for nucleic acids precipitation overnight at 4°C. Samples were centrifuged at high speed for 3 min and pellets were washed with cold, 95% ethanol. Nucleic acids were rehydrated in 50 ml TE buffer (10 mM Tris and 0.1 mM EDTA, pH 8.0). DNA purity and concentrations for each extraction were determined on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, LLC, USA).
**PCR amplification of genomic DNA, cloning, and sequencing**

Nearly full length 16S rRNA gene sequences were obtained by PCR amplification using the bacterial primer pair 8F (forward, 5'-AGAGTTTGATCCTGCGTCAAG-3') and 1510r (reverse, 5’-GTAAAACGACGGCCAG-3’) (Lane, 1991). The archaeal primers UA571F (forward, 5’-GCYTAAGSAGCCGTAGC-3') and UA1204R (Reverse, 5’-TGGGATATCCACCT-3') (Baker & Cowan, 2004) were also used, but amplifications were unsuccessful. Amplification was performed with a MJ Research Dyad Disciple thermal cycler with 5 U/ml ABGene Taq DNA polymerase (ABGene, Thermo Fisher Scientific, USA). Optimal PCR products for bacteria were achieved for three of the four extractions using the following conditions: an initial hot start at 94°C for 4 min, followed by denaturation at 94°C for 1 min, primer annealing at 47°C for 1 min, chain extension at 72°C for 1 min, repeated for 29 more cycles, followed by a final extension step for 20 min at 72°C.

Amplified PCR products were purified by using a 0.7% TAE low-melt agarose gel with a Wizard PCR prep DNA Purification kit (Promega Corp., USA), following manufacturer recommendations. Concentrations and purity were determined by spectrophotometry. Purified products were cloned using the TOPO Cloning Kit with the pCR2.1-TOPO vector, according to manufacturer instructions (Invitrogen Corp., USA); ligation reactions were run overnight at 14°C. Clones were screened by PCR with the M13-Forward (5’-GTAAAACGACGGCCAG-3') and M13-Reverse (5’-CAGGAAAACGCTATGAC-3') primers (Invitrogen Corp., USA). Amplified inserts were diluted and sequenced by capillary Sanger sequencing using High-throughput Sequencing Solutions (http://www.htseq.org/index.html). Sanger sequencing was chosen over other, more high-throughput methods (e.g., 454 tag pyrosequencing) in order to obtain near-full length 16S rRNA gene sequences for detailed phylogenetic analyses.

**Phylogenetic analysis and estimation of microbial diversity**

Resulting 16S rRNA gene sequences were assembled using ContigExpress, a component of Vector NTI Advance 10.3.0 (Invitrogen Corp., USA), and subjected to BLAST searches using GenBank (http://www.ncbi.nlm.nih.gov/) to establish gene sequence similarities to culturable and not yet cultured organisms. Sequences were screened for chimera and aligned using greengenes NAST-aligner (http://greengenes.lbl.gov) and using the program DECIPHER’s Find Chimera tool (http://decipher.cee.wisc.edu/FindChimeras.html) (Wright et al., 2011). The Ribosomal Database Project (RDP), release 10 (Cole et al., 2009), Library Compare online analysis tool (Wang et al., 2007) was used to test for statistical differences in the taxonomic representation between the two moonmilk bacterial clone libraries, at alpha = 0.01 and a 95% confidence threshold.

Designation of operational taxonomic units (OTUs) representing the ≥97% sequence similarity predicted for species-level relationships using 16S rRNA gene sequences (e.g., Stackebrandt & Goebel, 1994) was done in DOTUR using the furthest-neighbor clustering algorithm (http://www.plantpath.wisc.edu/faq/joh/dotur.html) (Schloss & Handelsman, 2005). If the DOTUR ≥97% sequence similarity groups were concordant with a monophyletic clade from the reconstructed phylogeny, then those groups were designated as OTUs. Rarefaction curves were generated using DOTUR. More than 400 clone sequences from this study and sequences identified from RDP and BLAST, including outgroup taxa, were used to reconstruct a phylogeny based on a GTR +G model of evolution. Branch support from 500 bootstrap replicates was constructed using the program MEGA (ver. 5.0) (Kumar et al., 2004) based on the Jukes-Cantor model (Tamura et al., 2004). The phylogenetic associations for the moonmilk clones were evaluated from RDP SeqMatch analysis tool, whereby the highest similarity scores (as S_ab) to environmental clones or cultured strains were compiled and specific habitat types were evaluated (e.g., cave, freshwater, soil, other). Scores of 1.0 would result for identical sequences; the closer the score is to 1.0, the more similar sequences are to each other. Scores >0.96-98 loosely correlate to genus-level associations.

**Nucleotide sequence accession numbers**

Representative 16S rRNA gene sequences from Grotta della Foos moonmilk clone libraries were submitted to GenBank under the accession numbers EU431666-EU431834 and KC255251-KC255375 for Bus della Genziana samples.

**RESULTS AND DISCUSSION**

**Moonmilk geochemistry**

The percolating waters flowing over Grotta della Foos moonmilk (Fig. 1B) had a pH of 8.3 at 8.8 °C, 200 μS/cm specific conductance, and 0.3 mmol/L dissolved oxygen (Table 1). The water associated with the Bus della Genziana moonmilk had a pH of 7.98 at 7.0 °C, 390 mS/cm specific conductance, and 0.24 mmol/L dissolved oxygen (Table 1). For both caves, the water types were characterized as Ca-HCO₃⁻ type (Table 1). The waters were supersaturated with respect to calcite (Saturation Index = +0.43) and undersaturated with respect to other mineral phases, confirming the mineralogy of each deposit, although the Bus della Genziana moonmilk appeared to be less hydrated and in a state of erosion rather than deposition.

The moonmilk from Grotta della Foos was soft, whitish to yellowish color, hydrated and of calcite mineralogy. The density of C. servadeii on the moonmilk was ~8-12 individuals per m² (Fig. 1B). The organic C composition of the moonmilk was 0.06-0.52 %, with 0.01-0.05 % organic N, and no detectable S but minor amounts of Al, Si, and Fe (data not shown). By contrast, the Bus della Genziana moonmilk was markedly thinner and patchier, but also of calcite mineralogy. Traces of chlorophyll and other photosynthetic pigments, as proxies for surface-derived (plant) material, were measured from Grotta...
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be specialized to the subsurface: Glaucoma scintillans (a benthic ciliate), flagellates belonging to the genera Anisonema, Bodo, and Cercomonas, Chironomidae larvae (possibly Eukiefferiella), and an annelid from the Lumbriculidae. Microbial cells were cocciod, bacilliform, or filamentous, and most were a few microns or less in diameter. Rare fungal hyphae were observed, suggesting that fungi do not comprise a significant portion of the moonmilk microbial communities. Cell counts from water were conservatively estimated to be $10^5 - 10^6$ cells/ml, which is similar to what has been reported previously as $10^3 – 10^6$ cells/ml for epikarst waters (e.g., Rusterholtz & Mallory, 1994; Laiz et al., 1999). We considered the counts to be a mixture of viable and nonviable cells from the system. We were unable to quantify the number of cells precisely from the moonmilk because high carbonate mineral content (Fig. 2A) interfered with the DAPI signal (Fig. 2B). Nonquantitative estimates revealed that cell numbers in the moonmilk could be 10 to 100 times greater compared to the percolating waters. These microbial biomass estimates would be comparable to those reported by other investigators. Braissant et al. (2012) estimate approximately $10^5$ colony forming units (CFUs) per gram of moonmilk from Swiss caves using culture-based and microcalorimetric analyses, and Curry et al. (2009) also describe $10^4 – 10^6$ cells as CFUs per gram of moonmilk from Alaska, depending on the type of moonmilk. Considering the abundance of moonmilk in both caves, there is noteworthy microbial standing stock to cycle organic carbon (e.g., del Giorgio & Cole, 1998; Eiler et al., 2003).

**Phylogenetic analysis of microbial communities from moonmilk**

16S rRNA gene sequences were retrieved from moonmilk to evaluate the microbial diversity, which was expected to be high based on previous research of natural, cave-wall habitats (e.g., Northup et al., 2003; Chelius & Moore, 2004; Van de Kamp, 2004; Barton et al., 2007) and cave-walls with Paleolithic paintings or system having been anthropogenically impacted (e.g., Groth & Saiz-Jimenez, 1999; Schabereiter-

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**Table 1. Geochemistry of percolating waters. Ions are listed in mmol/L concentrations.**

<table>
<thead>
<tr>
<th>Ion</th>
<th>Grotta della Foos</th>
<th>Bus della Genziana</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air temperature (°C)</td>
<td>8.6</td>
<td>6.2</td>
</tr>
<tr>
<td>Water temperature (°C)</td>
<td>8.8</td>
<td>7.0</td>
</tr>
<tr>
<td>pH</td>
<td>8.25</td>
<td>7.99</td>
</tr>
<tr>
<td>Conductivity (µS/cm)</td>
<td>225</td>
<td>390</td>
</tr>
<tr>
<td>Dissolved oxygen (mmol/L)</td>
<td>0.34</td>
<td>0.24</td>
</tr>
<tr>
<td>Na⁺</td>
<td>0.01</td>
<td>1.10</td>
</tr>
<tr>
<td>K⁺</td>
<td>&lt;0.001</td>
<td>0.1</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>0.02</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.13</td>
<td>1.15</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>&lt;0.002</td>
<td>0.04</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>0.03</td>
<td>1.65</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>2.45</td>
<td>2.16</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>0.04</td>
<td>&lt;0.0008</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>0.002</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>0.04</td>
<td>0.03</td>
</tr>
</tbody>
</table>

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**Fig. 2.** (A) Environmental scanning electron photomicrograph of moonmilk from Grotta della Foos showing cocciod, bacilliform, and filamentous cells intermixed with microcrystalline calcite. Scale bar = 50 µm (B) DAPI-stained cells (arrows) from a slurry of moonmilk. Other, larger bright spots are calcite crystals. Scale bar is 10 µm.
Comamonadaceae or the Oxalobacteraceae families, (Betaproteobacteria), and specifically either the Representatives from the Burkholderiales order della Foos and Bus della Genziana, respectively. Represented 22.2% and 15% of the clones for Grotta = 0.003) within the Betaproteobacteria, which p (Suppl. Fig. 1). Clones from Grotta della Foos formed a novel clade within the Acidovorax genus (Comamonadaceae), which are known to grow chemolithoautotrophically using hydrogen as an energy source, while others are capable of denitrification (Heylen et al., 2008). An isolate belonging to the Acidovorax was also retrieved from Ballynamintrna Cave moonmilk using minimal medium agar (Rooney et al., 2010), suggesting that there may be some common microbial groups in moonmilk from different caves. From the Italian moonmilk, at least three other novel clades were affiliated with the genera Janthinobacterium and Massilia spp. (Oxalobacteraceae), at 92-97% sequence similarities. These groups are strict aerobic chemooorganotrophs and are associated with nitrogen cycling, found in aquatic, soil, and metal-contaminated sites, and form plant associations, usually as pathogens (Suppl. Fig. 1). They have also been identified from naturally oligotrophic, but not associated with cave paintings, cave-wall surfaces (e.g., Northup et al., 2003; Barton et al., 2007). The general phylogenetic similarities, even in the absence of high sequence identities, may indicate that the cave habitat, irrespective of geochemical or mineralologic conditions, may be a common habitat for these betaproteobacterial groups.

Other prevalent proteobacterial groups from the moonmilk included the Gammaproteobacteria (Suppl. Fig. 2), Alphaproteobacteria Deltaproteobacteria (Suppl. Fig. 3), and members of the candidate division TM7. Among the Gammaproteobacteria (Fig. 4B; Suppl. Fig. 2), clones were predominately related to various pseudomonad species (Pseudomonadales order). Clones related to Acinetobacter spp. were retrieved from Grotta della Foos. Isolates belonging to this genus cause carbonate precipitation in cultures collected from carbonate speleothems from Cervo Cave, Italy (Cacchio et al., 2004). Other notable genera included Lysobacter spp., a predatory group (Jurkevitch & Davidov, 2006), and Perlucidibaca and Rhizobacter spp. Among the Alphaproteobacteria (Fig. 4B; Suppl. Fig. 3), retrieved clones were closely related (93-99% sequence identity) to groups that also cycle nitrogen, including the genera Sphingomonas, Sphingopyxis, and Novosphingobium (Sphingomonadales order) and Azospirillum (Rhodospirillales order) (Suppl. Fig. 3). These organisms are obligate aerobes and have the ability to fix N₂ (Addison et al., 2007). Several clades within the Sphingomonadales were comprised of clones from both moonmilk sites, and were more closely related to each other than to previously retrieved environmental clones or cultured strains. Schabereiter-Gurtner et al. (2004) retrieved similar alphaproteobacterial groups from cave-wall rock and Paleolithic paintings in Spain, and Northup et al. (2003) found these genera from caves in New Mexico.
These results suggest that the moonmilk and similar cave and karst habitats likely contain additional novel diversity. Deltaproteobacteria associated with the genera *Bdellovibrio*, *Pedobacter*, and *Haliangium* were retrieved from both caves (Suppl. Fig. 3), but had only previously been reported from the Movile Cave in Romania. *Bdellovibrio* spp. are also obligate predatory bacteria (Jurkevitch & Davidov, 2006).

The Bacteroidetes phylum represented the second most abundant group at the phylum-level (Fig. 4A), and comprised 16.7% and 14.9% of all clone sequences from Grotta della Foos and Bus della Genziana, respectively. Clones were affiliated with predominately the Sphingobacteria class, but representation among the Flavobacteria was also relatively high (Suppl. Fig. 4). Some of the retrieved clones affiliated with the *Flexibacter* spp. were related to strains previously identified as aerobic chemoorganotrophs from freshwater settings, including a novel clade of Bus della Genziana clones with a sequence from the Frasassi Caves, Italy, and another clade of Grotta della Foos clones and a karst soil clone. Several clones from both caves formed clades within the *Flavobacterium* genus and were related to known chemoorganotrophs, facultative aerobes, as well as *N*₂ fixers.

Three OTUs (at 97% sequence identity) belonged to the candidate division TM7, with one large novel clade of Grotta della Foos sequences and other clades with

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**Fig. 4.** (A) Summary of the percentage of total 16S rRNA gene sequence clones from Grotta della Foos (colored in green) and Bus della Genziana (colored in orange). (B) Phylogenic reconstruction using neighbor joining topology for 16S rRNA gene sequences of representative bacterial clones retrieved from Grotta della Foos and Bus della Genziana moonmilk samples. Branches linked to sequences retrieved from this study are colored in either green or orange, corresponding to each of the caves. The bar graphs located around the tree show the total percentage of clones affiliated with the specific taxonomic groups to the class level for Bacteroidetes and to the order level for the proteobacterial groups.
Bus della Genziana clones and other environmental sequences (Fig. 4B). The ecophysiology of the TM7 candidate division remains a mystery (Podar et al., 2007), but finding that these microbes occur in the moonmilk may add to the known metabolic capabilities and physiological requirements for members of this group. To our knowledge, sequences affiliated with the TM7 candidate division have not been retrieved from caves, suggesting that the presence in moonmilk from both caves requires further investigation.

Several culture-independent studies from cave and karst habitats have retrieved sequences belonging to the Verrucomicrobia, Planctomycetes, and Acidobacteria (e.g., Chelius & Moore, 2004; Ikner et al., 2007). Collectively, these groups were represented by <2% each of all of the clones from both Grotta della Foos and Bus della Genziana (Fig. 4A). Verrucomicrobia were only retrieved from Grotta della Foos. Among the Planctomycetes from both sites, none of the clones had high sequences similarities to cultured representatives, but one clone was related to an environmental clone from an ‘anammox’ reactor, suggesting that anaerobic oxidation of ammonium may be occurring in the moonmilk community. If anammox is occurring, although based on the measured dissolved oxygen concentrations of 0.3 mmol/L, this is unlikely (e.g., Strous et al., 1998), then this metabolism could impact nitrogen isotope systematics of the moonmilk because anammox is associated with large isotopic fractionation (Paoletti et al., 2011). Retrieving four different groups of Acidobacteria in both moonmilk sites is interesting because this group has also been found to have high abundance on cave-walls (Chelius & Moore, 2004; Ikner et al., 2007), as well as cave surfaces associated with paintings (Schabereiter-Gurtner et al., 2002a).

Other moonmilk clones were associated with the Actinobacteria, Firmicutes, Nitrospirae, Chloroflexi, Chlorobi, and candidate division WS3. Actinobacteria and Firmicutes have been identified from numerous cave settings (e.g., Groth & Saiz-Jimenez, 1999; Laiz, 1999; Schabereiter-Gurtner et al., 2002a; Northup et al., 2003; Chelius & Moore, 2004; Geric et al., 2004; Schabereiter-Gurtner et al., 2004; Van de Kamp, 2004; Barton et al., 2007; Ikner et al., 2007), and the candidate division WS3 has been previously identified from caves in Spain (Schabereiter-Gurtner et al., 2004). The significance of why there were relatively low numbers of clones retrieved from the moonmilk for these phyla is unknown, particularly for the Actinobacteria that have been shown to play a role in the formation of moonmilk by Cañaveras et al. (2006) and organic matter degradation (e.g., Laiz et al., 2000).

Although the Grotta della Foos and Bus della Genziana moonmilk bacterial diversity was statistically similar to each other, the bacteria appear to be distinct taxonomically, especially at the genus level, from previously examined natural and human-impacted cave-wall habitats (e.g., Groth & Saiz-Jimenez, 1999; Vlasceanu et al., 2000; Holmes et al., 2001; Northup & Lavoie, 2001; Northup et al., 2003; Barton & Northup, 2007; Barton et al., 2007; Ikner et al., 2007) (Fig. 5). Some of the groups retrieved from the moonmilk were more closely related to each other than to environmental clones or strains from other habitats. But, the overlying soil and percolation waters for both caves are considered to be the primary inoculation source(s) for the moonmilk communities. As such, there were also a high number of phylogenetic associations (at >93% sequence identity) between moonmilk clones and environmental clones or cultured strains from soil (e.g., permafrost, forest, grassland) or freshwater systems (e.g., ice, lakes, streams, groundwater), at almost equal representation for all sequence similarity levels (Fig. 5). In terms of the relative proportions of major taxonomic divisions, the community composition from the moonmilk more closely resembles the composition reported in several studies of freshwater systems not soils, featuring over 50% Proteobacteria, mostly from the Betaproteobacteria, followed by Bacteroidetes, and by rare Verrucomicrobia and Actinobacteria (e.g., Zwart et al., 2002; Newton et al., 2006). These associations provide a unique perspective on the microbial diversity of moonmilk because moonmilk is a potentially novel habitat type in karst and the relatively low diversity and dominance among certain bacterial groups could be due to the moonmilk being a specialized habitat that would favor a variety of metabolic specialists (e.g., Smith, 2007).

Although 6% of the clones had close affinities to sequences previously retrieved from organic-rich material, such as manure and sludge (Fig. 5), there is no such organic-rich contribution to the moonmilk from the surface. There is potential that the composition and structure of the moonmilk bacterial communities in both caves may be influenced from fecal material or gut flora from the beetles that graze the moonmilk. However, previous research of arthropod gut flora has indicated that guts contain an abundance of fermentative microbes, and lactogenic and acetogenic groups (Egert et al., 2003) that would likely not be sustained in the moonmilk geochemical system. Moreover, our recent examination of the Cansiliea gut flora (Paoletti et al., 2013) reveals that Firmicutes represented >58% of all retrieved 16S rRNA gene sequences from clone libraries constructed from guts from three individuals, and that Bacteroidetes represented ~16% of the sequences, Actinobacteria 11.5%, Deltaproteobacteria 8%, and <5% each of the alphaproteobacterial classes. Consequently, there was no similarity or affinity of the gut clones to sequences retrieved from the moonmilk, suggesting that there is little correspondence between the gut flora and the potential food source for the beetles.

Conclusions - implications for a moonmilk-based food web

Excluding sulfur-based cave ecosystems where chemolithoautotrophic microbes are primary producers (e.g., Sarbu et al., 1996; Porter et al., 2009), most microbes in cave ecosystems are generally considered to be degraders of allochthonous organic material, depending on the hydrologic connectivity of the cave to the surface, and/or food for higher level organisms. It is unlikely that the micro- and meiofaunal
Based on observations of densities are high enough to support higher trophic associations. Scores >0.96 loosely correlate to genus-level sequences. The closer the score to 1.0, the more similar sequences are to each other. Fig. 5. The number of phylogenetic associations for moonmilk clones to environmental clones and cultured strains from specific habitat types that are described in the literature according to similarity (as S_ab) scores determined by RDP. Scores of 1.0 are for identical sequences. The closer the score to 1.0, the more similar sequences are to each other. Scores >0.96 loosely correlate to genus-level associations.

densities are high enough to support higher trophic levels (e.g., Simon et al., 2003; Simon et al., 2007). Based on observations of *C. servadeii* foraging on the moonmilk (Paoletti et al., 2011; Paoletti et al., 2013), we consider that the beetles feed directly on the microbial biofilms incorporated within the moonmilk structure. We suspect that the high microbial biomass within the moonmilk alone could serve as the base of a food web, but future work will need to measure the rates of microbial primary productivity specifically from the moonmilk. Further support for our hypothesis may come from the average organic C:N ratio for the moonmilk being 9.4 (±1.3) (Paoletti et al., 2011). This ratio implies that the nutritional quality of the moonmilk is potentially greater than plant-derived food, which would be ~15 to >700 depending on plant species or soil type (e.g., Snowdon et al., 2005). In general, ratios >5 indicate an abundant carbon supply but a potential reduction in nitrogen availability due to storage of microbial biomass and nitrogen remineralization by microbes, which would have a C:N ratios for the biomass of ~2-8, depending on the cell type (Anderson, 1992).

Acknowledging that phylogeny does not yield unequivocal metabolic information (e.g., Achenbach & Coates, 2000; Rodriguez-Valera, 2002), and the clone libraries were not as exhaustively sampled as possible, there is still some indication from the 16S rRNA gene sequence data that the active metabolism of microbes within the moonmilk influences the geochemistry of the potential moonmilk-based food web. Specifically, the prevalence of microbes associated with the nitrogen cycle, and the detectable concentrations of nitrate, nitrite, and ammonium in the percolating waters, further point to an active nitrogen cycle that would affect how the trophic status is interpreted from stable isotope ratio analyses (Paoletti et al., 2011). Because the C:N ratio is >5, this would also force the animals to browse and process moonmilk constantly to achieve sufficient organic N. Browsing behavior by *Cansiliella* spp. would aerate the top of the moonmilk in contact with the percolating waters, and this could not only affect the precipitation dynamics of carbonate minerals within the moonmilk, but also affect the microbial community structure and system geochemistry associated with carbon and nitrogen cycling. These results provide unique insight into the types of food webs that can develop in cave and karst habitats and offer important implications regarding the evolutionary adaptations of animals such as *Cansiliella* that exploit a hygropetric habitat.

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Incision history of Glenwood Canyon, Colorado, USA, from the uranium-series analyses of water-table speleothems

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Abstract: Uranium-series analyses of water-table-type speleothems from Glenwood Cavern and “cavelets” near the town of Glenwood Springs, Colorado, USA, yield incision rates of the Colorado River in Glenwood Canyon for the last ~1.4 My. The incision rates, calculated from dating cave mammillary and cave folia calcite situated 65 and 90 m above the Colorado River, are 174 ± 30 m/My for the last 0.46 My and 144 ± 30 m/My for the last 0.62 My, respectively. These are consistent with incision rates determined from nearby volcanic deposits. In contrast, δ234U model ages (1.39 ± 0.25 My; 1.36 ± 0.25 My; and 1.72 ± 0.25 My) from three different samples of mammillary-like subaqueous crust collected from Glenwood Cavern, 375 m above the Colorado River, yield incision rates of 271 +58/-41 m/My, 277 +61/-42 m/My, and 218 +36/-27 m/My. These data suggest a relatively fast incision rate between roughly 3 and 1 Ma. The onset of Pleistocene glaciation may have influenced this rate by increasing precipitation on the Colorado Plateau starting at 2.5 Ma. Slowing of incision just before 0.6 Ma could be related to the change in frequency of glacial cycles from 40 to 100 kyr in the middle Pleistocene. This interpretation would suggest that the cutting power of the Colorado River prior to 3 Ma was smaller. An alternative interpretation involving tectonic activity would invoke an episode of fast uplift in the Glenwood Canyon region from 3 to 1 Ma.

Keywords: incision rate; Colorado River; mammillary; uranium-series; speleothem; folia

INTRODUCTION

Certain speleothem types form close to water tables, and our previous study of these water-table-type speleothems showed paleo-water tables in eastern and western Grand Canyon that supported the interpretation for pre-Colorado River western Grand Canyon development (Polyak et al., 2008). That study supported two stages of Grand Canyon evolution: an older Western Grand Canyon that was partially cut slowly by a more localized river, and a younger Eastern Grand Canyon that was carved by the modern-day Colorado River. By the same means, here we provide an incision history of the Colorado River in Glenwood Canyon, Colorado, USA, situated on the northeastern edge of the Colorado Plateau. Previous results based on volcanic rock data indicate an incision history for Glenwood Canyon that goes back 10 Ma (Bryant et al., 2002; Kunk et al., 2002; Aslan et al., 2010). These studies give conflicting results on whether the incision rates were stable (steady state) or dynamic (varying with time). Our speleothem-based results help resolve the conflict. Here, on the basis of radiometric ages of pre-existing basalt and ash, and supported by new speleothem uranium-series ages we propose an incision history that favors the dynamic interplay between tectonic uplift and climate-driven acceleration of incision.

Water-table-type speleothems used in this study, and in our previous work in Grand Canyon, include mammillary coatings, folia, cave rafts, and gypsum rings (Hill & Forti, 1997; Davis, 2012). In both of these study areas these are found in intimate association with each other in settings not associated with vadose dripstones and flowstones in caves and “cavelets” (caves too small for human entry) that have formed by hypogene speleogenesis, a process described by Klimchouk (2007) and Palmer (1991, 2007). In other words, these speleothem types are found in contexts that make them relevant to the dating of water-table levels.
Two sites along the Colorado River in Glenwood Canyon are Glenwood Cavern (375 m above the river) and surface-exposed “cavelets” near the city of Glenwood Springs (65 and 90 m above the Colorado River). Uranium-series (U-series) dating of samples from these sites has produced a record of incision that goes back about 1.4 My.

Glenwood Canyon and Glenwood Cavern

Glenwood Canyon is located in west-central Colorado where the south end of the broad, elongate dome of the White River uplift is intersected by the southwest-flowing Colorado River (Fig. 1) (Kirkham et al., 1996). Downcutting by the Colorado River incised Glenwood Canyon into the southern part of the uplift, exposing progressively older Paleozoic and Precambrian rocks toward its core. The west side of the White River uplift is bounded by the Grand Hogback monocline (Fig. 1), which marks the east edge of the Piceance basin and is also the boundary between the Rocky Mountain orogenic province and the Colorado Plateau. West of the city of Glenwood Springs, the Colorado River flows obliquely through the Grand Hogback monocline, cutting across progressively younger strata toward the Piceance basin to the west (Fig. 1), where Eocene strata are exposed. The Colorado flows into Utah where it is joined by the Green River, then continues southwestward through the Grand Canyon to the Gulf of California.

Iron Mountain, north of the town of Glenwood Springs, is a prominent topographic feature comprised of south-dipping, mostly pre-Pennsylvanian carbonate and clastic strata resting on Precambrian crystalline rocks. The dip slope on the south side of Iron Mountain is composed of Leadville Limestone and contains numerous caves, including Glenwood Cavern (Fig. 1). In this area, the Leadville Limestone is a 61-m-thick unit composed of gray limestone with abundant chert nodules in the lower part (Kirkham et al., 1997). In Iron Mountain, it is locally altered to hydrothermal dolomite (Inden & Humphrey, 2002; DuChene, 2011). The upper surface of the Leadville is an unconformity overlain by gray to black shale and altered to hydrothermal dolomite (Inden & Humphrey, 2002). The Leadville Limestone in Glenwood Canyon rests unconformably on the Gilman Sandstone conglomerate of the Pennsylvanian Belden Formation. The upper surface of the Leadville Limestone is an unconformity overlain by gray to black shale and is an underformation by the Grand Hogback monocline (Fig. 1), which marks the east edge of the Piceance basin and is also the boundary between the Rocky Mountain orogenic province and the Colorado Plateau. West of the city of Glenwood Springs (2,156 m (7,073 ft) elevation, 399 m (1,309 ft) above the Colorado River; this process is probably active at present in younger cavities at the base level. There are multiple hot springs and six seepage areas along the Colorado River on the north side of Glenwood Springs. These springs and seepage areas cumulatively discharge hot water at a rate that ranges from 15–19 m²/sec. Springs issue from either the Leadville Limestone or from overlying alluvium. The springs are supplied by water migrating up through fractures and faults and emerging from the Leadville Limestone (Barrett & Pearl, 1978; Golden, 1989, page 20-21). Water temperatures range from 44° to 47°C (Gelden, 1989, p 71). Water from two test wells on the north side of Glenwood Springs near the Colorado River contains dissolved H₂S ranging from 1.2 to 2.1 mg/L. A CO₂ concentration of 110 mg/L was reported in water from one of these wells (Gelden, 1989, Table 3, p. 32-33). Glenwood Cavern (formerly Fairy Cave) is developed in the Leadville Limestone near the top of Iron Mountain (Fig. 1). The historic Fairy Cave entrance is near the top of the mountain and the highest point in the known cave is at 2,156 m (7,073 ft) elevation, 399 m (1,309 ft) above the Colorado River. The cavern has 4.83 km (3.0 mi) of known passage and vertical relief of 84.7 m (278 ft) (Anderson & Barton, 2002). The Leadville Limestone in Glenwood Cavern dips 40° southwest (Kirkham et al., 1997). The cavern is of phreatic origin, formed primarily by dissolution of calcium carbonate by carbonic acid, but with a late-stage secondary overprint from sulfuric acid dissolution. Most of the limestone dissolution took place in rising water that contained carbonic acid from dissolved CO₂, as well as sulfuric acid from the mixture of hypogenic H₂S and O₂-rich meteoric water. Nearby examples of deeply corroded Leadville Limestone are located in quarry faces at the foot of Iron Mountain, north of the Colorado River, where joints have been enlarged by dissolution and later filled with river gravel.

While caves were enlarging at depth by calcite dissolution, much carbon dioxide was simultaneously lost to the atmosphere near the water table. This caused near-surface water to become supersaturated with respect to calcite, resulting in subaqueous deposition of calcite crusts that were mammillary-shaped and therefore referred to as mammillary layers. In the upper levels of the cave, fluctuations in water level and/or water chemistry interrupted calcite deposition, resulting in deposition of multiple stacked mammillary layers. Typical mammillaries in Glenwood Cavern range from 9 to 10 mm thick (Fig. 2).

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overlying cave atmosphere, to be reabsorbed into films of oxygenated meteoric and condensation water on gallery walls and other surfaces. Here, the H₂S was converted to sulfuric acid, which rapidly reacted with the calcite mammillaries and limestone and partially altered them to gypsum. Most of this gypsum was dissolved by water that partly filled passages and was transported out of the cave. In protected areas where there is little seepage of meteoric water, gypsum crust on passage ceilings, walls and floors is preserved (DuChene et al., 2003). This process has been documented in active sulfuric acid caves, such as Cueva de Villalobos in Tabasco, Mexico (Hose et al., 2000).

The mixing zone where most limestone dissolution occurred was at or near the water table. As the water table dropped in response to lowering of base level, the mixing zone (and zone of limestone dissolution) also dropped. Consequently, the oldest cave passages are topographically highest, and they become progressively younger with depth.

On the basis of U-series dating of wall crusts in Wind Cave, South Dakota, Ford et al. (1993) suggest that calcite deposition took place over a vertical range of roughly 70 m below the water table. However, this estimate is based on the downward increase in calcite solubility with pressure, rather than by CO₂ degassing. The mammillary crusts described in our paper show strong evidence for having formed in response to degassing of CO₂ in rising water, which would concentrate calcite deposition within a few meters of the water table.

“Cavelets” above Colorado River near Glenwood Springs

At the downriver end of Glenwood Canyon, just upstream from the town of Glenwood Springs, the Colorado River is cutting through the Leadville Limestone, where it is about 60 meters (200 ft) thick. At river level, thermal springs, issuing from the Leadville, are presently dissolving small phreatic and water-table caves such as those in the Yampah Spa Vapor Caves. Upriver, the limestone along the rims of Glenwood Canyon rises steeply in a series of cliffy southwestward-dipping blocks, offset by a series of faults. Along these faults and associated fractures are a number of such caves and associated smaller cavities, now abandoned by water flow, which increase in age upward in the canyon walls.

Our model, modified from Polyak et al. (2008), has these caves forming from water originally aggressive to calcite at moderate depths below the water table, with the water chemistry shifted to depositional near and at the water table before the water table dropped below the cavities, resulting in linings of subaqueous calcite mammillary coating below the water surface. As the water table descended through these cavities, calcite precipitated at the fluctuating water surface, accreting to the walls as small-scale calcite folia (downsloping, interleaved calcite shelves projecting up to ~2 cm out from the bedrock walls). At the same time, piles of calcite rafts accumulated. Examples of these speleothems are shown in Fig. 3.

Most of these cavities were solution pockets and crevices that never grew large enough for human entry.

![Mammillary coating in recesses preserved](image1)

**Fig. 2.** Mammillary calcite in Glenwood Cavern. a) remnant mammillary layer after partial collapse of the cave wall. The mammillary calcite coats recesses in the wall; b) mammillary coating of a vug in the artificial access tunnel to the cave (photos by V.J. Polyak).

![Cave mammillaries, folia, and rafts exposed on the surface in association with cavelets](image2)

**Fig. 3.** Cave mammillaries, folia, and rafts exposed on the surface in association with cavelets (photos by A.N. and M.V. Palmer).
before the time of speleothem growth—hence our designation as “cavelets.” However, many of them are now being exposed by cliff retreat and incision of steep gullies tributary to Glenwood Canyon. This erosion process is baring some of the previously hidden cave deposits at the present surface, where they are accessible in the partially dissected cavities. Those sampled for this study are along southwest-facing ledges of a steep ravine about 1 km northeast of Glenwood Springs and 65 to 90 m above present river level.

Incision History of Glenwood Canyon

Mechanisms that drive fluctuations in incision rates of rivers in the western United States are generally considered to be related to tectonic activity, climate change, or a blend of both (Dethier, 2001). Based on Lava Creek B ash deposition in the western United States, incision rates of rivers in the Glenwood Canyon region averaged 100 and 150 m/My over the last 0.6 My (Dethier, 2001). Dethier favored increased fluvial cutting power during the late Pleistocene due to increased glacial runoff rather than epeirogenic uplift as a way to explain these seemingly high incision rates. It has recently been suggested that mantle/crust processes (more specifically, removal of lithospheric crust below the Colorado Plateau from the margins inward), resulted in Pliocene uplift of the Plateau, explaining higher incision rates (>100 m/My) on the Plateau and volcanic activity around the margins of the Plateau during this period (Levander et al., 2011). Incision rate history of Glenwood Canyon is based on Miocene and Pliocene basalt flows and the Lava Creek B ash bed, all located near the head of Glenwood Canyon near the town of Gypsum (Fig. 1). Bryant et al. (2002) and Kunk et al. (2002) reported an incision history that covers the last 8 My based on a 7.8 Ma basalt that cap what they interpret to be Colorado River gravels on Spruce Ridge about 5 km south of Glenwood Canyon, as well as a 3.0 Ma basalt capping valley sediments on Gobbler Knob about 2 km east of the Spruce Ridge locality, and a 1.4 Ma basalt capping stream sediments on Triangle Peak towards Aspen, Colorado, near the town of Basalt (Fig. 1). The Gobbler Knob basalt is interpreted to have been deposited onto sediments close to Colorado River level. The Triangle Peak basalt is interpreted to have been deposited over sediments that were close to the level of the Roaring Fork River. These data produce a dynamic history of incision, with slow incision rates from 8 to 3 Ma (~25 m/My), and faster incision rates from 3 to 0.6 Ma (240 to 290 m/My). The Lava Creek B ash at the head of Glenwood Canyon (Dethier, 2001; Aslan et al., 2010) of 140 m/My indicates a slowing of incision over the last 0.64 My, relative to incision rates derived from the 3.0 and 1.4 Ma basalts. Aslan et al. (2010) point out that the 3.0 and 1.4 Ma basalts on Gobbler Knob Ridge and Triangle Peak are not capping actual Colorado River sediments, and seemingly favor a more static incision history (110 m/My) for the last 8 My. Independent dates in the 1-6 My interval would allow us to distinguish between the two competing models. In other parts of the Colorado Plateau, such as the Grand Canyon (Polyak et al., 2008), speleothems sensitive to water table position have provided decisive data. The caves and karst features of Glenwood Canyon contain these same speleothem types. Here we demonstrate the potential for determining incision-rate histories from these speleothem types using U-series analyses.

Cave sediments (chemical and clastic) as indicators of landscape evolution

Studies of caves are producing impressive records of river incision and landscape history. Absolute 26Al/10Be dating of cave sediments has been used in Mammoth Cave, Kentucky, USA, and in caves of the nearby Cumberland Valley in Tennessee, to record changes in base level of local rivers, and headward incision of river tributaries caused by a resistant sandstone bed (Granger et al., 2001; Anthony & Granger, 2004). A similar technique has been used to determine incision-rate histories in the Sierra Nevada Mountains of California and in the Bighorn Mountains of Wyoming and Montana, USA (Stock et al., 2004; 2006), and to reconstruct the valley incision history for Aare Valley, Switzerland (Haueuselmann et al., 2007). U/Pb dating of water-table-type speleothems was used to determine paleo-water tables in Grand Canyon, Arizona, USA (Polyak et al., 2008). Speleothems in Grand Canyon caves include mammillary calcite, folia, rafts, and gypsum rinds, which are all related to the final phases of hypogene speleogenesis. Glenwood Canyon exhibits the same suite of speleothem types.

Mammillary crusts

Of the water table type speleothems, mammillary crusts are the most essential to this type of study because they are thicker and therefore the calcite in their interior is more likely to be preserved. Preservation of calcite/aragonite is necessary to obtain the U/Pb chronology for these studies. Equally essential is the geochemical model that explains the origin of mammillary crusts and the near-water table environment in which they form.

Mammillary calcite crusts on cave walls are generally produced by the escape of CO₂ from rising groundwater at and just below the water table. The source of dissolved calcite can be anywhere along the groundwater flow path. As the water rises from depth it cools, and the solubility of calcite increases because its dissolution is an endothermic reaction. However, as the water approaches the water table, the hydrostatic pressure decreases enough to allow CO₂ degassing and calcite precipitation. Bubbling of the dissolved gas takes place when its partial pressure exceeds the static pressure of the surrounding water. The CO₂ partial pressure (pCO₂) in typical karst groundwater ranges from roughly 0.01 to 0.1 atm, but in deep groundwater values of 1.0 atm or more are possible. Spontaneous degassing of CO₂ is limited to a depth of roughly 10 m below the water table for each atmosphere of partial pressure. At typical values of pCO₂, therefore, calcite precipitation in rising groundwater is limited to a zone that extends no more than a few meters below the water table. Calcite precipitation can also be enhanced slightly by upward diffusion of dissolved CO₂ along the concentration gradient.
Calcite mammillaries deposited in this way can be considered a valid proxy for former positions of the water table. Their vertical positions along canyon walls of highly permeable carbonate rocks, as in Glenwood Canyon, should correlate closely with contemporaneous levels of fluvial entrenchment.

Globally, the most thoroughly studied cave mammillaries are those of Devils Hole, Nevada, a narrow tectonic fissure that extends at least 140 m below the water table. Over that entire depth the walls are lined with calcite mammillaries up to 40 cm thick, and one core from this deposit provided a continuous paleoclimate record beginning at 560 ka (Winograd et al., 1992). Deposition ceased at 60 ka, even though the fissure remained water-filled. Studies of additional cores have provided most of the missing 60 ka of record (Spötl & Dublyansky, 2012). Measurements by Plummer et al. (2000) show that the present groundwater has $P_{CO_2} = 0.016$ atm and is only slightly supersaturated with calcite (saturation index = +0.16 to +0.21, calculated as logion activity product/$K_{calcite}$). The $P_{CO_2}$ value is within the normal range for shallow groundwater but at least an order of magnitude lower than that of most rising hypogenic water (Palmer, 1991). Above the present water table are relict calcite mammillaries, as well as folia, flowstone, and evaporative botryoids (“cave popcorn”), which provide evidence for Pleistocene water-table fluctuations (Kolesar & Riggs, 2004). Mammillaries with such a large vertical extent are rare. However, contrary to our interpretation of those in Glenwood canyon, the mammillaries in Devils Hole appear not to have formed by rising groundwater, or that at present any rising component is minor and diluted. The Devils Hole fissure appears to be simply a window into a horizontal part of the flow system, rather than a conduit for rising high-$P_{CO_2}$ water. The Devils Hole recharge area is estimated to extend more than 100 km to the northeast, while the nearest discharge area is located about one kilometer to the southwest (Winograd et al., 1992). Thus Devils Hole does not provide a close modern analog to the Glenwood model for mammillary deposition, because its recharge is largely lateral, rather than upward as is probable for the recharge to Glenwood Canyon.

Calcite wall crusts can also be deposited in vadose cave pools above the water table. However, in contrast to phreatic mammillaries, these deposits show evidence for deposition by inflowing gravitational water, such as gravitationally-oriented stalactitic growths that have been engulfed by calcite deposited in perched pools. Vadose pool deposits also tend to be rimmed by prominent shelves of calcite that have grown inward from their shorelines along pool surfaces. Shelfstone of this type requires that water levels be stabilized by long-lived thresholds composed of sediment deposits, piles of collapse material, or barriers of chemical precipitates. Rising phreatic water seldom produces such shelves because of the relatively great amplitude and frequency of water-table fluctuations.

Folia

The presence of calcite folia in close proximity to mammillaries helps to support the water-table interpretation of the deposits analyzed for this study. All known examples of actively forming calcite folia are located at or near the surfaces of cave pools where $CO_2$ degassing is active (Hill & Forti 1996).

The origin of folia is considered to be the accretion of adherent particles at a fluctuating interface, and that interface is not strictly restricted to the water-table environment (Davis, 2012). Our setting necessitates modifying this definition to include chemical precipitation as a supplement to accretion of adherent particles as explained in Fig. 4. The folia in Glenwood Canyon, like those of Grand Canyon, are intimately associated with mammillaries, rafts, and gypsum rinds (in Glenwood Cavern). Furthermore, in hypogene speleogenesis in carbonate rocks, the folia fit the water-table environment. In Glenwood Canyon, the processes involved in forming the water-table-type speleothems are not far removed in both space and time from the Glenwood hot springs, which are an active example of the process that forms the caves and these speleothem types.

 Dating of these associated speleothem types can produce a history of paleo-water tables. This is possible if the samples are sufficiently thick, densely crystalline, and well preserved. Mammillary calcite is useful in this respect, but folia are rare and small, making them more vulnerable to alteration. The sample of folia used in this study is relatively young and still well preserved, as shown in Fig. 4. In Grand Canyon, the water-table speleothems are too old for typical U-series dating, so the U/Pb dating method was applied, in some instances along with the $^{234}U/^{238}U$ geochronometer (Polyak et al., 2008). However, our study of Glenwood Canyon incision was accomplished using U-series dating alone.

![Fig. 4. Thin section of Glenwood Canyon folia. Note the growth layering. Petrographic examination shows fibrous calcite. Dissolution of calcite in the lab revealed only trace amounts of detrital material (photo by V.J. Polyak).](image)
RESULTS

Results from "cavelets"

A sample of thick mammillary calcite located 65 m above the Colorado River was dated twice with $^{234}$U-$^{230}$Th (uranium-series) dating. See Asmerom et al. (2010) and Cheng et al. (2013) for methods and improved $^{234}$U and $^{230}$Th half-lives. The two subsample powders from this sample contained 0.5 to 1 parts per million (ppm) of uranium (U), and less than 500 parts per trillion (ppt) of thorium (Th), making the calcite very suitable for U-series dating. The ages were $357 \pm 8$, and $363 \pm 5$ ka. At 65 m ± 10 m above the river, the apparent incision rate of the Colorado River over this time period is $174 \pm 30$ m/My. A sample of folia at 90 m above the river produced a U-series age of $623 \pm 67$ -74 ka, and yielded an incision rate of $144 \pm 30$ m/My. A sample of cave rafts also at 90 m above the river yielded a U-series age of $462 \pm 23$ ka, and a maximum incision rate of $199 \pm 20$ m/My. The mammillary crust and folia are more likely linked to the paleo-water table, and incision rates generated by these agree with each other within their reported errors and show an average incision rate of Glenwood Canyon by the Colorado River of 160 m/My over the last 650,000 years.

A uranium evolution curve was generated for these data to test for open-system behavior. This test showed no obvious anomalies (Fig. 5), and the calcite of the samples chosen for U-series analyses shows no physical evidence of alteration or diagenetic effects. We avoided portions of samples that exhibit evidence of re-solution or surface hydration.

The incision rate estimated from the cave rafts is higher and just beyond the 2σ absolute error of the mammillary and folia incision rates. While cave rafts are considered in this paper to be water-table-type speleothems, they also commonly form in calcite-saturated pools of all types. Our anomalously higher incision rate from the cave raft data may indicate that they formed on vadose pools fed by infiltrating water rather than at the water table. Also, cave rafts are essentially two-dimensional speleothems and therefore more prone to alteration.

A coating of calcite with a slightly more brownish color was collected in the cavelet setting. Other than the slight color difference, the coating resembled a mammillary. For this sample we obtained two U-series ages, within the 2σ absolute error margin of each other, $148 \pm 1.4$ and $147 \pm 0.7$ ka. These ages are significantly younger than the mammillary crust and folia and suggest that this deposit is a thin flowstone or vadose pool deposit. The brownish color probably indicates soil-derived organic material in the calcite.

Results from Glenwood Cavern

Samples from Glenwood Cavern contained only 0.5 to 1.0 ppm U and too much lead (Pb) (1 ppm) for U-Pb dating of such young samples. However, U-series analyses of GC-2011-1, GC-HD1, and GC-HD2 produced $^{234}$U/$^{238}$U activity ratios (number of atoms of an isotope times its decay constant) of $1.040 \pm 0.001$, $1.044 \pm 0.001$, and $1.016 \pm 0.001$, also expressed as δ$^{234}$U in permil (%o), 40‰, 44‰, and 16‰, respectively, where $\delta^{234}$U = ($^{234}$U/$^{238}$U activity - 1) x 1000 (Cheng et al., 2000). The $^{230}$Th/$^{234}$U data indicate that the samples are too old for $^{230}$Th/$^{234}$U chronology, > ~600 ky. However, model ages can be generated using the $^{234}$U/$^{238}$U geochronometer if the initial δ$^{234}$U value can be estimated and used in the following relationship: $\delta^{234}$U$^{\text{measured}} = \delta^{234}$U$^{\text{initial}} \exp(\lambda_{234} t)$, where $\lambda_{234}$ = the decay constant for $^{234}$U, and t = years (Edwards et al, 1987). Using as endmembers the initial δ$^{234}$U of the mammillary and folia, 1766 and 3105 ‰, respectively, an age and absolute error can be calculated with the assumption that the Glenwood Cavern sample had a value similar to the more modern mammillary calcite samples from the cavelet sites. In single samples, phreatic and vadose, initial δ$^{234}$U values can show consistency over long periods of time (Ludwig et al., 1992; Woodhead et al., 2006). Our results are from different samples and sites, so a large possible variation in initial δ$^{234}$U values is needed. Using the largest variation in initial δ$^{234}$U values from the four samples yielding U-series ages, the δ$^{234}$U model age for sample GC-2011-1 is $1.39 \pm 0.1$ Ma. More conservatively, using lower and higher initial δ$^{234}$U values of 1000 and 4000 ‰ produces essentially the same age, but with greater uncertainty (1.39 ± 0.25 Ma). Applying the same procedure for GC-HD1 and GC-HD2 yields δ$^{234}$U model ages of 1.36 ± 0.25 Ma 1.72 ± 0.25 Ma. These ages are close to that reported by DuChene et al. (2003) for cave development at 1.34 to 1.69 Ma, with the assumption that these water-table speleothems formed soon after the cave did. The DuChene et al. (2003) value is obtained by projecting constant incision history based on the 3.03 My old Spruce Ridge basalt positioned 732 m above the Colorado River (Bryant et al., 2002; Kunk et al., 2002). Our mammillary-like speleothem from Glenwood Cavern, GC-2011-1, indicates incision rates of 271 +58/-41 m/My, 277 +61/-42 m/My, and 218 +36/-27 m/My, at 375 ± 20 m above the Colorado River. These compare well with the 241 m/My incision rate for the last 3.03 My obtained from the basalt dates.
Incision history of Glenwood Canyon (CO, USA), from speleothems

They are also close to the 291 m/My incision rate of the Roaring Fork River near Triangle Peak for the last 1.4 My, also based on a basal age. U-series results for this study are shown in Table 1. The concept of using these speleothem types for interpreting canyon incision is outlined in Fig. 6.

DISCUSSION

A 3 Ma basalt at Gobbler Knob, just south of Glenwood Canyon, caps a paleovalley that is interpreted to be at or very close to the ancestral Colorado River. This basalt is located 732 m above the Colorado River, and suggests an incision rate for the Colorado River in nearby Glenwood Canyon of 241 m/My for the last 3 My (Bryant et al. 2002; Kunk et al., 2002). Only 115 m above the Gobbler Knob basalt is the Spruce Ridge basalt, dated to 7.8 Ma (Bryant et al., 2002; Kunk et al., 2002). From this date, incision rates in Glenwood Canyon were interpreted to be very slow from 7.8 to 3.03 Ma (24 m/My), and then increased after 3 Ma up to at least 1.4 Ma. An incision rate of 132 m/My was reported for the Colorado River at the head of Glenwood Canyon (Dotsoro, Colorado) on the basis of the 640 ka Lava Creek B Ash (Aslan et al., 2010). The Dotsoro incision rate matches our 144 m/My almost exactly over the same time period (623 vs. 640 ka).

Aslan et al. (2010) question the accuracy of the incision history determined from the Gobbler Knob and Triangle Peak basalts, and suggest that they instead covered a valley fill that was situated at some significant height above the Colorado River at 3 Ma and Roaring Fork River at 1.4 Ma. They emphasize the fact that the Spruce Ridge basalt and other older basalts overlie what are interpreted to be actual Colorado River sediments, and propose that incision by the Colorado River could have been mostly steady at an approximate rate of 120 m/My over the last 8 or 10 My. However, our Glenwood Cavern SC-2011-1 model age produces an incision rate that is essentially the same as the incision rate generated by the Gobbler Knob and Triangle Peak basalts, and therefore supports the interpretations presented by Bryant et al. (2002) and Kunk et al. (2002). The Glenwood Cavern SC-2011-1 mammillary calcite would need to be 2 My older to fit the static (more steady-state entrenchment) model, and the 16-44 % values of the 623U indicates that the ages of the mammillary calcite samples are less than 2.5 Ma.

Our speleothem-based incision history of Glenwood Canyon, combined with results of other researchers, point to a dynamic rather than static incision-rate history of Glenwood Canyon. The results shown in Fig. 7 suggest that incision was faster between 3 and 0.6 Ma, and slowed with time. Such an increase in incision rate could be produced by accelerated regional uplift, and/or by climatic changes, either of which would affect the erosional power of the Colorado River.

A stage of similarly fast incision in eastern Grand Canyon for approximately the same time period, but not for western Grand Canyon (Polyak et al., 2008) would seem to support tectonic uplift as the cause of

Table 1. Glenwood Canyon speleothem U-series data.

<table>
<thead>
<tr>
<th>Sample</th>
<th>238U (ppb)</th>
<th>232Th (ppb)</th>
<th>238U/232Th activity ratio</th>
<th>230Th/238U activity ratio</th>
<th>measured</th>
<th>initial</th>
<th>δ234U</th>
<th>corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>cavelet flowstone 1a</td>
<td>4339.4 ± 10.7</td>
<td>317 ± 53</td>
<td>41134 ± 10759</td>
<td>0.984 ± 0.004</td>
<td>273 ± 3</td>
<td>416 ± 5</td>
<td>0.148 ± 0.001</td>
<td>-0.001</td>
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<tr>
<td>cavelet flowstone 1b</td>
<td>4443.9 ± 5.2</td>
<td>313 ± 42</td>
<td>42955 ± 6812</td>
<td>0.988 ± 0.002</td>
<td>262 ± 1</td>
<td>428 ± 2</td>
<td>0.147 ± 0.001</td>
<td>-0.001</td>
</tr>
<tr>
<td>cavelet mammillary 1a</td>
<td>1083.6 ± 2.5</td>
<td>435 ± 35</td>
<td>12860 ± 1023</td>
<td>1.720 ± 0.007</td>
<td>586 ± 1</td>
<td>1605 ± 36</td>
<td>0.357 ± 0.008</td>
<td>-0.007</td>
</tr>
<tr>
<td>cavelet mammillary 1b</td>
<td>560.7 ± 0.3</td>
<td>62 ± 39</td>
<td>49711 ± 31550</td>
<td>1.703 ± 0.004</td>
<td>638 ± 2</td>
<td>1776 ± 24</td>
<td>0.363 ± 0.005</td>
<td>-0.005</td>
</tr>
<tr>
<td>cavelet folia</td>
<td>671.6 ± 1.8</td>
<td>12158 ± 56</td>
<td>296 ± 2</td>
<td>1.755 ± 0.006</td>
<td>535 ± 2</td>
<td>3103 ± 645</td>
<td>0.623 ± 0.067</td>
<td>-0.047</td>
</tr>
<tr>
<td>cavelet rafts</td>
<td>647.3 ± 1.7</td>
<td>18616 ± 56</td>
<td>123 ± 0</td>
<td>1.160 ± 0.004</td>
<td>121 ± 1</td>
<td>462 ± 32</td>
<td>0.452 ± 0.023</td>
<td>-0.020</td>
</tr>
<tr>
<td>Glenwood Caverns mam1</td>
<td>11357 ± 1.2</td>
<td>514 ± 143</td>
<td>7175 ± 1998</td>
<td>1.062 ± 0.002</td>
<td>40 ± 1</td>
<td>model age = 1.39 ± 0.25</td>
<td>0.452 ± 0.023</td>
<td>-0.020</td>
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<tr>
<td>Glenwood Caverns mam HD1</td>
<td>578.8 ± 0.3</td>
<td>394 ± 47</td>
<td>4794 ± 577</td>
<td>1.067 ± 0.001</td>
<td>44 ± 1</td>
<td>model age = 1.36 ± 0.25</td>
<td>0.452 ± 0.023</td>
<td>-0.020</td>
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<tr>
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<td>59452 ± 67531</td>
<td>1.032 ± 0.001</td>
<td>15 ± 1</td>
<td>model age = 1.72 ± 0.25</td>
<td>0.452 ± 0.023</td>
<td>-0.020</td>
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Corrected ages use a calculated initial 230Th/238U atomic ratio 4.4 ppm ± 50%. Million years before present = Ma, where present is AD 2011. All errors are absolute 2σ. Subsample sizes range from 30 to 140 mg. Model age = δ234U model age.
the increased incision rates (Karlstrom et al., 2008). Perhaps the eastern Grand Canyon region and the Glenwood Canyon region experienced a phase of accelerated uplift between 3 and 1 Ma, similar to that of the Pliocene uplift of the Colorado Plateau proposed by Levander et al. (2011). If so, our evidence would suggest that the uplift was temporally and spatially non-uniform during the latest Pliocene and Pleistocene.

The onset of the Pleistocene at 2.5 Ma (Clark et al., 1999) may also have caused an increase in cutting power of the Colorado River via increased precipitation in the Colorado Plateau region, in combination with the descent of hydrologic base levels due to lower sea levels. The slowing of incision before 0.65 Ma could be due to the change in ice age frequency during the Middle Pleistocene at ~0.8 Ma, from 40-kyr glacial cycles before 0.8 Ma, to 100-kyr glacial cycles after 0.8 Ma (Clark et al., 1999). Glacially controlled incision rates for the Green River area, Kentucky, USA (Granger et al., 2001) and for the Aare Valley, Switzerland (Haueselmann et al., 2007) have been derived from analysis of cave sediment. These studies show that glacially induced changes in incision rate show considerable regional variation.

For the western United States, the onset of the Pleistocene alone could have triggered faster incision due to lowering of the hydrologic base via lower sea level. But this seems less likely in our study area because Glenwood Canyon is located far upstream along the Colorado River. A climate-driven explanation of faster incision at 3 to 1 Ma would tie the 40-kyr glacial cycle to increased effective precipitation on the Colorado Plateau. Furthermore, the slowing of incision could have been caused by the onset of the 100-kyr glacial cycle that pushed the storm track south of the study area, resulting in a slightly drier climate over the last 0.8 Ma. Another possible cause for decreased incision rate after 0.8 Ma could be that knickzones triggered by the onset of Pleistocene glaciation migrated through Glenwood Canyon before 0.65 Ma, and were diffused in softer sediments in the Eagle Collapse Center (DuChene et al., 2003) upstream from Glenwood Canyon.

**CONCLUSION**

The U-series chronology of water-table-type speleothems from caves and cavelets in Glenwood Canyon, Colorado, provides an incision history of the canyon by the Colorado River over the last 1.5 My. The speleothem-based incision rates match previously published rates derived from the Spruce Ridge, Gobbler Knob, and Triangle Peak basalts and the Lava Creek B ash. These data support a non-steady state history of incision in Glenwood Canyon. Tectonic mechanisms for faster incision from 3 to 0.65 Ma would invoke faster uplift of the region during the Pleistocene compared to the Pliocene. While climatic mechanisms would suggest that the onset of the Pleistocene ice ages was responsible for faster incision rates of the Colorado River in Glenwood Canyon, and that incision has slowed down since the middle Pleistocene.

**ACKNOWLEDGEMENTS**

We thank Ted Lappin for GPS coordinates at the cavelet sites near Glenwood Springs, Colorado. We are grateful to Paula Provenocio for helpful discussions. We especially thank Steve and Jeanne Beckley, owners of Glenwood Caverns Adventure Park, for permission to collect samples for this research. This research was made possible by NSF grants EAR-0518602 and EAR-0326902. Comments by reviewers David A. Richards, Stein-Erik Lauritzen, and an anonymous reviewer helped improve our manuscript.

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Spatial and temporal changes in invertebrate assemblage structure from the entrance to deep-cave zone of a temperate marble cave

Benjamin W. Tobin*, Benjamin T. Hutchins, and Benjamin F. Schwartz

Abstract: Seasonality in surface weather results in seasonal temperature and humidity changes in caves. Ecological and physiological differences among trogloxenes, troglophiles, and troglobionts result in species-dependent responses to this variability. To investigate these responses, we conducted five biological inventories in a marble cave in the Sierra Nevada Range, California, USA between May and December 2010. The cave was divided into six quadrats and temperature was continuously logged in each (humidity was logged at the entrance and in the deep cave). With increasing distance from the entrance, temperature changes were increasingly attenuated and lagged relative to surface temperature. Linear regressions were created to determine the relationship between measured environmental variables and diversity for cavernicoles (troglobionts and troglophiles) and trogloxenes cave-wide and in the transition zone. Diversity for cavernicoles and trogloxenes peaked in the entrance and deep cave zones, respectively. Quadrat, date, 2-week antecedent temperature average, 2-week antecedent temperature range, and trogloxene abundance explained 76% of cavernicole diversity variability. Quadrat explained 55% of trogloxene diversity variability. In the transition zone, trogloxene abundance explained 26% of cavernicole variability and 2-week antecedent temperature and 2-week antecedent temperature range explained 40% of trogloxene variability. In the transition zone, trogloxene diversity was inversely related to 2-week antecedent temperature average and 2-week antecedent temperature range, suggesting that species were moving into the transition zone when temperature was most stable. In a CCA of cavernicoles distribution data and environmental variables, 35% of variation in species-specific distributions was attributable to quadrat, and non-significant percentages were explained by date and environmental variables. Differences in assemblage structure among quadrats were largely due to differences between distributions of trogloxenes and cavernicoles, but responses varied among species. Differences are likely due to ecological niche width, physiological constraints, and competition.

Keywords: cave climate; troglobiont; troglophile; Sequoia National Park, California, USA; community response; species interaction

INTRODUCTION

Spatial heterogeneity in the distribution of species at local scales within caves and other subterranean habitats has long been documented (Racoviță, 2006). This heterogeneity has been attributed to resource availability (Barr & Kuehne, 1971; Poulson, 2005), microclimate variability (Barr & Kuehne, 1971; Howarth, 1980; Culver, 2005a; Pipan et al., 2006), invasion history (Fong & Culver, 1994), and species interactions (Culver, 1970, 2005b; Culver et al., 1991). However, despite a shift away from the old paradigm that subterranean habitats are temporally stable, little research has focused on quantifying and explaining temporal variability in the distribution of invertebrates found in caves. In most, if not all caves, seasonality on the surface affects the velocity, direction, and daily oscillations of barometric and chimney-effect airflow, resulting in seasonal trends in cave humidity and temperature (Howarth, 1980; Cigna, 2002). Cave morphology, vertical position of entrance/s relative to the...
rest of the cave, and changing surface conditions create seasonal drying near an entrance when air flows into the cave and a seasonal increase in humidity near an entrance when air flows out of the cave. Variability in these seasonal patterns is typically reduced deeper into the cave.

Based on environmental conditions and environmental variability, cave habitats can be divided into three primary zones: 1) an entrance area that is climatically variable and strongly affected by changes in the surface environment, 2) a deep cave zone that is characterized by relatively stable temperature, and humidity at or near saturation, and 3) a transition zone that seasonally displays characteristics of either the entrance or deep cave zone. The spatial extents of these zones vary among caves and are primarily determined by passage morphology and airflow patterns.

Troglobionts (obligate subterranean species) are typically assumed to be stenothermic (adapted to a narrow range of temperatures) and stenohygrobic (restricted to areas with nearly 100% humidity) (Barr & Kuehne, 1971; Howarth, 1980), probably because of thinner exoskeletons and longer, attenuated appendages which result in higher rates of desiccation compared to surface taxa (Howarth, 1980). Consequently, troglobionts often occur in greater numbers in the deep cave zone because of higher and more stable humidity relative to other zones (Peck, 1976), and Barr & Kuehne (1971) observed that troglobionts are largely absent from drier portions of Mammoth Cave in Kentucky under sandstone caprock and near entrances. However, caves represent only one component of a largely inaccessible but potentially extensive subterranean habitat that includes shallow subterranean habitats (SSH) (Juberthie et al., 1980), and Novak et al. (2012) suggest that there are two largely distinct groups of troglobionts; one specializing in SSH, primarily within 10 m of the surface, and one in deeper cave habitats. This explains bimodal distributions of troglobiont abundance that peak near the entrance (the SSH fauna) and in the deep cave environment. Relative to troglobionts, the distribution of trogloxenes (occasional and temporary residents of caves) and troglophiles (non-obligate cave inhabitants) has been less well studied by biospeleologists. A number of studies have shown that the distribution and composition of guanobiont communities (comprised of troglobionts, troglophiles, and trogloxenes) is influenced by the size of guano piles, distance from the entrance (influencing guano quantity and quality and colonization), and guano pile microclimate (Ferriera & Martins, 1998; Ferriera et al., 2000; Bahia & Ferriera, 2005; Ferriera et al., 2007). Because of constraints imposed by environmental conditions, nutrient availability and competition with troglobionts, troglophiles typically decrease in abundance with increasing distances into a cave, although both troglobionts and troglophiles may occur in all parts of a cave system (Sendra et al., 2012).

Cave transition zones are influenced by both deep cave and surface climates and are characterized by seasonal movement of a temperature and/or high-humidity front that changes the extent and location of favorable habitat for troglobionts, troglophiles, and trogloxenes, consequently affecting interactions between these groups. While other studies have focused on species responses to nutrient quality and availability, to our knowledge, movement of species within caves in response to seasonally changing environmental conditions has not been assessed. Variability in temperature and troglobiont distribution has been studied, however, in the milieu souterrain superficiel (MSS): the interconnected subterranean spaces between soil and loose rock in scree and talus. Crouau-Roy et al. (1992) showed that both temperature and abundance of troglobionts showed strong seasonal cycles in an MSS in the Pyrenees. However, daily oscillations in temperature were not correlated to changes in troglobiont species richness in the MSS in a lava field in the Canary Islands (Pipan et al., 2010).

We expected that troglobionts and troglophiles (collectively referred to as cavernicoles) move within caves in response to the movement of a temperature variability and humidity front in the cave because of the physiological limitations discussed above. This behavior would be evident in a correlation between temporal changes in the longitudinal distribution of cavernicoles, and temporal changes in longitudinal patterns of cave temperature and humidity. In contrast, while it is likely that trogloxenes also display a preference for optimal environmental conditions, their ability to move within the subterranean environment in response to changing environmental conditions may be limited by some other aspect of the cave environment (e.g. distance from an entrance, darkness, low nutrient availability, competition with cavernicoles) because of a lack of troglobomorphic adaptations. Consequently, we predicted that the presence of trogloxenes would decrease with increasing distance from the cave entrance as has previously been noted (Racoviţă, 2006), although seasonal changes in the distribution of trogloxenes, corresponding with seasonal changes in the distribution of cavernicoles could indicate competition between these two ecological groups.

An understanding of community responses to in-cave climate seasonality (which is dependent on surface environmental conditions) is essential for: 1) understanding how these systems will respond to climate change and environmental variability, and 2) improving management of rare and vulnerable species. Ecosystems, such as caves, which are characterized by high levels of endemism, small, isolated patches of habitat, and species that are intolerant of climatic variation are especially vulnerable to rapidly changing climate. Consequently, the objectives of this research were to: 1) quantify the seasonal variability of in-cave climate from the entrance to the deep zone in Clough Cave in the Sierra Nevada, California, USA, and 2) document whether the distributions and the movements of both cavernicoles and trogloxenes are affected by in-cave climate variability.

METHODS

Site description

Clough Cave is a 140 m long cave formed in a marble cliff 40 m above the Kaweah River at an elevation of 1000 masl in Sequoia and Kings Canyon National Parks (SEKI) (Fig. 1). This region has a Mediterranean climate marked by extreme seasonality in temperature and humidity, with low temperatures (<0° C) and high precipitation (700 mm) in winter (November – April) and seasonal drought and high temperatures (>40° C) in summer.
The main passage in Clough Cave is a highly decorated 2-4 m diameter abandoned stream meander passage (Fig. 1). A small crawlway side passage loop and an upper level passage at the back of the cave were not included in this study to reduce any potential effects that different passage morphologies might have on climate and species data. For this research, the main passage was divided into six approximately equal quadrats, beginning near the entrance and extending to the farthest reaches of the main passage (Fig. 1). Quadrats were assigned to either the entrance, transition, or deep cave zone based on physical characteristics (see results).

Clough Cave was chosen as a research site for multiple reasons. First, it is one of the most biologically diverse caves within SEKI, with over 50 documented invertebrate species (Krejca, 2006), including at least 10 cavernicoles. Secondly, the simple morphology of the cave provides an ideal study location with few confounding variables (e.g. multiple entrances, large sections of unfavorable habitat, etc.) that could obfuscate climatic effects on species distributions. Food sources for primary consumers appear to be evenly distributed throughout the cave, largely consisting of root mats from overlying trees and, to a lesser extent, vertebrate and invertebrate guano. Microhabitats such as breakdown, fractured bedrock, and wall habitat are also distributed evenly throughout the cave. Finally, as a small cave, it is similar to many caves world-wide, and results should be generalizable to other caves.

Cave climate data collection

In-cave temperature was recorded using Onset TidbiT® temperature loggers in quadrats 1 – 5, hidden from view to avoid theft. Additionally, Onset Hobo® temperature and humidity loggers were placed in the entrance (before quadrat 1) and in quadrat 6 (Fig. 1). Data were collected at 15 minute intervals. Temperature data were converted into four variables: daily temperature average, daily temperature range, 2-week antecedent temperature average (prior to each biological inventory), and 2-week antecedent temperature range. Temperature ranges were used rather than standard deviation because physiological functional constraints (including death) can occur at extreme temperature events, even if infrequent (Pörtner & Ferrell, 2008). Furthermore, temperature range has been used as a measure of environmental stability for predicting species diversity (Owen et al., 1989).

A linear relationship between temperature and relative humidity was established using data collected at the cave entrance. Surface temperature is highly negatively correlated to relative humidity and we assume that a similar relationship exists within the transition zone, due to influence from the surface environment. However, because of a lack of instrumentation, humidity data were not collected in quadrats 1-5, so we were unable to use humidity in statistical analyses.

Biological inventories

Between 10 May and 27 December 2010, five biologic inventories were conducted in Clough Cave. This time period spanned transitions from wet to dry seasons and back, and overlapped the period of highest variability in cave temperature and humidity. Each inventory consisted of 30 person-minutes of visual searches in each quadrat. Invertebrates were enumerated and identified to the lowest possible taxonomic level using picture keys derived from an existing species list for the cave (Krejca, 2006). To reduce potential researcher bias, inventories were conducted by the same people whenever possible. Although this method does not necessarily capture all species, it has been established as an acceptable monitoring method (Culver & Sket, 2002) and is in line with conservation ethics of the United States National Park Service, minimizing disturbance to the ecosystem.

Statistical analyses

For each quadrat and sampling date, diversity (calculated using the Shannon-Weaver index, H’), abundance, and species richness (S) were calculated separately for cavernicoles and troglobiones. Community-wide and species-specific responses were analyzed using multiple linear regression and canonical correspondence analysis, respectively. Cavernicole and troglobionte H’ were used as response variables, with cave quadrat, date, 2-week antecedent temperature average (antT), and 2-week antecedent temperature range (antTr) used as predictor variables. Additionally, cavernicole and troglobionte abundances were used as predictors for troglobionte and cavernicole diversity, respectively, to assess competitive interactions. Although humidity is important, temperature was used as a proxy for humidity based on the relationship between temperature and humidity established from the data logger in the entrance of the cave.

Although H’ is sensitive to small changes in community composition (i.e. the addition of one or a
Similarly, 2-week antecedent temperature averages from 15.54° C to 17.21° C over the course of the study. Quadrat 6 was relatively stable, with temperatures varying at the entrance varied from 6.8° C to 22.52 ° C. In contrast, and dry (May to October) seasons.

Climate data illustrate two major patterns: 1) with strong, negative correlation (r²= 0.88; Fig. 4). Humidity in quadrat 6 remained constant at over 100% over the course of the study, although this may be due, at least in part, to condensation forming on the logger. This nevertheless indicates a small humidity range that never dropped low enough to evaporate water that condensed on the logger. In addition to the humidity data collected in the entrance and quadrat 6, we qualitatively observed a drying front in the sediments in the cave that moved progressively further into the cave during the dry season and then back towards the entrance during the wet season. This drying front

RESULTS

Climate data

Data were log or log-square-root transformed for normality, if necessary, and sampling date was centered to reduce potential multicolinearity. Quadrat was modeled as a random categorical variable using five dummy variables. Interactions among predictor variables were not included in any models. Akaike information criterion (AIC) analysis for finite sample sizes was then performed to select the most likely model. Cut-off for significance was set at α=0.95 and corresponding p-values were calculated using the false discovery rate algorithm of Benjamini & Hochberg (1995) and Narum (2006) to account for multiple hypothesis testing. Regressions and post hoc tests of differences among groups (Tukeys HSD) were run in R (R Development Core Team, 2012).

Canonical correspondence analysis (CCA) was used to assess species-specific cavernicole distributions in response to the measured environmental variables. Taxa that comprised less than 1% of the total abundance of cavernicoles collected on all sampling dates were removed from analysis. Additionally, abundance data were log transformed and the effect of rare species was down-weighted during ordination. Predictor variables were the same as those used in linear regressions and were partitioned into date, quadrat, and environmental effects. Significance of pure effects was determined using a Monte Carlo test (9999 permutations). CCA was performed using Canoco 4.5 (Braak & Šmilauer, 2002).
Spatial and temporal changes in invertebrate assemblage structure

It appears to be related to increased temperature in quadrats 1–3, suggesting that temperature variability in these quadrats was high enough that humidity was periodically reduced to below saturation. These data suggest that temperature range may act as a proxy for humidity in the cave although there is not enough data to quantify this relationship.

Temperature data and proximity to the entrance were used to designate entrance, deep, or transitional zones. Quadrat 1 was assigned to the entrance zone because it is in the twilight zone and shows temperature and humidity fluctuations that are synchronous with the surface. Quadrats 2, 3, 4, and 5 were assigned to the transition zone because they are in the dark zone and, with the exception of quadrat 3, they show temperature profiles that suggest that they are influenced, at least seasonally, by surface temperatures. The lack of a similar pattern in quadrat 3 is probably due to its placement in an alcove with a stable microclimate than does not reflect the typical environmental pattern within the quadrat. Quadrat 6 was assigned to the deep zone because it is in the dark zone and displays relatively stable temperature profiles that are asynchronous with the surface.

Faunal distribution patterns

Flooding in the cave during the 27 December 2010 sampling event reduced the amount of searchable area by approximately 50%. As a result, trogloxene richness, cavernicole richness, and abundances were all significantly lower for this sampling date (results of MANOVA, data not shown). Because data from this date were not representative of species’ responses to temperature or humidity, they were excluded from analyses. Excluding the December data, 1117 individuals belonging to 31 taxa were recorded during biological inventories (Table 2, available online at http://dx.doi.org/10.5038/1827-806X.42.3.4). Seven taxa were represented by a single individual, and an additional 6 were represented by 2 or 3 individuals. Two taxa, the collembolan Tomocerus celsus and an unidentified pscopteran made up 68% of the total abundance. Chelicerates (spiders, mites, scorpions, and harvestmen) were an especially diverse component of the fauna, represented by 14 taxa comprising 19% of the total abundance (Table 2). The collembolan Tomocerus celsus was recorded from every quadrat during every sampling event, with one exception. Other cavernicoles only rarely occurred outside of quadrats 5 or 6 (e.g. the spiders Yorima sp. and Usofila sp., and the scorpion Uroctonus mordax sequoiae). Conversely, several species of trogloxenes are largely restricted to the entrance zone (e.g. Titiotus sp., Nesticus silvestrii, Arcuphantes sp., and Cybeaus sp.). The two subspecies of scorpion, Uroctonus mordax mordax and U. mordax sequoiae appear to be restricted to the entrance and deep cave zone, respectively. Two species, the isopod Brackenridgia sp. and the harvestman Calicina cloughensis appeared to have a bimodal distribution, occurring most frequently in quadrats 1 and 6, but rarely, if ever in the transition zone.

In general, trogloxenes and cavernicoles were present in similar numbers (average abundance per quadrat per sampling event = 19.7 and 17.6, respectively), although average trogloxene species richness (3.5...
spp.) was slightly higher than average cavernicole species richness (2.2 spp.). Variability among samples was also greater for trogloxenes (standard deviation of abundance = 37.7 versus 15.1 for cavernicoles) (Table 1). For both trogloxenes and cavernicoles, S and H’ were highly correlated (r² = 0.79 and 0.88, respectively), although no correlation between abundance and H’ was observed (r² = 0.09 and 0.02, respectively). Trogloxene diversity was typically highest in the entrance zone (quadrat 1), and to a lesser extent, in the deep cave zone, whereas cavernicole diversity was typically highest in the deep cave zone and, to a lesser extent, in the entrance zone. Transition zone quadrats had lower overall diversity and abundance values than quadrats near the entrance or back of the cave, although this pattern was more pronounced in cavernicoles than in trogloxenes. Cavernicole diversity and species richness appear to peak in the summer dry season for quadrats 1-3. This temporal pattern is not apparent in quadrats 4-6 (Table 1). Trogloxene diversity and species richness appear to decrease over the course of the summer dry season, although the pattern is not apparent in quadrats 3 and 6 (Table 1).

Regression analysis
To test for a relationship between diversity (H’) and climatic variables, 9 linear regression models containing between 1 and 5 predictor variables were run separately for cavernicoles and trogloxenes (Table 3). For cavernicoles, AIC suggested that, of the evaluated models, the full model (quadrat + date + 2-week antecedent temperature average + 2-week antecedent temperature range + log (trogloxenes abundance)) had a 57% chance of being the most likely model and was 2.0 times more likely than the next best model, although the quadrat + log (trogloxenes abundance) model was a competing model, with a 29% chance of being most likely. The full model explained 76% of the variance in diversity but included three non-significant parameters: intercept p = 0.09, quadrat 2 p = 0.58, and 2-week antecedent temperature average p = 0.06. The quadrat + log (trogloxene abundance) model explained
63% of the variability. For trogloxenes, AIC suggested that of the evaluated models, the quadrat model had a 69% chance of being the most likely, was 4.6 times more likely than the next best model, and explained 55% of the variance in diversity.

For both cavernicoles and trogloxenes, $H'$ was significantly affected by quadrat ($p = 0.04$ & $0.01$, respectively) and, contrary to our expectations, cavernicole $H'$ was positively correlated with trogloxene abundance ($p = 0.04$). Regressions predict diversity equally well for all sampling dates and quadrats (Fig. 5a-d). For cavernicoles, Tukeys HSD post hoc test revealed that quadrant 6 had significantly higher $H'$ values than quadrants 2-5 ($p \leq 0.05$), but not quadrant 1 ($p = 0.132$). For trogloxenes, Tukeys HSD post hoc test revealed that quadrant 1 had significantly higher diversity than all other quadrants, with the exception of quadrant 2. Quadrant 2 also had significantly higher diversity than the transition quadrant 5.

To investigate changes in diversity within the transition zone, linear regressions were run for cavernicoles and trogloxenes in quadrats 2-5 (Table 3). For cavernicoles in quadrats 2-5, AIC suggested that of the evaluated models, the date + log (trogloxene abundance) model and the log(trogloxene abundance) model were competing, having 29% and 56% chances of being the most likely.

### Table 3. Alternative linear regression models assessed using Akaike Information Criterion corrected for finite sample sizes (AICc). Only predictor variables are listed. The response variable is Shannon’s diversity index: $H'$ from Table 1. $k =$ the number of parameters included, $\Delta =$ AICc – AICc

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<th>$df$</th>
<th>$p$</th>
<th>AIC</th>
<th>AICc</th>
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<td>27.48</td>
<td>28.41</td>
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</table>
models, respectively. The log (trogloxene abundance) model was 1.9 times more likely than the date + log (trogloxene abundance) model. The two models explained 26% and 27% of the variance in diversity, respectively, although in the date + log (trogloxene abundance) model, the coefficient for date was not significant (p = 0.298). For trogloxenes in quadrats 2-5, AIC suggested that of the evaluated models, the 2-week antecedent temperature average + 2-week antecedent temperature range model and the 2-week antecedent temperature average model were competing, having 51% and 25% chances of being the most likely models, respectively. The 2-week antecedent temperature average + 2-week antecedent temperature range model was 2.1 times more likely than the 2-week antecedent temperature average model. The two models explained 40% and 28% of variance in diversity, respectively. Tukey’s HSD post hoc test did not reveal any significant pairwise differences in trogloxene H’ among quadrats. As in the models evaluating all quadrats, cavernicole H’ was positively correlated with trogloxene abundance in the transition zone models. Unlike in the all quadrats models, however, trogloxene H’ in the transition zone was positively correlated with 2-week antecedent temperature average.

**Canonical correspondence analysis of cavernicoles and environmental variables**

In a CCA of species distributions and environmental variables, 58.7% of variance in cavernicole distribution was explained by all canonical axes (F = 1.554, p = 0.0121) (Fig. 6). The first four canonical axes alone explained 49.7% of variance in cavernicole distribution. Explained variance was partitioned into quadrat, environment, date, and interaction effects. Quadrat alone explained 35% of variance in cavernicole distribution (F=2.058, p=0.003), date alone explained 11.5% (F = 0.113, p=0.344), and environment (temperature variables and trogloxene abundance) explained 11.1% (F=1.077, p=0.378). The remaining 1.1% was attributable.
to secondary and tertiary (interaction) effects. For five cavernicoles (Amplura sp., Tomocerus celsius, Brackenridgia sp., Usofila sp., and Yortina sp.), over 50% of variance in abundance was explained by the first four canonical axes. Axes primarily explained differences in community structure due to quadrat effect. However, weaker gradients explained by trogloxene abundance and date were also illustrated.

**DISCUSSION**

Caves and other subterranean habitats are not stable environments as is often assumed, but exhibit seasonal fluctuations in temperature and humidity that reflect a delayed response to changing climate on the surface. High frequency, continuous temperature monitoring showed that environmental conditions are most variable in the entrance and, with increasing distance into the cave, become increasingly stable and temporally lagged relative to surface conditions.

During the winter and spring wet season (beginning in late November), climate in the entrance of Clough Cave is influenced by surface conditions. Cool and humid surface air moves into the entrance and near-surface quadrats, resulting in decreased temperature, increased temperature range, and higher humidity (Fig. 4). These effects are attenuated and lagged in the deep cave, but temperatures begin to gradually decrease in deep-cave quadrats at the same time. At the end of the wet season (late May – early June) airflow direction reverses, and cave air begins to blow from the deep zone toward the entrance, raising temperatures in the cave. This, combined with more stable surface climate, leads to smaller temperature ranges (Fig. 3), although hot and dry conditions on the surface cause drying in the cave entrance.

As has been previously noted (Barr & Kuene, 1971; Peck, 1976; Howarth, 1980), troglobionts and trogloxenes are, in many cases, restricted to areas with the most stable temperature and humidity levels or, at least, to areas where temperature stability and humidity remain above some threshold. Consequently, cave-wide seasonal changes in temperature and humidity could exert major influences on cavernicoles distribution. As previously stated, humidity was not directly measured in this study, but preliminary observations suggest a negative correlation between temperature and humidity in Clough Cave.

Cavernicole distribution patterns exhibited significant cave-wide spatial and temporal trends but did not significantly respond to 2-week antecedent temperature average. Several mechanisms may explain the lack of a relationship between cavernicole diversity and temperature: 1) Vagility is so low for some species that despite intermittent periods of environmentally favorable conditions, unfavorable periods are too frequent in the transition zone to allow for colonization and persistence. 2) Favorable micro-habitats (e.g. under stones, mesovoids, etc.) were more prevalent in some quadrats, allowing the persistence of animals year-round. This could explain the higher diversity seen in quadrats 1 and 6, although no a priori physical differences among quadrats were observed. 3) Temperature and relative humidity stayed within tolerance ranges for the species detected during our sampling events. Troglobionts are often regarded as stenothermic and stenohygrotic although temperature and relative humidity tolerances have been determined for only a few species. Howarth (1980) reported 50% mortality in Hawaiian lavatube lycosid spiderlings after 15.4 days following exposure to 90% relative humidity. Bull and Mitchell (1972) reported less than 50% survivorship after 12 hr exposure to 50% relative humidity in a species of Texas cave millipede. Lencioni et al. (2010) reported lower lethal temperatures well below 0° C for two species of troglobitic leptodirin beetles from Italy although the beetles were collected from caves with mean temperatures of 9.3 and 9.8°C. The lack of correlation between trogloxene abundance and date and temperature supports our hypothesis that at least some trogloxenes are likely limited by other physiological constraints (e.g. dependence on sight, dispersal ability, etc.) and may thus be unable to migrate and/or persist with increasing distance into the cave. This is also supported by higher trogloxene diversity in quadrat 1.

Species did exhibit significant responses to temperature within the transition zone, where trogloxene H’ varied among transition zone quadrats due, in part, to differences in temperature among quadrats. Specifically, trogloxene diversity was inversely related to 2-week antecedent temperature average. The highest trogloxene H’ values from transition zone quadrats occurred in June and July, when the temperature was most stable and the atmosphere in the cave was dominated by cooler, more humid and thermally stable air from the deep cave zone.

Differences in assemblage structure among quadrats were largely due to differences between the distributional patterns of cavernicoles and trogloxenes. These differences are likely due to environmental variability within the cave. For trogloxenes, diversity was highest in the entrance, and for cavernicoles, diversity was highest in the entrance and in the deepest, most environmentally stable quadrat. The non-significant difference between cavernicole diversity in quadrat 1 and quadrat 6 may be due to a lack of power in our statistical analyses due to small sampling size. For every sampling event except July, richness and diversity of cavernicoles was higher in quadrat 6, and additional sampling may show that this trend is significant. Furthermore, several taxa analyzed in this study are currently undescribed and their ecology is unknown. Therefore, we conservatively grouped trogloxenes and troglobionts into a single ecological category (cavernicoles). Better ecological characterization of these species is needed to identify potential differences between the responses of trogloxenes and troglobionts to environmental variability in the Sierra Nevada. Peaks in cavernicole diversity in the entrance and deep cave zones parallel the observations of Novak et al. (2012) and may reflect distinct troglobiont communities (e.g. shallow epikarst, SSS, and MSS communities versus deep cave communities). In Clough Cave, diversity and
richness were lowest in the transition zone. This observation is in contrast to other systems in which diversity of the ecotone between terrestrial (dominated by surface taxa) and hypogean habitats (dominated by troglobiont taxa) was higher than in adjoining habitats (Prous et al., 2004). Correlation between $H'$ and species richness and lack of correlation between diversity and abundance suggest that changes in $H'$ reflect changes in species richness and species relative abundances, but not in absolute abundances.

It is important to note that additional cave-wide changes in community structure not related to temperature and humidity were also observed. Specifically, the December sampling event had significantly lower diversity, richness, and abundance values for cavernicoles and trogloxenes. This inventory occurred after a record precipitation event led to large changes in the distributional patterns of subterranean species (including extirpation) in response to both natural and anthropogenic changes in surface conditions have been noted in other systems, but mostly for aquatic species (Culver et al., 1992; Dole-Olivier & Marmonier, 1992; Culver & Pipan, 2009 and references therein).

The community-wide responses to spatial and temporal conditions discussed above do not apply to all species found within the cave, and some species-specific patterns are worth noting. Distributional patterns of species were non-random and responses to environmental conditions varied among species, possibly obscuring community scale patterns. For example, the ubiquitous collembola Tomocerus celsus appears to occupy a larger ecological niche than other cavernicoles: appearing in all quadrats whereas most other cavernicoles were found almost exclusively in quadrats 1, 5 or 6. The distributions of the predatory, cavernicolous spiders Yorima sp. and Usosifila sp. that occur primarily in the deep cave zone, may be limited by competition with the trogloxene spiders Titiotus sp., Nesticus silvestrii, Arcuphantes sp., and Cybeaus sp. that occur near the cave entrance. Yorima sp. and Usosifila sp. are hunting and web-building spiders, respectively, and these different predation strategies may allow them to co-exist in the deep cave zone where animal abundances are generally lower. Competitive exclusion may also limit the distribution of the two subspecies of scorpion, Uroctonus mordax mordax and U. mordax sequoiae which are a trogloxene (occurring in the near-entrance quadrats) and a cavernicole (occurring in the deep-cave quadrats), respectively. Reasons for the apparently bimodal distribution of the isopod Brackenridgia sp. and the harvestman Calcina cloughensis are unclear.

Factors other than meteorological conditions also influence the distribution of cavernicoles and trogloxenes, but these confounding factors are minimized in Clough Cave. Although not measured, nutrient availability and substrate appear to be relatively evenly distributed among quadrats, suggesting that these variables play a minor role in controlling species distribution in Clough Cave. However, the positive correlation between cavernicole richness and trogloxene abundance may suggest that both groups are responding to some unmeasured variable. The relation also suggests that competition between these groups is not controlling distributions.

**CONCLUSIONS**

Trogloxene diversity is highest near the entrance of caves, likely because of physiological limitations. Conversely, in Clough Cave, cavernicole diversity was highest in quadrat 6 where humidity and temperature were most stable and humidity is highest. Diversity was always low for cavernicoles and trogloxenes in the transition zone, even though climate in the transition zone was periodically similar to both entrance and deep cave conditions. This suggests that conditions within the transition zone are unfavorable for both groups because of environmental variability (cavernicoles), distance from the entrance (trogloxenes), or some unmeasured variable.

Climate change models for the Sierra Nevada predict an increase in temperature and minimal change in precipitation for the region (Cayan et al., 2008). Increased surface temperature is likely to cause an increase in the temperature difference between the deep cave and surface environments, increasing the length and variability of the transition zone. This in turn may further separate trogloxene- and cavernicoles-dominated communities and potentially reduce allochthonous resources in the deep cave environment. Because many cave adapted species are likely stenothermic and stenothermal, they are potentially susceptible to both seasonal and long-term climate changes that would, at least periodically, increase temperature variability and decrease humidity in cave entrances and transitions zones in temperate regions.

Our understanding of subterranean community responses to climate variability is incomplete. High frequency, long-term monitoring of community structure and inclusion of humidity data from throughout the cave is needed to detect changes that may not have been detected in this study. Additional studies should focus on 1) microhabitat (i.e. under stones, small alcoves, areas affected by drips) that may sustain favorable microclimates for stenothermic species, and 2) how cave morphology affects temperature, relative humidity, airflow patterns and the size of the transition zone. Furthermore, trogloxenes and troglophtes deserve more attention as they are an important component of the ecological community of caves. Finally, the basic ecology of all cave inhabitants needs to be better understood in order to predict both species-specific and community wide responses to environmental variability.

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Forty years of epikarst: what biology have we learned?
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Abstract: Epikarst is not only an important component of the hydrogeology of karst and an active site of speleogenesis, it is habitat for a number of species adapted to subterranean life. Water in epikarst, with a residence time of days to months, is a highly heterogeneous habitat, and the animals are primarily sampled from continuously sampling dripping water or collecting from residual drip pools. While the subterranean fauna of cracks and crevices has been known for over 100 years, it is only in the past several decades that epikarst has been recognized as a distinct habitat, with reproducing populations of stygobionts. Dissolved organic carbon in epikarst drip water is a primary and sometimes the only source of organic matter for underlying caves, especially if there are not sinking streams that enter the cave. Typical concentrations of organic carbon are 1 mg L⁻¹. The fauna of epikarst is dominated by copepods, but other groups, including some terrestrial taxa, are important in some areas. Most of the diversity is β-diversity (between drips and between caves). In Slovenia, an average of nearly 9 stygobiotic copepod species were found per cave. In studies in Romania and Slovenia, a number of factors have been found to be important in determining species distribution, including ceiling thickness, habitat connectivity and habitat size. In addition to eye and pigment loss, epikarst copepod species may show a number of specializations for life in epikarst, including adaptations to avoid displacement by water flow. Several geoscientists and biologists have challenged the uniqueness and importance of epikarst, but on balance the concept is valid and useful. Fruitful future research directions include development of better sampling techniques, studies to explain differences among nearby epikarst communities, phylogeographic studies, and assessing the possible role of copepods as tracers of vadose water.

Keywords: adaptation; cave ecology; Copepoda; stygobionts; subterranean biodiversity

INTRODUCTION

Forty years ago, in 1973, Mangin coined the phrase “epikarst” for the uppermost layer of karst, an active zone of transfer between karst and the overlying soil. Since that time, epikarst has become recognized as an important zone of water storage, of speleogenesis, and an important biological habitat (Pipan, 2005; Williams, 1983). It was the subject of an interdisciplinary symposium organized by the Karst Waters Institute (Jones et al., 2004), and a review by Williams in this journal in 2008 provided a hydrogeological perspective on epikarst. In this review we propose to provide a biological perspective on epikarst, the fortieth anniversary of the coining of the term epikarst being an appropriate marker.

We begin with an overview of the physical and chemical aspects of the habitat, summarize what is known of the biology of epikarst, evaluate challenges, both biological and hydrogeological, that have be posed to the importance of epikarst, and conclude with some suggestions for future research.

The epikarst habitat

Wherever there is soil covering base rock, there is a zone of contact between the rock and the soil, typically consisting of an unconsolidated layer of rock mixed with soil—the regolith. This zone often has spaces larger than the soil above. When the base rock is water soluble at the pH of water in the area, these spaces are greatly enhanced by the dissolution of rock into small channels and cavities. It is the shallow part of karst areas, where stress release, climate, tree roots, and karst processes fracture and enlarge rock joints and cracks, creating a more permeable and porous zone over the carbonate rock in which only a few vertical joints and cracks occur (Bakalowicz, 2012).
The word epikarst came into widespread use in the 1990s following the definition by Mangin (1973) of an epikarst aquifer as a perched saturated zone within the superficial part of the karst that stores a part of the infiltrated water (Bakalowicz, 2012). Typically 3 to 10 m thick, epikarst overlies the water infiltration zone. Participants in a workshop on epikarst (Jones et al., 2004) decided upon the following definition of epikarst:

**Epikarst is located within the vadose zone and is defined as the heterogeneous interface between unconsolidated material, including soil, regolith, sediment, and vegetative debris, and solutionally altered carbonate rock that is partially saturated with water and capable of delaying or storing and locally rerouting vertical infiltration to the deeper, regional, phreatic zone of the underlying karst aquifer.**

According to Williams (2008), the typical porosity (per cent open space) of unweathered limestone is 2 percent while that of epikarst typically exceeds 20 percent. More generally, water storage in epikarst is the reason why cave streams typically have water for long periods of drought.

What we know about epikarst mostly comes from the study of the outflow of dripping water in caves. This outflow has a complex connection with precipitation (Kogovšek, 2010), and also includes water from other parts of the vadose zone. Typically, output spikes after several precipitation events, which cumulatively fill the cavities in epikarst, but different cavities fill at different rates. Based on continuous monitoring of three drips for three years in Postojnska jama (Slovenia), Kogovšek was able to estimate total surface catchment area of an individual drip using precipitation and drip rate data (Table 1). Even the largest catchment area of a drip (I in Table 1) was quite small, approximately 200 m². These catchment areas are to a certain extent virtual because of the high concentration of Ca²⁺ ions. Meleg et al. (2011b) did a similar analysis for three Romanian caves for pH, conductivity, and NO₂⁻ as well as the concentrations of several heavy metals—Al³⁺, Cr³⁺, and Fe³⁺ (Table 3). Compared to Slovenian caves (Table 2), pH was consistently higher in the Romanian caves, above 8.0; conductivity, except in Peștera Vadu Crișului, was lower and nitrites were much higher in Romanian caves than in Slovenian caves. Differences in parent rock and anthropogenic impacts are likely the reasons for the discrepancy between the two countries.

Two consistent themes emerge from the studies of geochemistry of dripping water. One is that drip water has high concentrations of the ions associated with CaCO₃ dissolution, e.g., Ca²⁺, the result of water being in contact with carbonate rock for significant periods of time, i.e., weeks to months. The second theme is that there is considerable temporal and spatial variability in geochemistry, even at scales on the order of 10 meters.

Organic carbon in drip water is especially interesting because the fauna of epikarst and caves is likely carbon-rich rather than nutrient-rich (nitrogen or phosphorus) limited (Simon & Benfield, 2002; Simon et al., 2007). The source of organic carbon in epikarst water is the soil. Rainwater does not contain organic carbon, but because of biological activity in the soil, water leaching from the soil into epikarst cavities has organic carbon.

Simon et al. (2007) measured dissolved organic carbon (DOC) concentrations in both Organ Cave, West Virginia, U.S.A. and Postojna Planina Cave System (PPCS) in Slovenia, as did Ban et al. (2008) in Shihua Cave, China. In Organ Cave, mean DOC concentrations in the unsaturated zone were 1.10 mg/L while in PPCS they were 0.70 mg/L (Table 4). The differences are likely the result of different land uses. The land above Organ Cave is mostly pasture and the land above PPCS is forest. In addition to water entering...
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Table 2. Average values and coefficients of variation (standard deviation X 100/mean) for pH, conductivity, and nine ions for six Slovenian caves. Ionic concentrations are in mg/L. Data from Pipan (2003, 2005).

<table>
<thead>
<tr>
<th>Cave</th>
<th>pH</th>
<th>Conductivity (μS/cm)</th>
<th>NH4⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Na⁺</th>
<th>Mg²⁺</th>
<th>NO₃⁻</th>
<th>NO₂⁻</th>
<th>SO₄²⁻</th>
<th>Cl⁻</th>
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<td>74.83</td>
<td>52.18</td>
<td>40.42</td>
<td>91.25</td>
<td>63.14</td>
<td>190.16</td>
<td>345.22</td>
<td>80.64</td>
<td>135.08</td>
</tr>
<tr>
<td>n</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
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<td>45</td>
<td>45</td>
<td>45</td>
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<td>45</td>
</tr>
<tr>
<td>Pivka jama</td>
<td>7.84</td>
<td>416.36</td>
<td>0.11</td>
<td>0.83</td>
<td>42.93</td>
<td>2.17</td>
<td>1.14</td>
<td>11.81</td>
<td>0.012</td>
<td>5.44</td>
<td>2.37</td>
</tr>
<tr>
<td>Coef. Var.</td>
<td>3.39</td>
<td>27.73</td>
<td>69.95</td>
<td>50.41</td>
<td>61.69</td>
<td>94.82</td>
<td>60.56</td>
<td>122.62</td>
<td>399.73</td>
<td>46.81</td>
<td>69.84</td>
</tr>
<tr>
<td>n</td>
<td>69</td>
<td>69</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Postonska jama</td>
<td>7.81</td>
<td>342.57</td>
<td>0.07</td>
<td>0.42</td>
<td>39.00</td>
<td>0.99</td>
<td>0.86</td>
<td>3.09</td>
<td>0.001</td>
<td>5.73</td>
<td>1.26</td>
</tr>
<tr>
<td>Coef. Var.</td>
<td>2.38</td>
<td>27.28</td>
<td>52.63</td>
<td>32.31</td>
<td>36.42</td>
<td>42.80</td>
<td>31.25</td>
<td>74.57</td>
<td>433.17</td>
<td>47.79</td>
<td>36</td>
</tr>
<tr>
<td>n</td>
<td>219</td>
<td>219</td>
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<td>69</td>
<td>69</td>
<td>69</td>
<td>69</td>
<td>69</td>
</tr>
<tr>
<td>Škocjanske jame</td>
<td>7.87</td>
<td>308.40</td>
<td>0.12</td>
<td>0.45</td>
<td>23.64</td>
<td>1.40</td>
<td>0.88</td>
<td>1.05</td>
<td>0.004</td>
<td>6.40</td>
<td>0.37</td>
</tr>
<tr>
<td>Coef. Var.</td>
<td>2.14</td>
<td>28.43</td>
<td>104.46</td>
<td>53.11</td>
<td>68.55</td>
<td>77.70</td>
<td>52.56</td>
<td>95.29</td>
<td>655.74</td>
<td>43.66</td>
<td>54.9</td>
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<td>43</td>
<td>43</td>
<td>43</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>Županova jama</td>
<td>7.65</td>
<td>371.29</td>
<td>0.10</td>
<td>0.46</td>
<td>47.83</td>
<td>0.88</td>
<td>1.09</td>
<td>0.31</td>
<td>0.001</td>
<td>8.74</td>
<td>1.4</td>
</tr>
<tr>
<td>Coef. Var.</td>
<td>3.09</td>
<td>20.89</td>
<td>44.75</td>
<td>45.85</td>
<td>30.05</td>
<td>68.72</td>
<td>17.10</td>
<td>47.64</td>
<td>343.60</td>
<td>21.26</td>
<td>46.69</td>
</tr>
<tr>
<td>n</td>
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<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
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</tr>
</tbody>
</table>

Table 4. Estimates of dissolved organic carbon in mg/L from Organ Cave, West Virginia (USA) and Postojna Planina Cave System (Slovenia). From Simon et al. (2007). Used with permission of the National Speleological Society (www.caves.org).

<table>
<thead>
<tr>
<th>Cave System</th>
<th>Organ Cave</th>
<th>Postojna Planina Cave System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Input:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sinking streams</td>
<td>7.67±1.03</td>
<td>4.36±0.46</td>
</tr>
<tr>
<td>percolation water</td>
<td>1.10±0.15</td>
<td>0.70±0.04</td>
</tr>
<tr>
<td>In cave:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>streams</td>
<td>1.08±0.32</td>
<td>4.75±1.57</td>
</tr>
<tr>
<td>Output:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>resurgence</td>
<td>0.90±0.17</td>
<td>2.67±0.80</td>
</tr>
</tbody>
</table>

Fig. 1. A conceptual model of energy flow and distribution (as organic carbon) in a karst basin with estimates of fluxes and standing crops for Organ Cave, West Virginia, USA. Standing stocks are particulate (POM) and dissolved (DOM) organic matter in the water column and fine (FBOM) and coarse (CBOM) benthic organic carbon and microbial films on rocks (epilithon). Solid and dashed arrows represent fluxes. Data are standing stocks of carbon except for respiration flux, shown by the wavy arrow. Values for FBOM, CBOM and microbial film are taken from Simon et al. (2003), the whole-stream respiration rate (wavy arrow) is from Simon and Benfield (2002), and the remaining values are from Simon et al. (2007). Modified from Simon et al. (2007). Used with permission of the National Speleological Society (www.caves.org).
Simon et al. (2010) measured specific UV absorbance (SUVA) at 254 nm, a standard measure of the frequency of aromatic compounds (McKnight et al., 2001), for Organ Cave and Postojna Planina Cave System (PPCS). Higher SUVA values tend to mean the compounds are less reactive and less easy to metabolize but there are numerous caveats (Weishaar et al., 2003). The pattern, which is very similar for both caves, is shown in Fig. 2. Soil, one of the sources of DOC in epikarst, had relatively high SUVA and percolating water drips had relatively low SUVA. SUVA values suggest that the organic carbon in epikarst is more metabolically accessible than that of the soil, or at least with lower percentages of aromatic and humic compounds.

**History of biological studies of epikarst**

Beginning with Racoviţă’s 1907 classic “Essai sur les problèmes biospéologiques”, biologists have recognized that much of the fauna observed in cave passages accessible by humans often occurs more frequently in cracks and crevices. In Racoviţă’s time, the epikarst zone was completely unknown to hydrogeologists, and he can scarcely be faulted for not identifying it as a separate habitat. By the mid 20th century, several biologists, such as Petkovski (1959), became aware that there were stygobiotic copepods in caves with no streams and only percolating water. He recognized that the accumulation of water from above depended on fractured rock, and that there was water in tiny fissures and cracks which slowly flowed down from the ceiling. He believed that this habitat was the realm of copepods like Speocyclops as well as many harpacticoids. Thus he didn’t recognize the infiltration zone as a habitat per se, but as a source of water that filled small depressions in walls, the “realm of Parastenocaraida”. Holsinger (1971) came to similar view with respect to a population of the amphipod Crangonyx antennatus living in Molly Waggle Cave in Virginia. Part of the population was in an old trough used for saltpetre mining during the American Civil War. He concluded that the only way the individuals could have gotten there was via what we would now call epikarst, but he reviewed it more as a dispersal corridor than a habitat.

In his study of copepods in the Baget karst basin in France, Rouch (1968, pers. comm.) recognized that the small number of individuals occurring in pools in Grotte de Sainte-Catherine was much too small to constitute a viable population and concluded that there were populations in perched, i.e., epikarst, aquifers. Rouch had the advantage of collaborating with Mangin (1973), one of the discoverers of epikarst. Other French biologists, notably Delay (1968) and Gibert (1986) studied the fauna of percolating waters, and included terrestrial species in their study, but did not distinguish different components of the zone of percolation.

Brancelj’s (2002) discovery of a rich copepod fauna in drip pools in the shallow Slovenian cave Velika Pasica, which has no other water, but with relatively few reproducing individuals, led him to conclude that reproduction was occurring in “small cracks around the cave”. Although he did not use the phrase epikarst, the cave is so shallow that most of the ceiling is epikarst. Unlike Petkovski and Holsinger, he held that reproduction was not occurring in the cave but in crevices in the cave ceiling and walls. Pipan (2003, 2005) championed the idea that there was an epikarst habitat and fauna distinct from other subterranean habitats, and developed innovative techniques to sample dripping water continuously (Pipan & Brancelj, 2001). Camacho et al. (2006) extensively sampled epikarst pools (but not the drips directly) and found a large number of stygobiotic species in Ojo Guareña cave in Spain. Culver and Pipan (2011) argued that epikarst was one of several aquatic shallow subterranean habitats each of which harbors a unique, trogloborphic, stygobiotic fauna.

Moldovan, and her colleagues initiated an intensive study of Romanian caves in the mid 2000’s, emphasizing community structure and diversity (Meleg et al., 2011a, 2011b), as well as effects of habitat fragmentation and quality (Moldovan et al., 2011). During the same time period, Simon and colleagues (2007, 2010) integrated epikarst into models of organic carbon flux in caves, and provided the first measurements of dissolved organic carbon.

**Overview of the epikarst fauna**

The epikarst fauna, best collected directly from dripping water rather than pools (see below), is typically dominated by copepods (Pipan, 2005; Pipan & Culver, 2005; Meleg et al., 2011b). Other groups may be present in considerable numbers, including amphipods, ostracods, and even syncarids, depending on the location. Terrestrial species are also sometimes common in dripping water, presumably having been flushed out of their air-filled cavity. Nonetheless, the bulk of biological studies have emphasized the copepod fauna.

Not all species found in epikarst are epikarst endemics, or even stygobionts (aquatic species limited to subterranean waters). For the 35 drips in six caves studied by Pipan (2005), the number of (1) epikarst endemic copepod species, (2) non-epikarst endemic stygobiont copepod species, and (3) non-stygobiont copepod species was about the same, ranging between 10 and 15 (Fig. 3). For all Slovenian records, non-epikarst endemic stygobionts and non-stygobiont species were approximately equal in number, while the number of epikarst endemics was approximately half of the other two categories.

![Fig. 2. Specific UV absorbance (SUVA) for DOC samples from soil extracts, epikarst drips, sinking streams, cave streams, and resurgences at PPCS and Organ Cave karst aquifers in September 2007. From Simon et al. (2010).](image-url)
The reduced relative frequency of epikarst endemic species is not surprising since only seven caves have been thoroughly investigated.

It is interesting to consider the relative abundance of the different categories of species—epikarst endemic, non-epikarst endemic stygobiont, and non-stygobiont, because one could hypothesize that the three categories represent different stages of adaptation, with non-stygobionts being the least adapted. For the six caves where drips were sampled directly (Pipan, 2003, 2005), it is possible to test this directly. Using overall abundance of each species in drip samples as the variable, the mean abundance of non-stygobionts was the lowest of the three groups, as expected (Table 5), but the presumably specialized epikarst endemics were less than half as abundant as the other stygobionts. Overall however, the ANOVA was not significant (p=0.0516, Table 5), indicating no significant differences in abundance among the ecological groups.

**Copepod species diversity and richness in epikarst**

Malard et al. (2009) analyzed data on stygobiotic groundwater species on a European wide scale and found that local α-diversity (in their case a local aquifer) contributed less than ten percent to overall species richness, and suggested that among sites β-diversity was characteristically high in subterranean systems. Sampling data on epikarst drips allows for the analysis of even finer spatial scales. The same pattern of low α-diversity and high β-diversity demonstrated by Malard et al. (2009) for the stygofauna as a whole on a European wide scale was found for epikarst copepods for smaller geographic scales—ranging from individual drips to the south central region of Slovenia (Pipan & Culver, 2007a). The three components of total epikarst copepod diversity were partitioned as follows:

- 12 percent (3.20 species) of the total species richness was within-drip diversity (α-diversity)
- 20 percent (5.47 species) of the total species richness was among drip diversity within a cave (a component of β-diversity), for an average of 8.67 species per cave
- 68 percent (16.33 species) of total species richness was among cave (a component of β-diversity).

Meleg et al. (2011a, 2011b) report between 3 and 6 epikarst copepod species per cave and drip pools in Romania (Table 6) and a total of 11 species among their five study caves, lower numbers than those recorded for the Slovenian caves. The best estimate of total species richness was 16 species for the five Romanian caves (Meleg et al. 2011a). Based on this limited evidence, it seems likely that Slovenia and perhaps the Dinaric karst in general is a hotspot for epikarst copepod species, as it is for other subterranean aquatic groups (Sket 1999).

Not only are species numbers high in general in the Dinaric karst of Slovenia, but nearly all the species are stygobionts, in contrast to well studied caves in Romania and West Virginia, USA (Table 7). An interesting and unusual situation occurs in two non-Dinaric caves in Slovenia which had very low diversity (as opposite to the general high diversity recorded for the Dinaric karst); in drips in both Huda lučnja, occurring in an isolated karst area and Snežna jama na planini Arto, an ice cave in the Kamnik-Savinja Alps, only two copepod species were found in each cave, but they all were stygobionts.

**Ecology of epikarst fauna**

Pipan et al. (2006a) used Canonical Correspondence Analysis to search for patterns and connections between individual species and environmental parameters. In this study, it was only the samples with copepods that were used, and the emphasis was on niche differences among species. Figure 4 is a two-dimensional plot of the 12 chemical and physical parameters of epikarst drips from five Slovenian caves (Postojnska jama was excluded because species were rare and more than half of the drips had no fauna).

Table 6. Species richness of epikarst copepods for five Romanian caves. The estimated total includes observed and unobserved species, using Chao 1 estimate in EstimateS. Data from Meleg et al. (2011a).

<table>
<thead>
<tr>
<th>Cave</th>
<th>Number of Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peștera Ungurului</td>
<td>6</td>
</tr>
<tr>
<td>Peștera Vadu Crișului</td>
<td>5</td>
</tr>
<tr>
<td>Peștera cu Apă din Valea Leșului</td>
<td>5</td>
</tr>
<tr>
<td>Peștera Ciur Izbuc</td>
<td>5</td>
</tr>
<tr>
<td>Peștera Dobos</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>11</strong></td>
</tr>
<tr>
<td><strong>Estimated total (Chao estimate)</strong></td>
<td><strong>15.5</strong></td>
</tr>
</tbody>
</table>
The most important parameters which separated the different drips were NO$_3$ concentration and ceiling thickness. Each cave formed a relatively compact cluster, with Županova jama both having the largest cluster and being the most distinct. When species were superimposed on the two-dimensional plot, three clusters of species could be distinguished. One is represented by the single species Parastenocaris cf. andrejii, and largely separated by low concentrations of NO$_3$ and high concentrations of Na$^+$ and Cl$, and was only found in Dimnice drips. A second cluster — Moraria varica, Maraenobiotus cf. bruuci, Bryocamptus dacicus, and Bryocamptus sp.— was separated by high concentrations of NO$_3$. These species were only found in Pivka jama. A third cluster comprised all of the other species. Because the first two clusters were related not only with particular environmental conditions but also with a particular cave, it was impossible to distinguish which factor (physical-chemical or geographic separation) was important. Nevertheless, each species occupied a distinct set of sites, and even species within a cluster slightly differed in their preferential conditions. Given the highly fragmented character of epikarst and the high levels of heterogeneity of physical and chemical conditions, there are many possibilities for niche separation, both along geochemical and spatial axes. It may be this heterogeneity makes possible the high β-diversity of epikarst habitats (see above).

Meleg et al. (2011a) and Pipan et al. (2006b) report similar analyses for epikarst communities in Romania and USA, respectively. Using pH, temperature, conductivity, and precipitation are predictor variables, Meleg et al. (2011a) produced correspondence plots for the entire epikarst community, including amphipods, isopods, ostracods, and copepods, the latter being the dominant taxa as number of species and individuals. They found that, unlike Pipan et al. (2006a), differences in conductivity were correlated with abundance of different species and that the stygobiotic taxa were usually found in water with higher conductivity. Higher conductivity implies longer residence time of the water in epikarst as the water becomes saturated with CaCO$_3$ (Covington et al., 2012).

Moldovan et al. (2011) have made important steps in understanding why different epikarst communities are different, not just that they are different. Working in Peștera Ciur Izbcu in Romania, they used a combination of stable isotopes, drip rates, and species composition in abundance, to understand differences between the epikarst community in two sections of the cave 300 m apart. They concluded that the downstream epikarst section had smaller, well connected voids that allowed for rapid transport of animals and water from the surface. The upstream section had lower secondary porosity but larger spaces (and larger animals). Finally, they also demonstrated that the surface-dwelling copepod Bryocamptus caucasicus was able to penetrate the epikarst and had a negative impact on the stygobiotic copepods, even though B. caucasicus likely did not maintain permanent populations in subterranean habitats.

An analysis of the relationship between geographic distance and community structure in the Organ Cave (West Virginia, U.S.A.) drip community highlights the importance of spatial heterogeneity and patchiness. The highly dissected nature of epikarst may constrain dispersal. If this is the case, then community similarity should decline rapidly with distance. Using the Jaccard index, Pipan et al. (2006b) showed a striking relationship between community differences and distance (Fig. 5). There is an increase in community dissimilarity with distances up to 100 m, after which point there is an increase in the variability of community dissimilarity.

Table 7. Frequency of stygobiotic copepods taken from water in drips.

<table>
<thead>
<tr>
<th>Site</th>
<th>No. of species</th>
<th>Percent troglobions and stygobionts</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crna jama, Slovenia</td>
<td>8</td>
<td>100</td>
<td>Pipan (2005)</td>
</tr>
<tr>
<td>Dimnice, Slovenia</td>
<td>8</td>
<td>100</td>
<td>Pipan (2005)</td>
</tr>
<tr>
<td>Huda luknja, Slovenia</td>
<td>2</td>
<td>100</td>
<td>Pipan et al. (2008)</td>
</tr>
<tr>
<td>Šežna jama na planini Arto, Slovenia</td>
<td>2</td>
<td>10</td>
<td>Papi and Pipan (2011)</td>
</tr>
<tr>
<td>Županova jama, Slovenia</td>
<td>14</td>
<td>93</td>
<td>Pipan (2005)</td>
</tr>
<tr>
<td>Škocjanske jame, Slovenia</td>
<td>9</td>
<td>89</td>
<td>Pipan (2005)</td>
</tr>
<tr>
<td>Postojnska jama, Slovenia</td>
<td>5</td>
<td>80</td>
<td>Pipan (2005)</td>
</tr>
<tr>
<td>Pivka jama, Slovenia</td>
<td>11</td>
<td>73</td>
<td>Pipan (2005)</td>
</tr>
<tr>
<td>Peștera Dobog, Romania</td>
<td>33</td>
<td>67</td>
<td>Meleg et al. (2011b)</td>
</tr>
<tr>
<td>Peștera Ciur Izbcu, Romania</td>
<td>5</td>
<td>60</td>
<td>Meleg et al. (2011b)</td>
</tr>
<tr>
<td>Cave cu Apă din Valea Leșului, Romania</td>
<td>4</td>
<td>50</td>
<td>Meleg et al. (2011b)</td>
</tr>
<tr>
<td>Organ Cave, W.Va., USA</td>
<td>10</td>
<td>40</td>
<td>Pipan et al. (2006b)</td>
</tr>
<tr>
<td>Peștera Ungurului, Romania</td>
<td>6</td>
<td>33</td>
<td>Meleg et al. (2011b)</td>
</tr>
<tr>
<td>Peștera Vâlu Crului, Romania</td>
<td>5</td>
<td>20</td>
<td>Meleg et al. (2011b)</td>
</tr>
</tbody>
</table>
Thus, drips within several hundred meters of each other tend to have similar composition and there is an expected decline in similarity with distance. This relationship between distance and dissimilarity breaks down when drips greater than 1 km apart were considered, and communities are substituted by new ones. The “new” communities may or may not be similar to the adjoining communities, as evidenced by the high variability of the Jaccard coefficients. Epikarst copepod communities thus form a patchwork of communities, with variation on a scale of 100 m.

Morphological characteristics

Brancelj (2007, 2009) gave a number of intriguing suggestions about uniquely convergent features of epikarst copepods. Working with the genera *Morariopsis* and *Paramoriopsis*, he pointed out that animals living in the epikarst must have some morphological adaptations to prevent or minimize their transport downward. Combined with what he considers a low supply of organic carbon, he proposes that the following convergent features are present in specialized epikarstic copepods to avoid being displaced by water flow:

- Reduction in endopodal segmentation to two or one;
- Reduction in number of spines and setae on the terminal segments of both endopods and exopods;
- Reduction in number of spines and setae on the caudal rami to one terminal seta;
- Tips of the terminal setae of the caudal rami are far apart;
- Short and robust setae on the endopodal lobe of fifth leg (P5) as well as very strong spinales at the base of the caudal rami.

Brancelj (2009) demonstrated that the genus *Elaphoidella* shows similar convergent features, especially the last two. He also proposed that there is convergent reduction on length of the antennules and that robust setae are probably an adaptation for moving through small spaces in fractured rock as well as a protection against washout.

## Faunal differences between drips and drip pools

Most data on epikarst fauna comes from collections of individuals from drip pools. Drip pools are not necessarily passive collectors of the epikarst fauna in drips, and Pipan et al. (2010) specifically addressed the question of whether the fauna of drip pools reflected the drip community. Overall, the frequency of stygobionts was 1.5 times higher in drips than in pools, and the frequency of stygobionts that were epikarst specialists was three times higher in drips compared with pools. The frequency of immature individuals, suggestive of reproduction at the site, was also higher in drips than in pools, with the exception of one artificially enlarged pool in Skocjanske jam. Pipan et al. (2010) suggest that there is increased juvenile mortality in pools and reduced reproduction, indicating that pools are not “source populations”, i.e., populations that are self-sustaining in the absence of migrants (Pulliam, 1988).

## CRITIQUES AND CHALLENGES

Several criticisms and challenges have been posed to the reality and utility of the concept of epikarst. Two of them are hydrogeological, those of Šušteršič (1999) and Kresic (2013), and one is biological, that of Sket et al. (2004).

Based on his geological studies of the Dinaric karst where there is both rapid uplift and rapid denudation of karst, Šušteršič (1999) sees epikarst as a zone of destruction of karst, which he calls speleothanatic space. There is little doubt that in circumstances of both rapid uplift and erosion, caves become unroofed and a destruction zone can be identified. Even in this case, epikarst can still have major hydrological function for water storage, a point that Šušteršič did not address. Šušteršič’s work does point out the transitory nature of epikarst, more so than the underlying caves.

Kresic (2013) offers two objections to the widespread use of the term epikarst. First, he argues that epikarst is absent in many places. This is an empirical question, and its presence or absence in flank margin caves and hypogenic caves, for example, is unknown (Jones, 2013). Curiously, Kresic (2013) uses the presence of vertical shafts and sinkholes as evidence for epikarst, but they are not an integral part of epikarst, and Bakalowicz (2004) even argues that they are not even part of epikarst, since it represents rapid transmission routes through upper karst layers. Secondly, Kresic (2013) quite correctly points out that epikarst is not a usable aquifer by humans, but that speaks to its size, not its presence.

Skeč (2004) hold that there is no distinct epikarst fauna, and consider it part of the upper vadose fauna, which is also called the percolation zone. The method of collecting epikarst water necessarily means that it includes water in both epikarst and the percolation zone below it. However, it is likely that most of the animals are in the epikarst rather than the percolation zone, for two reasons, one hydrological and the other based on species occurrence patterns. As the pioneering work of the hydrogeologists Bakalowicz, Mangin, and Williams demonstrates, most of the water is stored in the epikarst.
zone. This volumetric relationship suggests most individuals are in the epikarst rather than other areas, especially since the upper vadose zone is a region of vertically moving water. In addition, Pipan et al. (2006a) found that the abundance of all stygobiotic copepod species except *Elaphoidella cvetkae* was negatively correlated with ceiling thickness (see Fig. 4). If the upper vadose zone were the major habitat, then most species should have a pattern like that of *E. cvetkae*. If the upper vadose zone were the major habitat, it is difficult to explain the high diversity in Velika Pasica and other caves with thin ceilings and essentially no upper vadose zone. Because all sampling is indirect, it cannot be known with certainty what the distribution of species in the epikarst and upper vadose is. While it is highly likely that, for the reasons given above, the major habitat is epikarst, many species may well occur in other part of the upper vadose zone. Thus, strictly speaking, it is likely that most epikarst species occur, albeit in much smaller numbers, in the upper vadose, percolation zone. Nevertheless, epikarst endemic is a very useful phrase to describe these species, just as riparian species may occasionally occur away from stream margins.

**DIRECTIONS FOR FUTURE RESEARCH**

So much is not known about epikarst that it is difficult to prioritize research needs. However, the following directions seem to be most promising. First, part of the difficulty of studying epikarst is that all sampling is indirect, and we can only infer habitat structure and subdivision. Any method of direct observation or direct sampling, perhaps through pumping, should yield important results. Second, understanding the reasons for the differences in species composition and richness of epikarst communities, especially in small scale, is an obvious next step forward. The promising start of Moldovan et al. (2011), one that combines hydrological, biological, and stable isotope data, should be expanded. Third, none of the epikarst fauna has been put in a phylogenetic context. Are these species basal to species in deeper subterranean habitats, or do they tend to form distinct clades? Phylogenetic studies would also provide some initial estimates of the age of the epikarst fauna. Fourth, the suggestion of Pipan and Culver (2007b) that copepods could be used as epikarst water tracers should be pursued.

**ACKNOWLEDGEMENTS**

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INTRODUCTION

Caves are part of a larger karst ecosystem. They have unique physical and biological characteristics and are dynamic ecosystems of landforms, life, energy, water, gases, soils and bedrock. In general, caves are highly sensitive and vulnerable to disruption/degradation. Since all caves are subject to natural and human-induced disturbance, they should be carefully managed in all of the above-mentioned fields in order to prevent any degradation of these ecosystems (Watson et al., 1997; Russell & MacLean, 2008; White & Culver, 2012). Many caves also represent important resources for tourism. The impact of tourism on a cave is much more evident in a low-energy environment where any human presence, such as visitors to the cave, may be reflected in considerable changes to the ecosystem no matter how carefully the cave is managed. A high-energy environment (e.g. a cave with a large throughput of water), on the other hand, is generally much less sensitive to any changes (Watson et al., 1997; de Freitas, 2010). Anthropogenic impact on caves has been studied from many perspectives including studies of heavy metals in different matrices (e.g. Gutiérrez et al., 2004; Dongarra et al., 2010; Cuculić et al., 2011; Zhou et al., 2011), but such studies are relatively rare. In general, various sources may contribute harmful substances such as heavy metals to an environment. A proper identification of sources that release these substances would not just provide a means for their most effective control but also allow prioritisation of the most significant substances and/or sources that would ultimately

Source assessment of deposited particles in a Slovenian show cave (Postojnska jama): evidence of long-lasting anthropogenic impact

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Abstract: Postojnska jama (Postojna Cave) is one of the most famous karst caves in the world and has been a well-known tourist attraction for nearly 200 years. It is particularly famous for its unique double-track railway. Eight heavy metals – aluminium (Al), chromium (Cr), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), strontium (Sr), and zinc (Zn) – were determined in dust deposits by ICP-MS in order to assess sources of deposited particles on the cave walls. The samples were collected along the main passage in the cave, at different horizontal and vertical levels, in order to test horizontal homogeneity and study vertical distribution of the particles. It seems that the railway is an important anthropogenic source of particles, reflected in increased concentrations of Cu, Pb, and Zn, as well as of Fe and Mn in dust deposits at individual sampling sites. The maximum concentrations of Cu (217 µg g⁻¹), Pb (4.940 µg g⁻¹), and Zn (1.060 µg g⁻¹) considerably exceeded their natural abundance and were explained by anthropogenic impact. The three heavy metals are markers for vehicles, engine oil and brake wear. On the other hand, mixed sources could prevail for Fe and Mn. The maximum concentrations of Fe (85,900 µg g⁻¹) and Mn (682 µg g⁻¹) in dust deposits were similar to the concentrations determined in fragments of the railway tracks (97,100 µg g⁻¹ for Fe and 821 µg g⁻¹ for Mn) and were explained by track wear and/or corrosion. In most other parts of the cave, Fe and Mn concentrations were, however, below the concentration of their natural abundance. Al, Sr, and Cr seem to be predominantly of natural origin. They generally exhibited concentrations lower than their natural abundance.

Keywords: dust; heavy metals; source apportionment; tourism; Postojna Cave

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Postojnska jama (Postojna Cave) is one of the most famous karst caves in the world. It is the cave with the greatest diversity of endemic cave fauna, one of the oldest and largest show caves in the world, and the largest managed cave in the Dinaric karst (Gams, 2003). As such, it has received much scientific attention across various fields. Physical (Šebela & Turk, 2011), chemical (Vokal et al., 1999; Zupančič et al., 2011), and biological parameters (Sket, 2003; Mulec et al., 2012), as well as radioactivity (Gregorič et al., 2011), have all been included in past research activity. Postojnska jama has also been a well-known tourist attraction for nearly 200 years. Development of the cave for tourism began in as early as 1818 and the number of visitors then grew consistently. The cave is particularly famous for its railway; it is the only cave in the world with a double-track railway. Petrol-powered locomotives began operating on a single track in 1924. Between 1964 and 1968 the single track was upgraded to a circular, double-track railway covering 3.7 km of the 5 km section of the cave that is open to visitors. Along with the reconstruction of the tracks in the 1960s, battery-powered locomotives were introduced (Čuk, 2003). In the following years, an enormous increase of visitors was observed. The peak period of cave visits was recorded between 1970 and 1990. In this period, more than 16 million visitors from all over the world came to see the cave, including as many as ~942,000 visitors in the peak year of 1985 and up to ~12,000 visitors per day (on 8 July 1978). In recent years, the number of visitors has stabilised at approximately a half a million tourists per year (Hrovatin, 2006).

The goal of this paper is to assess sources of deposited particles on the walls in the cave system of Postojnska jama and to examine whether the abundance and composition of the particles has been affected by natural processes or anthropogenic impact due to the cave’s tourist facilities. A railway has been operating in the cave for the last 89 years, and this may represent a substantial environmental burden on the cave. The cave receives massive number of visitors resulting in frequent train rides into and out of the cave. Deposited particles were scraped from the walls in the main passage of the cave system and chemically characterised. Concentrations of eight heavy metals – aluminium (Al), chromium (Cr), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), strontium (Sr), and zinc (Zn) – were determined in the wall deposits, sprinkling sand and parts of the railway track. The mineral composition of the fresh sprinkling sand was also identified. Finally, relations among the parameters and the sampling sites were studied.

**MATERIALS AND METHODS**

**Site description**

Postojnska jama (45°46'58" N, 14°12'13" E) is over 20 km long and is situated under the Postojna karst plateau. The cave is developed in upper Cretaceous limestone and limestone with chert lenses and was formed by the sinking Pivka river, which still flows through passages of the lower level of the cave. The passages of the upper level of the cave are no longer within the flood zone of the river and are mostly covered with calcite flowstone. The cave has several known entrances at different elevations, but there are also other less-known accesses from the surface, e.g., small openings and fissures (Gabrovšek & Mihevc, 2009; White & Culver, 2012).

As a result of the several entrances and openings at different elevations and differences in temperature between the cave and the ambient air, strong air currents occur in the cave. Air temperature in a cave depends on the temperature of the rock, which in karst areas is usually close to the mean annual temperature of the surface (Badino, 1995). In the warm part of the year, when the air in a cave is colder than the outside air, the cave air moves towards the lower entrance of the cave and out. Warmer ambient air enters the cave through the upper openings on the plateau, cooling down as it enters, which results in an increase of relative humidity and subsequent condensation of water vapour on the cave walls, so that the walls get wet. This process also affects the adhesion of particles to the cave wall surface, since wetter surface increases hydraulic retention of particles (Dredge et al., 2013). In winter, the air circulation is reversed. The warmer cave air exits through upper openings and draws colder ambient air in through the lower entrance. The ambient air warms in contact with the cave walls, resulting in a decrease of relative humidity and causing the walls to dry. The velocity of the air current through the main entrance of the cave, which has a profile of 30 m², can reach 1.5 m s⁻¹ in winter, desiccating the floor and walls of the main passage for a distance of about 1.5 km in the direction of Velika Gora (Fig. 1). Furthermore, the air current is likely to be responsible for the transport of dust particles in the cave in the winter (e.g. Pashchenko et al., 1993; Christoforou et al., 1996; Kertész et al., 1999; Gabrovšek & Mihevc, 2009; Drege et al., 2013). Clearly visible dust deposits can generally be found only in the main passage (called ‘Stara Jama’; Fig. 1, Supplementary Material 1) stretching between the main entrance and Velika Gora. The passage is approximately 10 m wide and 10 m high, with some wider segments. In contrast, in narrow side passages where there is no air circulation no desiccation is observed, the temperature is higher and there are practically no dust deposits (Gams, 2003; Gabrovšek & Mihevc, 2009; Supplementary Material 2).

**Sampling**

In February 2011, fourteen samples were collected from the walls at five locations in the main passage of the cave (Fig. 1). All sampling sites were located along the railway tracks, and the location of sample 1 was closest to the entrance. This sample was collected in the black part of the cave (referred to as the “black section” hereafter) approximately 1 metre above the tracks. The sample was coded as 1.1 in which the first figure in the sampling site code represents the sampling location and the second the sampling height.
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Three samples from location 2 were collected at different elevations (~1, 10, and 20 metres above the tracks) in order to study the vertical distribution of dust deposits. These samples were coded as 2.1, 2.10, and 2.20, respectively. Six samples were collected in Kongresna Dvorana (‘Congress Hall’; location 3) in order to study the horizontal distribution of dust deposits. Samples 3.1A, 3.1B, and 3.1C were all collected at approximately 1 metre above the tracks, but at different places in the chamber. A character has been added to the sampling site code to distinguish between the samples. A second group of samples (3.3A, 3.3B, and 3.3C) at this location was collected on a horizontal level approximately 3 metres above the tracks. The sample at location 4 was collected in the brown part of the cave (referred to as the “brown section” hereafter) approximately 1 metre above the tracks and coded as 4.1. The last three samples (5.1, 5.20, and 5.40) were taken in the interior of the cave, i.e., Velika Gora (location 5) where the railway ends, again at different elevations (~1, 20, and 40 metres above the tracks, respectively). At each sampling site, a sample was collected for the purpose of chemical characterisation. In contrast, samples for gravimetric analysis were only obtained at selected sampling sites where a sufficiently large flat area was found on the cave walls. No vertical or horizontal distributions of the particles were studied in this case.

Chemical and data analysis

Heavy metals in the deposited particles were determined according to the EN 14902 (2005) and EN ISO 17294-2 (2005) standards. Samples were first oven-dried for 24 h at 105°C. Subsequently, approximately 100–120 mg of dry sample was weighed into a high pressure Teflon vessel. A mixture of nitric acid (65% w/w, Suprapure) and hydrogen peroxide (30% w/w, Suprapure) in the ratio 4:1 was added into each vessel. The vessels were closed and the samples digested in a microwave oven (Milestone, Ethos 1). The microwave system provided 1200 W of power, ensuring complete digestion at a temperature of 220°C (for 25 minutes). After cooling, the digestate was transferred into a 50 ml volumetric flask, a mixture of internal standards (scandium, germanium, rhodium and gadolinium) was added and the digestate diluted with Milli-Q water to the mark. Each sample was prepared in five replicates. Additionally, blank and control samples were performed with each set of seven samples for quality control.

Concentrations of selected heavy metals were determined by ICP-MS (Perkin Elmer, Sciex Elan 6100) equipped with a Gem-Tip cross-flow nebulizer and a Scott-type spray chamber. The following m/z were selected for measurements: 27Al, 53Cr, 55Mn, 57Fe, 63Cu, 66Zn, 88Sr, and 208Pb. Each digestate was measured in two replicates. The limits of detection were mostly from 0.01 to 0.1 µg g⁻¹, while those of Al, Fe and Zn were a bit higher (0.5-2 µg g⁻¹). Instrumental precision of the measurements was up to 5% and a certified reference material (NIST 1648a) was used to control the trueness of the analytical procedure.

The mineral composition of the fresh sprinkling sand was characterised by X-ray diffractometer (Philips, PW 1820) carried out on unoriented powder mounts. Qualitative estimation was based on peak intensity measurement of X-ray patterns (Cu Kα / Ni 40 kV, 30 mA) using the X’Pert HighScore software program.

Basic descriptive statistics were used to describe the data variability. Average and standard deviation was calculated for each parameter and sampling location. In addition, the strength of relation between heavy metals across the sampling sites was ascertained using the Pearson correlation coefficient.
RESULTS

The concentrations of heavy metals in the deposited particles are presented in Table 1 and Fig. 2. Fe was the most abundant heavy metal at eleven out of fourteen sampling sites, and it exhibited a wide range of concentrations (546-85,900 µg g⁻¹), with the highest concentration observed at sampling site 4.1. Al was the most abundant heavy metal at the remaining three sampling sites, while at most of the other sampling sites it was second highest in abundance. Its concentration ranged from 1,200 to 32,900 µg g⁻¹ (at sampling site 2.10). All other measured heavy metals were considerably less abundant. Only Pb, Sr, and Zn exceeded 1,000 µg g⁻¹. The highest concentration of Pb was 4,940 µg g⁻¹ at sampling site 3.3C (and approximately 2,000 µg g⁻¹ at two other sampling sites), while that of Sr was 1,300 µg g⁻¹ at sampling site 3.3B and that of Zn was 1,060 µg g⁻¹ at sampling site 3.3C. At all other sampling sites, the concentrations of Pb amounted to a few hundred µg g⁻¹, while those of Sr and Zn were generally even lower. The concentrations of Mn were all below 1,000 µg g⁻¹, while the concentrations of Cr and Cu did not exceed 200 µg g⁻¹.

The concentrations of heavy metals in the fresh sand that is used for sprinkling the railway tracks are shown in Fig. 3. Fe exhibited the highest concentration (2,340 µg g⁻¹), followed by Sr and Al (438 and 298 µg g⁻¹, respectively). All other heavy metals were considerably less abundant, with concentrations lower than 18 µg g⁻¹. According to X-ray diffractometry, the fresh sprinkling sand is composed solely of calcite (Fig. 4). On the other hand, Fe, Al and Mn concentrations increased markedly in sand collected beside the railway tracks. Their concentrations amounted to 4,170, 578 and 46 µg g⁻¹, respectively.

Fragments of the railway tracks were also analysed. Their main heavy metal constituents were Fe (97,100 µg g⁻¹), Mn (821 µg g⁻¹), Al (383 µg g⁻¹) and Cr (276 µg g⁻¹).

The mass of deposited particles per unit area was highest in the black section (sampling site 1.1), amounting to 443 g m⁻² (Fig. 5). In Kongresna Dvorana, the mass dropped to 211 g m⁻² (sampling site 3.3B) and decreased even further in the deeper parts of the cave (sampling sites 4.1 and 5.1) ranging from 12 to 24 g m⁻².

Pearson correlation coefficients between the measured parameters are presented in Table 2. The strongest correlation was observed between Cu and Zn (0.87), followed by Pb and Zn (0.74). The two correlations were significant at p < 0.05. A relatively high and significant correlation was also observed between Mn and Al, Mn and Cr, and Mn and Fe ranging from 0.66 to 0.69. All other correlations were not significant at p < 0.05.

DISCUSSION

Variability of the data

The concentrations of heavy metals in the deposited particles varied considerably throughout the cave (Table 1, Fig. 2). Deposits from all sampling sites were quite homogeneous. Repeatability mostly ranged from 5 to 15%, based on replicate analyses of the same deposit. Marked changes in concentration were observed at sampling sites where the vertical
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distribution of heavy metals was studied, e.g., sampling sites 2.1, 2.10, 2.20, and 5.1, 5.20, and 5.40. At sampling sites 2.1, 2.10, and 2.20, concentrations were generally higher at higher levels, while maximum concentrations were observed at either sampling site 2.10 or 2.20. In contrast, Pb and Sr showed almost no vertical change in concentration. The opposite was observed at sampling sites 5.1, 5.20, and 5.40 where most parameters exhibited higher concentrations at lower levels. Sr again showed almost no vertical change in concentration. Samples collected from the same horizontal level, e.g. sampling sites 3.1A, 3.1B, 3.1C, and 3.3A, 3.3B, 3.3C mostly showed some degree of heterogeneity. Sampling sites 3.1A, 3.1B, and 3.1C presented relatively similar distributions of Fe, Sr, and Zn, suggesting homogeneity of the samples, but the distributions of Al, Cr, Mn, and Pb differed noticeably between the samples, suggesting a reverse relationship. Even greater variations in concentrations were observed at a higher horizontal level (sampling sites 3.3A, 3.3B, and 3.3C). 

Faimon et al. (2011) demonstrated the relationship between particle size and chemistry; the varying particle sizes and subsequent size controlled distribution effects could explain variations in concentration among the sampling sites in the cave system of Postojnska jama. Nevertheless, we believe that the varying distributions of heavy metals more likely indicate that different sources of particles could be present in the cave. Dust deposited on the walls and floors of the cave has a characteristic colour. For example, between the entrance and the chamber of Kongresna Dvorana, dust deposits are black. Deeper in the cave (from Kongresna Dvorana towards Velika Gora), brown-coloured dust deposits prevail, supporting our supposition of the presence of different sources of particles in the cave.

Source assessment of dust deposits

The black section

In the black section (sampling point 1.1), which is situated approximately 500 m from the entrance (Fig. 1) and thus is the most susceptible to outer factors, very low concentrations of all heavy metals were observed (Table 1, Fig. 2). The overall lowest concentrations of Al, Cr, Mn and Sr were observed in this part of the cave. In contrast, the highest mass of deposited particles per unit area was observed in this part of the cave (Fig. 5). These deposits were studied in detail by Zupančič et al. (2011). They found that concentrations of Fe and Mn oxides were lower than 2–3%, equalling 20,000–30,000 µg g⁻¹, and concluded that the source of these deposits was not the two oxides that would explain black colouring of the particles. Their conclusion is also supported by our data. We determined quite low Fe and Mn concentrations in deposited particles at this...
sampling site (Table 1, Fig. 2). In addition, Zupančič et al. (2011) found that black deposits contained a high content of C. Lack of a significant amount of any other element except O and some Ca suggested to the authors that black deposits are mainly composed of carbonaceous particles originating from the explosion of gasoline in 1944. During a fuel explosion and a fire, a large quantity of black carbon particles is emitted into the space (Goldberg, 1985). The fuel explosion thus explains the high mass of deposited particles per unit area that was observed in the black section and is between two and thirty-six times higher than in other parts of the cave (Fig. 5). On the other hand, large amounts of carbonaceous particles released in the fuel explosion would also “dilute” other particles, thus explaining the low concentrations of all heavy metals observed in this part of the cave.

It has been shown that in the part of a cave that is close to its entrance, the cave temperature is still influenced by seasonal variations (Leutscher & Jeannin, 2004). In the black section, temperature variations are thus much lower than at the entrance but still higher than in the interior of the cave, where the temperature usually varies within one degree Celsius (Šebela & Turk, 2011). The ambient air that enters the cave during the winter period and flows along the main passage (Gams, 2003; Gabrovšek & Mihevc, 2009) thus has a potential to impact on the cave (Christoforou et al., 1996). Since the ambient air usually contains particulate matter that is polluted with various heavy metals (e.g. Dongarra et al., 2010), it could be expected that this air might influence/alter the composition of heavy metals in Postojnska jama. It seems, however, that this factor is not so important, since the observed concentrations of all heavy metals in the black section were so low. In addition, if particles from the ambient air had been the major source of particles deposited on the walls, then the concentrations of heavy metals should have decreased toward the inner parts of the cave in accordance with the decreasing concentration of particles in the air. This is not the case, and therefore we believe that other factors exist and/or are more important.

The railway

Zn and Cu exhibited the highest correlation among all parameters, while the correlation between Zn and Pb was only slightly lower (Table 2). The highest concentrations of all three parameters were observed in the upper part of Kongresna Dvorana (sampling site 3.3C; Table 1, Fig. 2). Although the correlation between Cu and Pb was only significant at \( p < 0.1 \) (0.46), we may conclude that the distribution of the three heavy metals was quite similar throughout the cave, suggesting that they may have the same origin. Cu, Pb, and Zn can be naturally found together in minerals, but their abundance is low (Hester & Harrison, 1997). The common concentration of Zn in the Earth’s crust is around 75 µg g\(^{-1}\), with that of Cu around 50 µg g\(^{-1}\) and that of Pb around 14 µg g\(^{-1}\) (Emsley, 2001). The concentration ranges observed in Postojnska jama were clearly higher, suggesting that these three heavy metals could be mostly of anthropogenic origin. These heavy metals have been identified as markers of vehicles, engine oil and brake wear (Bruinen de Bruin et al., 2006), thus indicating that the railway probably has an impact on the cave. On the other hand, the highest concentrations of Cu, Pb and Zn were observed in Kongresna Dvorana, where human presence is evident. This hall is frequently used for the many social events that are held in the cave.

Source assessment for Fe and Mn is not as straightforward as for Cu, Pb and Zn. Natural abundances of Fe and Mn reach up to 41,000 and 1,000 µg g\(^{-1}\), respectively (Emsley, 2001). The concentrations observed in Postojnska jama were mostly considerably lower (Table 1, Fig. 2). Only in the brown section (sampling site 4.1) and outside Kongresna Dvorana (sampling sites 2.1, 2.10, and 2.20) did Fe exceed its natural abundance, by a factor of approximately 2, while Mn was roughly three times higher than in other parts of the cave. Railway tracks are typically made of ordinary steel consisting mainly of iron, but in many alloys manganese is also added (Askeland et al., 2011). Since Fe and Mn are the major heavy metal constituents of the railway tracks in Postojnska jama, enriched Fe and Mn concentrations in the sand collected beside the railway tracks (Fig. 3; also see the discussion below) indicate that the railway tracks wear out and/or corrode slowly. It is likely that fine particles containing Fe and Mn are formed in these processes. In accordance with substantial air currents that have been observed in the fairly narrow main passage along the railway tracks in winter (Gabrovšek & Mihevc, 2009), the

Table 2. Pearson correlation matrix for measured parameters in deposited particles.

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Correlation coefficients significant at \( p < 0.05 \) are marked in bold.
finer and lighter fraction of the particles could easily be transported away. If we presume that only these fine particles are transported along the cave away from the railway tracks, the rather high Fe and Mn concentrations in the deposited particles in the brown section and outside Kongresna Dvorana would be explained. This supposition is further supported by our data. The maximum concentrations of Fe and Mn in the deposited particles were similar to the concentrations determined in fragments of the railway tracks. Furthermore, a significant and relatively high correlation was observed between Fe and Mn (Table 2). Although the two heavy metals are also naturally found together in minerals (Emsley, 2001), we nonetheless believe that high concentrations of Fe and Mn in the brown section and outside Kongresna Dvorana resulted from train operation. Mixed sources could thus be assumed for Fe and Mn. They could be mostly of natural origin, but in several parts of the cave anthropogenic origin also seems to be relevant.

**Sprinkling sand**

A comparison of heavy metal concentrations in the fresh sprinkling sand, whose function is to obtain a better adhesion of the train wheels to the railway track, and the sand that was collected beside the railway track showed that concentrations of several heavy metals were significantly higher in the latter fraction. An approximately twofold increase in concentrations of Al and Fe for example was observed in the sand collected beside the railway track, while in the case of Mn the concentration was up to four times higher (Fig. 3). Mn concentration was nevertheless one and two orders of magnitudes lower than Al and Fe, respectively. Frequent train rides into and out of the cave cause wear of the railway tracks over time. Therefore, the sprinkling sand is likely to be mixed with the constituents of the railway tracks as they wear and/or corrode. This explains the relative enrichment of heavy metals in the sand collected beside the railway tracks. However, the sprinkling sand may also act as an abrasive, grinding tiny particles containing metals from the railway tracks. Nevertheless, the only mineral present in the fresh sprinkling sand (Fig. 4), calcite, is softer than steel, thus negating the role of sprinkling sand as an abrasive. Although Cr was also determined in the railway tracks, no enrichment was observed in the sand collected beside the railway tracks. This can be explained by the fact that Cr usually clings to the steel layer and is not transferred elsewhere (Askeland et al., 2011).

Sprinkling sand is ground into fine particles due to the weight of the train and could be transported away from the railway tracks along the cave. Nevertheless, it seems that fine sand particles are still too large and/or too heavy to be transferred away from the railway tracks. Fe and Al concentrations in the deposited particles were approximately one order of magnitude higher than in the fresh sprinkling sand (Figs. 2 and 3). If fine sand particles had been the major source of the particles deposited on the walls, then the concentrations of Fe and Al in the deposited particles should have been much lower. Furthermore, Sr was found in increased quantities in the sprinkling sand (Fig. 3). In all parts of the cave except at sampling sites 3.3A and 3.3B, Sr concentration in the deposited particles was at least two times lower than in the sprinkling sand (Table 1, Fig. 2). This again supports our conclusion that sprinkling sand is not a relevant source of particles in the cave.

**Natural origin**

Al concentration in fragments of the railway tracks was one to two orders of magnitude lower than the concentrations observed throughout the cave. It therefore seems that other, probably natural sources are more important for Al, especially as Al is the third most abundant element in the Earth’s crust with concentrations of up to 82,000 µg g⁻¹ (Emsley, 2001). The very low correlation between Al and Fe (Table 2) could indicate that their sources are different. On the other hand, Al was significantly correlated with Mn. By comparing the distribution of the two heavy metals throughout the cave, we found that both distributions were remarkably similar, with the only exception observed in the brown section (Table 1, Fig. 2). At sampling site 4.1, Mn concentration was among the highest and was attributed to train operation, while Al concentration was among the lowest observed in the cave. Since we assumed mixed sources for Mn, we concluded that natural sources could prevail for Al.

In most parts of the cave, Sr concentration was rather low, with almost no variations. Furthermore, concentrations lower than the common concentration in the Earth’s crust (370 µg g⁻¹; Emsley, 2001) were mostly observed. Sr is one of the trace elements that can be found in limestone. It readily substitutes for Ca in carbonates and the concentration range observed in Postojnska jama corresponds to concentrations commonly observed in ancient limestone, i.e. from a few hundred to 2,000 µg g⁻¹ (Tucker & Wright, 1999), thus suggesting a natural origin. There was, however, a significant increase in Sr in the upper part of Kongresna Dvorana, with concentrations that were much higher than in the other parts of the cave. We could only speculate that Sr at sampling sites 3.3A and 3.3B might be of anthropogenic origin. Sr is frequently used as a constituent in pyrotechnics and in the production of flares (Daintith, 2004). The latter use in particular may have some effect, since flares were frequently used for illumination at events held in Kongresna Dvorana.

Cr exhibited the lowest concentrations obtained in this study. They were similar to the concentrations commonly found in the environment, i.e. around 100 µg g⁻¹ (Emsley, 2001) and we could thus conclude that sources of Cr are most probably natural.

**CONCLUSIONS**

Tourist facilities in Postojnska jama result in an evident anthropogenic impact on the cave. The distribution and composition of heavy metals in dust particles throughout the cave indicate that several sources could exist for the particles. Some particles seem to be of natural origin, but anthropogenic sources
are also likely and important for some parts of the cave. The railway itself, as well as wear and/or corrosion of the railway tracks seem to be the most important anthropogenic sources of the deposited particles in the cave, while the use of sprinkling sand for a better adhesion of the train wheels is probably of negligible importance. Furthermore, specific characteristics of Postojnska jama, such as multiple entrances/openings at different elevation and temperature differences between the cave and the ambient air enable more intensive particle transportation along the cave, above all in winter. This seems to be one of the most important factors regarding dust deposits, since literally no dust was observed in side passages with no air currents. Dust deposits that darken the cave walls and flowstone can thus be attributed to the infrastructure in the cave. The deposits deteriorate the natural appearance of the cave and diminish its value as a show cave. The most serious issue, however, is that the deposits may also have a negative impact on cave fauna, since several heavy metals were observed in the particles in concentration levels that are much higher than their natural abundance. This aspect is of particular importance and should be included in future research. Finally, transportation of the particles along the main passage in the cave could, in our opinion, be substantially diminished by employing such simple measures as closing the entrance doors during winter (e.g., Garcia-Anton et al., 2012), in this way diminishing/disabling the supply of cold ambient air into the cave that subsequently impels air currents. The importance of draughts through a cave on the abundance and distribution of particles was also pointed out by Grgić et al. (pers. comm.). They recently studied the concentration of the particles in the cave air of the nearby Skocjanske jame (Skocjan Cave) and found that at times when the entrance door was opened for visitors, significantly higher concentrations of smaller size particles were observed in the cave air than at times when the entrance door was closed.

ACKNOWLEDGEMENTS

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temperature, which leads to a progressive increase in CaCO₃ solubility, and also a drastic drop of solubility near the water table due to the loss of CO₂. As a result a geochemical zone of carbonate dissolution and zone of carbonate precipitation appear (Dublyansky, 2000a).

Where H₂S rich waters mix with shallower oxygen rich waters, sulfuric acid forms at or near water table, which rapidly dissolves the carbonate rocks (Egemeier, 1981). This process is known as sulfuric acid speleogenesis; thermal carbonic speleogenesis; hypogenic cave; Provalata Cave, Macedonia

Abstract: Provalata Cave (Republic of Macedonia) is a small but remarkable hypogenic cave, developed in Cambrian marbles by successive thermal carbonic and sulfuric acid speleogenesis. The cave has a thick partly corroded calcite crust, abundant gypsum deposits, with cupolas, ceiling and wall channels, feeders and replacement pockets as some of the most characteristic morphological features. Distribution of morphology and deposits suggest a hypogenic origin in two distinct speleogenetic phases: the first by thermal CO₂ rich waters, the second by sulfuric acid dissolution, which were separated by complete infilling of cave passages with pyroclastic-derived clays. In the first phase of speleogenesis, cave passages were formed by dissolution along fractures due to cooling of rising carbonated thermal waters. These phreatic morphologies were later covered with a thick calcite crust deposited in a shallow phreatic environment. In Early Pleistocene the cave was completely filled with clays due to deposition of pyroclastic rocks in a lacustrine environment in the nearby Mariovo Basin. Mariovo Lake sediments were later incised by the Buturica River, which cut down into Cambrian marbles, creating its superimposed valley. Incision lowered the water table and allowed removal of the clay deposits in Provalata Cave. The second phase of speleogenesis started after introduction of H₂S associated with rising thermal waters. Oxidation produced sulfuric acid, which rapidly dissolved first calcite crust, then marble host rock. Condensation-corrosion by sulfuric vapors replaced carbonate rock with gypsum producing replacement pockets as well as second generation of pockets and cupolas. The contact of sulfuric acid with the clay deposits formed alunite, jarosite, and natroalunite. ⁴⁰Ar/³⁹Ar dating gave maximum ages of 1.6 Ma (alunite) and 1.46 Ma (jarosite) for this last stage of speleogenesis, thus making it the second ⁴⁰Ar/³⁹Ar dating of a sulfuric cave in Europe (after Kraushöhle in Austria), and the first dated cave in the Republic of Macedonia.

Keywords: sulfuric acid speleogenesis; thermal carbonic speleogenesis; hypogenic cave; Provalata Cave, Macedonia

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INTRODUCTION

Two major classes of hypogenic caves form due to dissolution of carbonate rocks: by CO₂ and H₂S rich waters with frequent but not systematic thermal characteristics (Palmer, 1991; Dublyansky, 2000a).

Rising thermal carbonic waters cool down along their flow path, increasing their aggressiveness due to the inverse relationship between solubility and temperature, which leads to a progressive increase in CaCO₃ solubility, and also a drastic drop of solubility near the water table due to the loss of CO₂. As a result a geochemical zone of carbonate dissolution and zone of carbonate precipitation appear (Dublyansky, 2000a).

Where H₂S rich waters mix with shallower oxygen rich waters, sulfuric acid forms at or near water table, which rapidly dissolves the carbonate rocks (Egemeier, 1981). This process is known as sulfuric
acid speleogenesis and has been recognized as a significant cave forming process in many caves around the world, such as Carlsbad and Lechuguilla caves in New Mexico (Hill, 2000), Frasassi Caves in Italy (Galdenzi & Menichetti, 1995), Cueva de Villa Luz in Mexico (Hose & Pisarowicz, 1999), Chat Cave in France (Audra, 2007), Cerna Valley caves in SW Romania (Onac et al., 2011), and Kraushöhle in Austria (Plan et al., 2012). Above the water table H$_2$S escapes in the cave air and redissolves in water condensation droplets on cave walls. There it oxidizes to sulfuric acid, which attacks the carbonate rock and converts it to gypsum by forming replacement gypsum crusts on cave walls and ceiling. Replacement gypsum crusts may detach and form mounds or blocks of massive gypsum on the floor (Egemeier, 1981; Galdenzi & Maruoka 2003). Massive gypsum can also form below water table as a result of sulfate supersaturation of the groundwater, as documented in the caves of the Guadalupe Mountains in U.S.A where the rising water contained much sulfate that escaped reduction, which gave the water a head start in depositing subaqueous gypsum (Palmer & Palmer, 2000); however such conditions for subaqueous gypsum remain seldom. In contact with alumino-silicates, sulfuric acid produces a characteristic suite of minerals such as alunite, jarosite, natroalunite, hydrated halloysite. The K-rich sulfate minerals formed during the speleogenesis, and Ar-Ar dating of these minerals have potential to yield the age of the cave forming process (Polyak and Provencio, 2001).

This paper presents results of a morphological study, combined with X-ray and stable isotope analyses of cave deposits, to determine the origin and evolution of Provalata Cave, a small cave in the southern part of the Republic of Macedonia (Fig. 1), formed on the edge of a Neogene coal bearing basin. The cave was first explored by caving clubs Peoni and Ursus Speleos from Skopje, and first published in the scientific literature (as Gulabinka Cave) by Kolčakovski et al. (2004). They noted the presence of gypsum in the cave and suggested that the origin of the cave was connected with dissolution of marble by hydrothermal waters enriched with H$_2$S, although concluded that the cave was a fossil ponor cave.

Our results suggest an origin in two recognizable speleogenetic phases, a first thermal carbonic phase responsible for the thick calcite crust and a second sulfuric acid phase creating abundant gypsum deposits.

**LOCATION AND GEOLOGICAL SETTING**

Provalata Cave is located near Melnica and Vitolšte villages in Mariovo, in the southern part of Republic of Macedonia, on the eastern edge of a Neogene tectonic basin (Mariovo Basin), at the top of the southern slope of the superimposed valley of the Buturica River (Fig. 2). In a wider tectonic sense this area is part of the Pelagonian Massif, a pre-Cenozoic major tectonic unit, which consists of Precambrian gneiss and schist rocks, covered by a thick section of dolomitic and calcitic marble in the upper part, and with abundant granitic plutons (Dumurdžanov et al., 2005).

The cave is mostly formed in Cambrian marbles with some (mostly upper) parts formed in marble breccia, which together with the underlying Precambrian dolomitic marbles are part of the Vepřčani Monocline that has a NNW-SSE direction and dips to the ENE by 25 to 50°. In the northern parts these Cambrian marbles have lenses of phyllito-micaschists, and to the south they lie directly over Cambrian phyllito-micaschists (Dumurdžanov et al., 1976). One such lense is located just west of the cave (the cave is formed near the contact of marbles and phyllito-micaschists).

To the south and east, these Pre-Cenozoic rocks are covered by Neogene and Quaternary lacustrine and pyroclastic sediments of the Mariovo Basin (Dumurdžanov et al., 2004) and are described as follows:

- **Nerezi Formation (Upper Miocene)** - gravel and sandstone; siltstone and silty claystone that grades upward into claystone and coal; and siltstone and sandstone followed by a hiatus.
- **Solnje Formation (Pliocene)** - poorly stratified gravel and sandstone, overlain by:
  - **Vitacevo Formation (Pliocene)** - stratified tuff overlain by sandstone and gravel interbedded with beds of diatomite, tuff, and sandy claystone; travertine deposits, tuff-agglomerate and sandstone.
  - **Mariovo Formation (Pleistocene)** - pyroclastic rocks with nine travertine layers and a 20-m-thick travertine deposit on top.

Dumurdžanov et al. (2004, 2005) interpret the evolution of the Neogene basins in Macedonia to consist of 5 cycles, with the Mariovo Basin forming in cycle II (late Miocene: late Sarmatian-Meotian), with a hiatus of deposition at the end of cycle III (Pontian). This hiatus may be a result of the Messinian Salinity Crisis, which influenced the Vardar zone at the end of the Nerezi Formation (Pliocene Gilbert-type delta was found in Dračevo, near Skopje; Clauzon et al., 2008). In

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Fig. 1. Location of Provalata Cave (red cross) in the Republic of Macedonia. Karst rock outcrops are shown in grey shades. Major tectonic (pre-Cenozoic) units in Macedonia: I: Chukali-Krasta unit; II: Western Macedonian unit; III: Pelagonian Massif; IV: Vardar zone; and V: Serbo-Macedonian Massif.
Pliocene – cycle IV (Solnje Formation) a transgression occurred, accompanied by volcanic activity in Kožuf and Kozjak Mts. The Pliocene-Pleistocene Vitačevo and Mariovo Formations contain volcanic material and travertine. Draining of the lake systems in central Macedonia is a result of subsidence of the Aegean Sea (which started probably as late as Middle Pleistocene; Dumurdžanov et al., 2005). Mariovo Lake also drained, thus Crna Reka established its fluvial basin, including the superimposed valley of Buturica River in which Provalata Cave has formed.

**RESEARCH METHODS**

We made a detailed survey of the cave, combined with morphogenetic analyses in order to determine the presence of hypogenic morphologies. Characteristic cave deposits were sampled for X-Ray analyses. This includes samples from gypsum, grey clay, yellow sand, pinkish clay, pale yellow sand and a dark crust under the gypsum crust. X-ray analyses were carried out at the CEREGE, France. X-ray powder diffraction (XRD) patterns were recorded on a Philips diffractometer using Cobalt radiation (λ = 1.79 Å) with a secondary graphite monochromator. The diffractometer optic used to record all samples was a front fixed slit of 1°, a scattered radiation slit of 1° after the sample, and a 0.2 mm detector slit. 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° 2θ using a step interval of 0.05° 2θ and a counting time of 2.5 s/interval.

Calcite crust, with a presumed thermal origin, was sampled for stable isotope analyses (δ¹³C, δ¹⁸O). The sample was collected from the ceiling of a small channel emerging from the north part of the First Room, and a 0.2 mm detector slit. 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° 2θ using a step interval of 0.05° 2θ and a counting time of 2.5 s/interval.

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The sulfur isotope ratio of cave gypsum was analyzed at the Institute of Mineralogy and Geochemistry of the University of Lausanne using a Carlo Erba 1108 elemental analyzer (EA) connected to a Thermo Fisher (Bremen, Germany) Delta V isotope ratio mass spectrometer (IRMS) that was operated in the continuous helium flow mode via a Conflo III split interface (EA-IRMS). The stable isotope composition of sulfur is reported in the delta (δ) notation as the per mil (‰) deviation of the isotope ratio relative to known standards: δ = [(Rsample - Rstandard)/Rstandard] x 1000, where R is the ratio of the heavy to light isotopes (34S/32S). The sulfur standard is the Vienna Cañon Diablo Troilite (VCDT). The reference SO2 gas was calibrated against the IAEA-S-1 sulfur isotope reference standard (Ag-S) with a δ34S value of +0.3‰. The overall analytical reproducibility of the EA-IRMS analyses, assessed by replicate analyses of three laboratory standards (barium sulfate, with a working δ34S value of +12.5‰; pyrite Ch, +6.1‰; pyrite E, −7.0‰) is better than ±0.2‰ (1 SD). The accuracy of the δ34S analyses was checked periodically by analyses of the international reference materials IAEA-S-1 and IAEA-S-2 silver sulfides (0.3‰ and +22.7±0.2‰, respectively, values from IAEA-Catalogue and Documents) and NBS-123 sphalerite (+17.09 ± 0.3‰, value from NIST-Catalogue and Documents).

Samples of alunite and jarosite (pale yellow sand) were dated by the 40Ar/39Ar method at the New Mexico Geochronology Research Laboratory in Socorro, New Mexico, USA. Two aliquots of each sample were analyzed, one unwrapped and one wrapped in Ag with a platinum crimp. The second analysis was not a complete degassing. Results were similar for both the alunite and jarosite, but some higher step ages were apparent at the end of degassing, indicating minor to trace amounts of contamination from another older phase. Because of this, the results are considered maximum ages. Actual crystallization ages could be slightly younger.

Water from the small thermal spring below Provalata was sampled and analyzed for basic physical and chemical parameters, at the Center for Public Health in Prilep, Macedonia. Field measurements of pH, EC, and temperature were made with a HI 98129 (Hanna Instruments) multi-parameter tester.

RESULTS

Cave morphology

Provalata is a small cave with a total length of about 230 m and total depth of 24 m. There are two major rooms (First and Second) with more or less vague (due to collapse) outlines, and two main passages (Lower and Upper) with more distinct (fracture guided) morphology (Fig. 3).

The most representative and common morphological features of the cave are cupolas and solution pockets (Fig. 4 A, D). Cupolas vary in form and size, often combining several cupolas overlain on another. They are most abundant in the Lower Passage as well as in the Upper Passage, but the highest ones (up to 7 m) are in the First Room, in marble breccia, as well as the entrance shaft which is a cupola formed in marble breccia opened to the surface by collapse. Two generations of cupolas and solution pockets can be clearly detected in the cave: one formed in marbles (or marble breccia) and covered with calcite crust, and the other formed in the calcite crust covering the first cupolas, or completely cutting through the calcite crust to the first generation cupula, and creating a secondary cupula or solution pocket.

Half tube wall channels are found in the Lower Passage, starting from feeders and leading to ceiling channels and cupolas (Fig. 4 C-F). They are 10-15 cm wide, mostly vertical and sometimes bifurcating. They rise from discrete small feeding channels along the floor sides, and lead to a central ceiling channel. The ceiling channel is curvilinear in plan view, 30-40 cm in diameter, and continues to SW to the neighbor passage rising in a big cupula. The small feeding channels have similar dimensions as the wall channels they lead to. The passage wall between rising wall channels is covered with white calcite popcorn speleothems. The popcorn distribution starts from the central ceiling passage downwards, with popcorn speleothems covering some of the half tube passages as well. Small gypsum crusts are also covering lower parts of passage walls with detached crust covering the floor near the walls. Along some prominent fractures the half tube wall channels lead to cupolas.

The genesis of this set of small feeding channels rising to half tube channels, which converge in a central ceiling channel is attributed to condensation corrosion, with the feeding channels representing vents supplying the rising moist air. Rising vapor from the vents cools and condenses at the contact with wall in upper parts producing film runoff due to accumulation of condensation. Cooler air that sinks warms up and produces evaporation. This leads to condensation-corrosion in the upper part, and evaporation-deposition in the lower part (Audra et al., 2007). The condensation-corrosion is largest at the ceiling producing the central ceiling channel, with popcorns and replacement gypsum deposited bellow the ceiling channel along the passage walls due to evaporation. The ceiling channel then continues to SW to the neighbor passage rising to a big cupula developed in calcite crust.

Gypsum replacement pockets (cf. Galdenzi and Marouka, 2003) can be found at several places with gypsum deposits already removed. In the Upper Passage they are developed in marble between two cupolas (Fig. 4 A, B), but replacement pockets formed in calcite crust can be also seen in the First Room. Their size ranges from few centimeters up to few decimeters in diameter.

Cave deposits

A calcite crust, black to transparent, up to 0.5m thick and highly corroded (Fig. 5 E), is covering the walls and ceiling throughout the cave. In places it is completely corroded.

It covers various channel features with phreatic morphologies, such as cupolas, and solution pockets.
The crust has mammillary morphology with mostly acicular calcite crystals. The morphology and thickness suggest formation by carbonic degassing in shallow thermal waters (Palmer, 1991; Dublyansky, 2000b; Audra, 2009a) preceding the clay infilling and sulfuric acid phase.

Five samples from a ~20 cm cut through the calcite crust and marble host rock (Fig. 6) were collected for δ¹³C and δ¹⁸O analyses, two of which were from the weathered marble host rock (C1, C2) and three from the calcite crust (C3, C4, C5). All of the five samples have light δ¹⁸O ratios (-12.7 to -10.4‰), but quite heavy δ¹³C ratio (2.8 to 7.2 ‰). The values of δ¹⁸O are within the typical range for thermal calcite (Dublyansky, 2000b), but the values for δ¹³C are quite high for the calcite.

Grey clays (Fig. 5 B) cover the lower parts of cave passages, but small patches of grey clay can be found...
Gypsum deposits are the most characteristic cave deposits. They are found in every part of the cave, except some places, which have periodic vadose percolation, where gypsum most likely was dissolved. Gypsum deposits are present as replacement crusts up to 40 cm thick (Fig. 5 F), or detached replacement gypsum crusts (Fig. 5 G) that accumulated in gypsum blocks (Fig. 6 H). Preliminary stable isotopes analyses (Table 3) of sulfur from cave gypsum (2 samples) gave δ^{34}S values of -2.3 to -1.9‰.

The origin of H_2S involved in the sulfuric acid speleogenesis of Provalata Cave might be attributed to the coal deposits, if we consider the proximity of the coal deposits in Mariovo Basin to Provalata Cave, and the fact that no sulfate rocks have been found in the stratigraphy in this part of Macedonia. Sulfur in the coals from Mariovo Basin is found as organic sulfur, pyrite and gypsum (Lerouge et al., 2007). Gypsum in coals can be a weathering product of pyrite when occurring closely connected with pyrite, while gypsum in fine layers associated with calcite is considered to be formed by crystallization of calcium and sulfate ions dissolved in the pore water during the sedimentation (Lerouge et al., 2007). Total sulfur
Fig. 5. Deposits in Provalata Cave. A: Schematic cross-section of sediments, showing stratigraphic relationships; B: Grey clay (montmorillonite, kaolinite, sanidine, albite, muscovite, quartz); C: Pale yellow sand (alunite, jarosite, muscovite, quartz); D: Pink clay (halloysite, natroalunite, muscovite, quartz); E: Corroded calcite crust; F: Replacement gypsum crust; G: Gypsum crust, detached from the wall; H: Detached gypsum crust piled as gypsum blocks.

Table 1. Mineralogical composition of clays from Provalata Cave and caves in the nearby Vitačevo Plateau.

<table>
<thead>
<tr>
<th>Cave</th>
<th>Sediment (sample)</th>
<th>Mineralogical composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Provalata</td>
<td>Grey clay (PR04)</td>
<td>montmorillonite, kaolinite, sanidine, albite, muscovite, quartz</td>
</tr>
<tr>
<td>Provalata</td>
<td>Pink clay (PR08)</td>
<td>natroalunite, halloysite, muscovite, quartz,</td>
</tr>
<tr>
<td>Provalata</td>
<td>Pale yellow sand (PR10)</td>
<td>alunite, jarosite, muscovite, quartz,</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Caves in nearby Vitačevo Plateau</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dragoželska Propast</td>
</tr>
<tr>
<td>Aramiska Peštera</td>
</tr>
<tr>
<td>Čulejca</td>
</tr>
</tbody>
</table>

content in the coals is low, 1.18 to 1.3% (Raleva et al., 2012), with high sulfur content in macerals (1.2 to 2.3%), showing that measured sulfur can be attributed to organic matter (Lerouge et al., 2007). δ³⁴S values are ranging from +8.5 to +17.3‰ for gypsum in coal seams and from +5.4 to +13.2‰ for vitrinite.

Considering the complexity of sulfur isotopic evolution that can derive from generation of H₂S by bacterial sulfate reduction or thermochemical sulfate reduction, depending on paucity and/or supply of hydrocarbon electron donors and sulfate (Wynn et al., 2010), as well as modification due to contribution of secondary source of S (e.g., pyrite, Onac et al., 2011), further stable isotope analyses of cave gypsum, coal and sulfate of Melnica spring are necessary to determine the origin and evolution of sulfur participating in the sulfuric speleogenesis of Provalata Cave.
Calcite popcorns are covering the corroded calcite crust at various places. Also small flowstone deposits are developing in a few places in the cave as a result of late vadose percolation.

**Remnant features on the surface**

Remnants of cupolas and solution pockets are also found on the surface, in the small gully in the gorge, which follows the contact of marbles and the phyllito-micaschist lens. Remnants of a similar calcite crust can be found on the surface, near the entrance of the cave (Fig. 2 B; Fig. 8).

**Melnica spring**

In 2010, a small spring in the riverbed of the Buturica River, just below Provalata Cave (Fig. 2 B), reemerged after (according to the local population) ~40 years of inactivity. The spring is discharging at several locations along the left bank of Buturica River, near the contact of Cambrian marbles with a lens of phyllito-micaschists. Considering that both Provalata Cave and the spring are located along the contact of marbles with phyllito-micaschist lens, and remnants of cupolas with calcite are found in between, along the same contact, the spring might be the present discharge point of the same system that formed Provalata Cave.

Preliminary analysis of some physical and chemical parameters show slightly thermal waters (20-22°C), with a high amount of dissolved solids, EC values from 891-972 μS/cm, and slightly acidic pH ranging from 6.62-6.9 (Table 4).

If Melnica spring is considered as a late stage phase of Provalata Cave evolution due to incision of Buturica River, then the question of cease of sulfuric acid dissolution remains. If origin of H₂S for Provalata Cave sulfuric acid speleogenesis is attributed to the nearby coal deposits, then the sulfuric phase can be a temporal manifestation, which ceased after lowering of water table below the elevation of the coal field. Further analysis of Melnica spring chemistry, cave gypsum and Marivo coal deposits are needed for this hypothesis to be tested.

**DISCUSSION**

The thick mammillary calcite crust was deposited in shallow phreatic environment by thermal waters, filling previously formed passages and convectional features (cupolas, pockets) formed in a previous phase in deeper parts of the thermal system due to cooling effect of rising carbonated thermal waters.

The cave was later completely filled with clay deposits (Fig. 9), originating from weathering of pyroclastic sediments most likely from Marivo Formation, which were deposited in a lacustrine environment in Early Pleistocene (Dumurdžanov et al., 2004).

Presence of alunite, jarosite, natroalunite in altered clay deposits, combined with large deposits of gypsum found as replacement crusts or gypsum blocks, and morphological features such as gypsum replacement pockets point towards dissolution by sulfuric acid.

Gypsum replacement crusts in sulfuric caves are considered to be of sub-aerial origin by condensation of H₂S rich vapors on carbonate rock (Galdenzi and Marouka, 2003; Palmer, 2007, 2013). In Provalata Cave they are found covering walls in several locations, with best examples found in the Second Room and Upper Passage, where detached crust are found piled below still standing wall crusts.

Morphological features such as pockets and cupolas developed in calcite crust, as well as trough the calcite crust in the marble host rock suggest second phase of dissolution after the deposition of the calcite crust. Based of their association with gypsum deposits (gypsum replacement crusts and replacement pockets found on calcite crust), their origin is attributed to dissolution by sulfuric acid. At places these second generation cupolas are also connected to feeders and wall and ceiling half tube channels. Such features are often indicative of convective cells in phreatic conditions (the “morphological suite of rising flow”, Klimchouk, 2007), but can be also formed by condensation corrosion above the water table, by highly corrosive vapors (Audra, 2007; Audra et al., 2007, 2009b; Palmer, 2013). The rising suite in the Lower Passage was produced by condensation corrosion with the small feeding channels representing vents supplying rising moist air, with condensation-corrosion producing half tube wall and ceiling channels and popcorn speleothems and gypsum crust depositing in the lower parts due to evaporation.

### Table 2. Summary of ⁴₀Ar/³⁹Ar results.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lab #</th>
<th>Irradiation</th>
<th>Mineral</th>
<th>Age analysis</th>
<th>Steps/analyses</th>
<th>Age (Ma)</th>
<th>±2σ</th>
<th>MSWD</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Provalata-a</td>
<td>60983</td>
<td>247</td>
<td>alunite</td>
<td>laser step-heat</td>
<td>3</td>
<td>1.60</td>
<td>0.05</td>
<td>1.03</td>
<td>maximum age</td>
</tr>
<tr>
<td>Provalata-j</td>
<td>60984</td>
<td>247</td>
<td>jarosite</td>
<td>laser step-heat</td>
<td>3</td>
<td>1.46</td>
<td>0.03</td>
<td>1.19</td>
<td>maximum age</td>
</tr>
</tbody>
</table>

Table 3. Stable isotope analyses results from marble, calcite crust (δ¹⁸O, δ¹³C), gypsum and vitrinite (δ³⁴S) from Provalata Cave and nearby coals.
The sulfate minerals (alunite, natroalunite, and jarosite) are clear evidence of alteration of clay by sulfuric acid (Polyak and Provencio, 2001). As pointed by Palmer (2007, 2013) alunite is formed by alteration of clay by low pH (less than 4) sulfuric acid. Sulfuric acid is most likely to reach such low pH needed to produce the alunite only in vadose moist droplets on clay (Palmer, 2007, 2013). The distribution of deposits and morphology connected with the sulfuric acid speleogenesis indicates that most of the dissolution in the sulfuric phase was above the water table by condensation-corrosion. Typical water table morphology such as corrosion tables, flat roof notches (Audra, 2007; Audra et al., 2009b) should be expected to develop in such environment. This was not evident in Provalata Cave and the absence of such morphology might be due to the presence of the formerly deposited grey clay. Covering the passage floors, the clay might have shielded the calcite crust or marble host rock from aggressive sulfuric waters or such morphology is located in a lower (presently not accessible) part of the cave.

$^{40}$Ar/$^{39}$Ar dating of alunite and jarosite from the First Room gave maximum age of 1.6 and 1.46 Ma. Considering their formation in a vadose environment, such condition could have been achieved only after the draining of Mariovo Lake and incision of the Buturica River Valley.

Since last layers of tephra can be found in the travertine layers deposited in lacustrine environment as end part of Mariovo formation, with volcanic activity in Kozjak (Kožuf) Mt. active from
4.0 ± 0.2 to 1.8 ± 0.1 Ma (Kolios et al., 1999), and with the oldest maximum age of cave alunite at 1.6 Ma we can place the draining of Mariovo Lake, and onset of fluvial drainage somewhere between 1.8 and 1.6 Ma.

With further incision of Buturica River, cave features (cupolas, pockets, calcite crust) were exposed on surface on the valley slope, due to slope retreat. The low thermal Melnica Spring found below the cave in the river bed is likely the present discharge point of the system.

Considering this, the calcite crust and the cave features covered by it, which represent the first thermal carbonic phase, formed before deposition of Mariovo Formation and are probably from Late Pliocene-Early Pleistocene age.

**CONCLUSIONS**

Provalata Cave is a small but remarkable cave in the southern parts of Republic of Macedonia, with two successive phases of thermal carbonic, then sulfuric acid speleogenesis, separated by deposition of pyroclastic-derived clay deposits. It is also the second ⁴⁰Ar/³⁹Ar dated sulfuric acid cave in Europe, after Kraushöhle in Austria, and the first dated cave in Republic of Macedonia.

Two sets of morphologies associated with different sets of deposits were identified and attributed to two separate speleogenetic phases: phreatic morphologies (cupolas) formed by cooling of rising carbonated thermal waters, covered with mammillary calcite crust (first phase); vadose morphologies (replacement pockets, second generation cupolas and pockets, vents, half tube wall and ceiling channels), formed by condensation-corrosion by sulfuric vapors (second phase), with second phase morphologies partly or completely imprinted onto the first phase morphologies.

In the first phase (Pliocene-Early Pleistocene?), cave passages were formed by dissolution of marbles due to cooling of thermal carbonated waters, and were later covered with thick calcite crust deposits due to shift in shallower phreatic environment. These passages were then completely filled with clay deposits, originating from weathering of pyroclastic sediments of Mariovo Formation, which were deposited in lacustrine environment (Early Pleistocene). Between 1.8 and 1.6 Ma BP, Mariovo Lake drained and Buturica River incised first in deposits of Mariovo Formation, then forming superimposed valley and cutting trough the Cambrian marbles. This lowered the water table, and allowed removal of clay deposits. After introduction of H₂S in the thermal waters (formed probably by reduction of sulfates in the nearby coal basin), oxidation produced sulfuric acid that rapidly dissolved first calcite crust, then marble host rock, with condensation-corrosion by sulfuric vapors producing replacement of carbonate rock with gypsum. At contact of sulfuric acid with clay

**Table 4. Some basic physical and chemical parameters of the Melnica thermal spring.**

<table>
<thead>
<tr>
<th>Date</th>
<th>T (°C)</th>
<th>pH</th>
<th>EC (µS/cm)</th>
<th>Hardness (dH)</th>
<th>HCO₃ (mg/l)</th>
<th>Ca (mg/l)</th>
<th>Mg (mg/l)</th>
<th>NO₃ (mg/l)</th>
<th>Cl (mg/l)</th>
<th>Fe (mg/l)</th>
<th>SO₄ (mg/l)</th>
<th>Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>21/07 2012</td>
<td>22</td>
<td>6.9</td>
<td>891</td>
<td>18.6</td>
<td>634.4</td>
<td>105.7</td>
<td>34.6</td>
<td>3.18</td>
<td>8</td>
<td>0</td>
<td>14.7</td>
<td>lab</td>
</tr>
<tr>
<td>24/11 2012</td>
<td>21.5</td>
<td>6.64</td>
<td>945</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>field</td>
</tr>
<tr>
<td>27/01 2013</td>
<td>21</td>
<td>6.74</td>
<td>892</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>field</td>
</tr>
<tr>
<td>09/02 2013</td>
<td>20.6</td>
<td>6.62</td>
<td>914</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>field</td>
</tr>
<tr>
<td>24/02 2013</td>
<td>21.1</td>
<td>6.7</td>
<td>972</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>field</td>
</tr>
</tbody>
</table>
deposits, alunite, jarosite, and natroalunite were formed, giving a maximum age of 1.6 Ma (alunite) and 1.46 Ma (jarosite) by ⁴⁰Ar/³⁹Ar dating. As Buturica River incised, the water table lowered and the sulfuric acid dissolution shifted downward. Today Provalata Cave is 100 m above the riverbed of Buturica River, where a small low temperature thermal spring (Melnica Spring) is located.

ACKNOWLEDGEMENTS

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REFERENCES


Unusual polygenetic void and cave development in dolomitized Miocene chalks on Barbados, West Indies

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2Department of Geosciences, Mississippi State University, Mississippi State, Mississippi, USA
3Department of Earth and Atmospheric Sciences, University of Alberta, Edmonton, Alberta, Canada

Abstract: Barbados provides an unusual case of polygenetic cave development within dolomitized chalks and marls of the Miocene Oceanics Group. These diagenetic processes are driven by a succession and interplay of tectonic uplift, fracturing, hypogene fluid injection, overprinting by mixing zone diagenesis, and mechanical and biological erosion in the current littoral zone. The significance of the voids and caves within the chalks on Barbados are: 1) these appear to be the first dissolution caves documented in dolomitized chalk, and 2) these features show a polygenic origin documenting the diagenetic changes in lithology that allowed the development and preservation of these cave types.

Keywords: dolomitized chalk; polygenic caves; Barbados; multi-stage dissolution

INTRODUCTION

Globally, dolomitized chalks are rare and relatively small in extent; however dolomitization in what is typically an impermeable unit is important when considering their diagenetic and hydrologic history. Wolfe (1970) reported dolomite locally in the Senonian chalks of Northern Ireland, derived from modified seawater. Joergensen (1983) reported dolomite cements in chalks from the North Sea Central Graben. Dolomitization of Late Cretaceous chalk in the southeastern Paris Basin was attributed to seawater entering the chalk as groundwater in structural depression areas (Thiry et al., 2003). On Barbados, dolomite in the Miocene chalk is fine-crystalline and replacive, attributed to replacement by methane-bearing fluids in cold seep environments (Machel et al., 2012).

Caves in chalk are relatively rare. Several chalk caves are found in the English chalk (Bradshaw et al., 1991). In France and Belgium large chalk caves are more common, and caves over a kilometre in length have been explored (Waltham, 1975; Rodet, 1985). Small conduits (50-300 mm in diameter) have been reported in cliff exposures and quarry faces in England (Maurice et al., 2006). However, there are apparently no reported caves currently known in dolomitized chalks.

Barbados

Barbados is an uplifted carbonate island located on a forearc bulge, formed on the crest of an accretionary prism, about 125 km east of the Lesser Antilles volcanic island arc. The island is approximately 23 x 32 km, with 432 km2 of area (Fig. 1). Glacioeustasy and episodic tectonic uplift have created a series of limestone terraces fronted by cliffs (e.g. Humphrey, 1997). The island conforms to the Carbonate Island Karst Model (CIKM) classification of a composite island (Mylroie & Mylroie, 2007).

The strata of the island from oldest are: 1) Eocene, tectonically deformed deep-sea clastics of the Scotland Formation; 2) flat-lying Miocene chalks and marls of the Oceanics Group; and 3) a series of Pleistocene carbonate terraces that are pervasively karstified (e.g., Speed, 1990; Taylor & Mann, 1991; Machel, 1999; Jones & Banner 2003). The clastics are exposed in the Scotland District (Fig. 1), and stream caves have developed in the Upper Coral Reef Terrace. The most conspicuous surface and near-surface post-depositional attributes of the Pleistocene carbonates are karst features, which include several dozen deeply incised gullies (dry valleys), abundant sinkholes, and caves of various sizes and origin (Day, 1983; Machel, 1999, 2011; Machel et al., 2012).

Geological framework

The ‘basement’ of Barbados is a structurally complex accretionary prism complex composed of terrigenous turbidites and gravity-flow deposits interbedded with hemipelagic and pelagic radiolarians of Eocene age (Speed, 1990). The Scotland Sandstone, which is part of
Tectonic diapirs consisting of a mélange of organic mud matrix intrude the pelagics of the Scotland District units. Emplacement of these diapirs likely continues today and is possibly responsible for the elevation of Barbados above the rest of the accretionary prism (Speed, 1990). Throughout most of the Neogene, Barbados has experienced tectonic uplift at rates averaging approximately 0.3 to 0.4 m ky\(^{-1}\) (Speed, 1990).

A layer of Miocene chalks and marls, up to 50 m thick, called the Oceanics Group, overlies the deformed clastics of the Scotland Formation (Senn, 1946). The presence of natural oil seeps indicates that the chalks are likely breached by fractures, or missing as a local confining unit in a few locations.

The Oceanics Group is overlain by a series of Pleistocene carbonate terraces fronted by cliffs. These cliffs were formed by the interplay of glacioeustasy, episodic tectonic uplift, and erosion. The three major terraces, which consist of multiple smaller-scale terraces and sedimentary parasequences, are the Upper Coral Rock terrace, Middle Coral Rock terrace, and Lower Coral Rock terrace (Fig. 1). The ages of these terraces are approximately 800-500 ka, 490-250 ka, and 120-60 ka, respectively (Humphrey, 1997; Machel, 1999, 2011; Schellmann & Radtke, 2004), and they cover about 85% of the island. Additional information about the geotectonic and sedimentary evolution of the island can be found in Jukes-Browne & Harrison (1891), Speed (1983, 1990, 2002), Torrini et al. (1985), Humphrey (1997), Machel (1999, 2011), Schellmann & Radtke (2004); and Machel et al. (2012).

Karst

Broadly, karstification and karst features fall into two genetic groups: epigene (also called epigenetic or epigenic), whereby dissolution is coupled to surface water hydrology; and hypogene (also called hypogenetic or hypogenic), whereby dissolution is decoupled from surface hydrological processes, and dissolutional aggressivity is developed at depth (Palmer, 2000; Klimchouk, 2007). According to Palmer (2000), carbonate hypogene caves are the product of dissolution in water in which the aggressiveness has been produced at depth beneath the surface, independent of surface or soil CO\(_2\) or other near-surface acid sources. In addition to the geochemical definition above, there is another approach at defining hypogene speleogenesis, based on the hydrogeologic setting. This hydrogeological approach defines hypogene speleogenesis as the formation of solution-enlarged permeability structures by water that recharges the cavernous zone from below, independent of recharge from the overlying or immediately adjacent surface (Ford, 2006; Klimchouk, 2007, 2013). Flank margin caves are a unique subset of coastal caves considered hypogene by the geochemical approach (Mylroie & Mylroie, 2007; Palmer 2007), but not by the hydrogeologic approach. Flank margin caves form at the flank of an enclosing landmass in the distal margin of a freshwater lens by the mixing of saline and fresh waters. Flank margin caves were originally described from eogenetic rocks in the Bahamas; however, the terms have been expanded to other coastal and diageneric settings globally (Mylroie & Mylroie, 2007).

Fig. 1. Simplified geologic map of Barbados showing sampling sites (modified from Machel, 1999). Chalks of the Oceanics Group are not resolvable at this scale. They are found as localized outcrops or as parts of cliffs capped by Pleistocene limestones.

Fig. 2. One of the most prominent oil seeps at Conset Bay. Oil can be seen seeping from joints within the chalk (photo by J. Mylroie).
Unusual cave development in dolomitized chalks on Barbados

Detected in the chalks of the Oceanics Group were calcite and dolomite. Calcite was the dominate carbonate mineral in most outcrops of the chalk; however, the outcrop at Cove Bay West contained significantly higher dolomite percentages. No other minerals were detected. Dolomitized chalks were typically harder and more resistant to weathering. Chalk outcrops also contained a series of caves large enough to be mapped and other smaller voids that were photodocumented (Figs. 5 and 6).

The δ13C values of dolomite from all locations vary from +4.9 to -23.0 ‰ PDB and for calcite vary from +7.3 to -22.6 ‰ PDB (Table 2 and Fig. 7). See Table 2 for isotopic values of all samples.

Objectives
The objectives of this study are documentation of the unusual caves developed within a relatively rare geologic occurrence of dolomitized chalk on Barbados and the development of a genetic mechanism for the caves.

Methods
Fieldwork for this study was conducted in December 2009 and December 2011. A total of thirty-three rock samples were taken from the Miocene chalk at Cove Bay, Gent’s Bay, Pico Teneriffe, Conset Bay, and Corben’s Bay (Fig. 1). Bulk samples, ranging from a few grams to about 1 kg in mass, were collected for petrographic and geochemical analyses from outcrops preferentially along joint and fracture planes, and from the walls of voids.

Samples were powdered in the laboratory for X-ray diffraction (XRD) and geochemical analyses using a low speed dental drill assembly. Powder XRD patterns were obtained using a Rigaku Ultima IV diffractometer. X-ray diffraction patterns were obtained as follows: continuous mode, 0.02° 2θ per step, 4° 2θ per minute, 3-70° 2θ, CuKα radiation. Mineral percentages were estimated from relative intensities of peak heights of X-ray diffraction lines using JADE 9. Quartz powder was added to powder mixtures as a standard due to its lack of interfering peaks with calcite and dolomite. Duplicate analyses indicate a reproducibility of ±5% of relative calcite/dolomite mixtures. Standard thin sections of 30 μm were used for petrographic analysis with the assistance of staining by Alizarin Red-S for identification of carbonate species.

Carbon and oxygen isotope analyses were performed according to standard procedure (McCrea, 1950) at the University of Calgary. The results were reproducible within ± 0.1 ‰ for δ18O and δ13C. The dolomite δ18O values were corrected by the –0.82 ‰ phosphoric acid fractionation (Scharma & Clayton, 1965). Samples of mixed calcite-dolomite mineralogy were separated by standard differential acid digestion (1-3 hours for calcite, 2-4 days for dolomite; Schrama & Clayton, 1965). The caves were mapped using a tape or Disto® laser rangefinder and Suunto® compass. Field sketches were scanned and rectified using the cave mapping software Compass®. Cave dimensions were measured from the resulting map using the NIH freeware Image J.

Results
Outcrops of the chalks and marls of the Oceanics Group were investigated on Barbados. An outcrop at Cove Bay was the focus of the majority of this study (Figs. 1 and 3). Cove Bay is a crescent-shaped embayment on an otherwise relatively straight eastern coastline, opening into the Atlantic Ocean. The north face of the bay exposes chalk over a distance of more than 100 m. This outcrop has been divided into east and west sections based on the presence of a rocky spur in the western portion.

In thin section, samples of the chalk are typical mudstones with abundant microfossils (Fig. 4A). Narrow fractures are present, typically lined with microsparitic dolomite cement, sometimes containing residual dead oil (Fig. 4B). The mineralogy calculated from X-ray diffraction is presented in Table 1. The two main minerals
detected in the chalks of the Oceanics Group were calcite and dolomite. Calcite was the dominate carbonate mineral in most outcrops of the chalk; however, the outcrop at Cove Bay West contained significantly higher dolomite percentages. No other minerals were detected. Dolomitized chalks were typically harder and more resistant to weathering. Chalk outcrops also contained a series of caves large enough to be mapped and other smaller voids that were photodocumented (Figs. 5 and 6).

The δ¹³C values of dolomite from all locations vary from +4.9 to -23.0 ‰ PDB and for calcite vary from +7.3 to -22.6 ‰ PDB (Table 2 and Fig. 7). See Table 2 for isotopic values of all samples.

As previously mentioned, the chalks with higher dolomite concentrations were hard, and these outcrops of chalk contained abundant fractures (Fig. 8). As indicated by the presence of hydrocarbon seeps, hydrocarbons have exploited and migrated up these fractures (Fig. 2). Fractures are further coincident with caves, and these caves are aligned along a horizontal datum (Fig. 3).

Table 1. Table of relative dolomite percentages from Barbados samples.

<table>
<thead>
<tr>
<th>Sample:</th>
<th>Dolomite %</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5 DOL</td>
<td>100</td>
<td>Cove Bay(NW)</td>
</tr>
<tr>
<td>A6 DOL</td>
<td>100</td>
<td>Cove Bay(NW)</td>
</tr>
<tr>
<td>A7 DOL</td>
<td>75</td>
<td>Cove Bay(NW)</td>
</tr>
<tr>
<td>A8B DOL</td>
<td>50</td>
<td>Cove Bay(NW)</td>
</tr>
<tr>
<td>A8C DOL</td>
<td>40</td>
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</tr>
<tr>
<td>A8D DOL</td>
<td>60</td>
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</tr>
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<td>A9 DOL</td>
<td>50</td>
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</tr>
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<tr>
<td>Z2 DOL</td>
<td>95</td>
<td>Cove Bay(NW)</td>
</tr>
<tr>
<td>Z7A DOL</td>
<td>30</td>
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</tr>
<tr>
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Fig. 5. Cove Bay West caves. A) Dissolutional pockets in the ceiling of largest cave; B) Tubular, blind passage pinching out with cuspatate morphology along the right side of the photograph.

**DISCUSSION**

**Dolomitized chalk**

The range of δ¹³C values (Fig. 7 and Table 2) of dolomite from Barbados can be attributed to three genetic mechanisms:

1) Microbial methane from the Barbados mud volcano field ranges in δ¹³C from about -60 to -65 ‰ PDB, with one exceptional sample as low as -113 ‰ PDB (Martin et al., 1996);

2) Thermogenic methane with typical values of δ¹³C ranging from -25 to -40 ‰ PDB (Schoell, 1988);

3) Soil processes whose δ¹³C values tend to range between zero and -12 ‰ PDB (Burns & Rossinsky, 1989).

Martin et al.’s (1996) data suggest input of carbon from oxidation of microbial methane, whereas δ¹³C from Barbados dolomite is as low as -23 ‰ PDB. It is likely that the isotopic range of Barbados dolomite is
related to thermogenic methane. Variable water-rock ratios and proportions of mixing between the ambient marine pore water and the expelled hypogene fluids best explain the spread in calcite $\delta^{13}$C and $\delta^{18}$O values. The few highly positive calcite $\delta^{13}$C values (+4.0 to +7.3 ‰ PDB) are interpreted as being derived from CO$_2$ in equilibrium with methane (Hudson, 1977). A complete diagenetic and petrographic history of the dolomitization, including the dolomitized chalks has been addressed (Machel et al., 2011).

In thin sections, samples of chalk are typical carbonate mudstones with abundant microfossils

**Chalk karstification**

Karstification of the chalks on Barbados follows a three-stage genetic mechanism. The first stage of karstification is the initial dissolution from upward rising fluids along fractures. This accounts for caves and other voids that are associated with fractures (Figs. 3, 5, and 9). These rising fluids likely are hydrocarbon-rich and result in dolomitization. These two phases are possibly syngenetic.

The second stage of karstification is the utilization of the early voids and fractures to host a freshwater lens. A similar utilization of fracture porosity has been documented in telogenetic limestone of New Zealand where flank margin caves developed within fracture networks along a horizontal datum interpreted as a paleosea level (Mylroie et al., 2008). While in the lens margin position, overprinting of the original voids by flank margin mixing zone dissolution occurs, which is considered a “geochemical hypogenic” environment. Also, dolomitization ‘hardens’ the chalk providing structural support of larger void by increasing the apparent shear strength.

The third stage comprises further modification of the void by pseudokarst processes when the void is exposed to littoral conditions by uplift and cliff retreat. The importance of the hardening of the rock and increased shear strength by dolomitization carries over from the previous stage by providing the mechanical support necessary for a large void to exist, which in an undolomitized equivalent would likely not persist.

The unique geologic situation of uplift and rising hydrocarbon-enriched fluids on Barbados allowed chalk dolomitization and initial hypogene dissolution. The dolomitization produced brittle rock behavior in the chalks, creating fracture networks that would be invaded by freshwater that lead to the formation of a particular geochemical environment in which dissolution (mixing corrosion) cause flank margin

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**Fig. 6.** Small oblong voids developed along fractures at Cove Bay (A) and Gent’s Bay (B).

**Fig. 7.** Oxygen-Carbon isotope plot of all data from this study. See text for further explanation.
Cove Bay West Caves
Located within the dolomitized sections of the Oceanics Group
Suunto and Disto Survey
by: M. Lace, P. Kambesis, and J. Sumrall

Plan View

Profile View

Fig. 9. Cave maps of the dolomitized outcrop at Cove Bay West. These caves occur sporadically along the cliff face just above sea level.
ACKNOWLEDGEMENTS

We would like to thank our field team (Patricia Kambesis, Mike Lace, Jeanne Sumrall, Athena Nagel,
REFERENCES


Unusual cave development in dolomitized chalks on Barbados


INTRODUCTION

The alpine karst, formed in the high mountain areas, is characterized by colder conditions due to high altitude, by restricted vegetation, seasonal snow and snow melt, and presence of past and present glaciers (Smart, 2004). Alpine karstic aquifers are invaluable reservoirs of drinking water and an important potential source for the future as well (Petrič, 2004). In karstic areas, the water from rainfall and snow melt flows through highly heterogeneous flow paths formed in the fractured rock (vadose zone with epikarst) until it reaches a saturated or phreatic zone (Gibert et al., 1994; Williams, 2008). At the contact zone between the fractured and less fractured rock or between permeable and impermeable layers, the groundwater discharges into temporary or perennial springs, which in the alpine areas usually occur at the feet of steep walls (Gams, 2003).

Karstic aquifers are inhabited by diverse hypogean aquatic fauna. Many of the hypogean species are endemic, occurring only in the particular locality or region (Culver and Sket, 2000; Gibert and Deharveng, 2002). Intensive studies of hypogean aquatic fauna over the central and southern Europe demonstrated that one of the richest regions in the number of stygobionts (i.e. strictly hypogean species) is the karstified peri-Mediterranean region and a wider Dinaric region (SE Europe) (Sket, 1999; Dole-Olivier et al., 2009b). Less information exists regarding hypogean aquatic fauna from the Alpine areas in Europe (Stoch, 2000; Sambugar et al., 2008; Cristian and Spötl, 2010; Papi and Pipan, 2011). A study from the northern Italian Alps, where high altitude caves were sampled, demonstrated that stygobionts are not as rare as expected for this region (Sambugar et al., 2008). Possible reasons for that could be either re-colonization following the retreat of Würmian glaciers, or their survival in the deep aquifer during the Quaternary glaciations (Sambugar et al., 2008).

Karstic aquifers are geomorphologically and hydrologically highly heterogeneous and difficult to...
filtering of the spring water and collecting invertebrate drift was conducted several times over two years during different hydrological conditions. Species richness and composition were expected to be affected by hydrological conditions during the sampling, when the springs will be fed either by stored water from the phreatic zone during low-water level conditions, by the water flowing from the epikarst and vadose zones or by the mixed water discharging from both zones during high-water level conditions. Concurrently, the sampling of the benthos from the adjacent spring mouth and the spring brook was carried out simultaneously with an objective to compare both sampling methods (drift versus benthos) in order to assess aquatic hypogean diversity and species composition. It was predicted that filtering of spring water will provide a better estimate of hypogean species richness than collecting the benthos from the spring mouth due to the fact that in the first case the fauna is collected directly from the water discharging from the aquifer while the sampling of benthos encompasses all the species living in the benthos, including surface species that normally never colonize hypogean habitats.

MATERIALS AND METHODS

Study site

The Lipnik spring complex is situated in the Julian Alps (NW Slovenia) which are part of the Southern Limestone Alps. The water discharges at the bottom of steep slopes of the Pokljuka plateau (altitude of 1300 m a. s. l.), and continues as a short stream to the Radovna river, flowing in the upper Sava river catchment (Fig. 1). The karstic aquifer of the Pokljuka is composed mainly of massive limestone and thick-grained dolomite of the Triassic age (Buser, 1987) and is fed from the surface by diffusive recharge only.
The water discharges through two temporary springs (T1 - 46°23′03″ N, 14°01′36″ E; T2 - 46°23′04″ N, 14°01′35″ E, altitude 692 m a.s.l.) and one perennial spring (46°23′05″ N, 14°01′38″ E, altitude 665 m a.s.l.) situated within a distance of 60 m and along altitudinal difference of 30 m. In 2009 and 2010, the annual precipitation in the area was 2204 and 2277 mm, respectively (the Radovna meteorological station; Environment Agency of RS). The overall discharge of the perennial spring measured by data logger continuously from June 2009 to June 2010 was between 6.4 and 7.3 °C. The age of the spring water was estimated to be around 3.8 years (Kanduč et al., 2012).

Sampling methodology
Filtering nets (circle opening, diameter 0.2 m, mesh size 100 µm, net length 1 m) were placed in the mouths of three springs (P – perennial; T1, T2 – temporary) for 24 hours during three seasons (spring, summer, autumn) over two years (2009, 2010) (Fig. 2). The data from the last sampling campaign (Oct 2010) for the two temporary springs (T1, T2) were not included in the data analyses due to accidental dislocation of the nets during the filtering. Occasionally, the water from the two temporary springs discharged up to 60 m downstream from the usual site placed at the foot of steep rock wall as shown on Fig. 1. During the extreme low flow conditions the water from temporary springs discharged at the same altitude as the water from the perennial spring. Concurrently with drift sampling, the benthos from the perennial spring mouth (SM) and from the spring brook (SB) at the site approximately 10 m downstream from the spring mouth were sampled by a kick sampling method. A substrate of approximately 0.04 m² (20 x 20 cm) was kicked by foot for 1 min and a hand net (mesh size 100 µm) was used to collect the animals. Additionally, larger stones were picked and washed out into the hand net.

Biological samples were preserved in 70 % ethanol and taken to the laboratory for further processing. Prior to collecting the biological samples, oxygen (WTW Multiline P4, CellOx 325), temperature and conductivity (WTW Multiline P4, TetraCon 325) were measured in the perennial spring mouth. The water levels and flow velocities were measured using an OTT ADC flow meter on every sampling occasion and each spring.

For the laboratory analyses, 250 ml of water was collected in polyethylene bottles (for the measurements of pH, alkalinity, total nitrogen and total phosphorus) from the mouth of the perennial spring. Sample aliquots collected for cation and anion analyses were passed through a 0.20 µm nylon filter into High Density Polyethylene (HDPE) bottles and kept refrigerated until analyzed.

Laboratory analyses
In the laboratory, pH was measured using a WTW pH 540 GLP, with a TetraCon 325 probe. Alkalinity was determined using titration after Gran, and was expressed as CaCO₃ equivalent per liter. Total nitrogen and total phosphorus were measured spectrophotometrically (Clesceri et al., 1998). Cations (Na⁺, K⁺, Ca²⁺, Mg²⁺) and anions (SO₄²⁻, PO₄³⁻, NO₃⁻) were measured by ion chromatography (Metrohm, 761 Compact IC). Biological samples were sorted, the animals counted and microcrustaceans identified to the species level using identification keys (Einsle, 1993; Janetzky et al., 1996; Meisch, 2000). The number of the animals in drift samples was standardized to the number of specimens per 1000 m³ where the absolute numbers were divided or multiplied by a correction factor obtained from the actual volume of filtered water.

Data analyses
The differences in microcrustacean species composition were investigated by non-metric multidimensional scaling (NMDS) based on a distance matrix computed with a Bray-Curtis similarity index as a distance measure. Prior to the analysis, the data were standardized by expressing the number of specimens of each species as a percentage of total specimens in the sample. SIMPER (Similarity Percentage) was carried out to calculate which taxa are primarily responsible for an observed difference between groups of samples. The overall significance of the difference was assessed by ANOSIM (Analysis of Similarity analyses). The analyses were performed by using the software PAST version 1.95 (Hammer et al., 2001).

The sample-based rarefaction curves (Sobs or Mao Tau) were calculated using analytical formulas to perform a quantitative comparison between assemblages (Gotelli and Colwell, 2001; Colwell et al., 2004; Mao et al., 2005). The species accumulation curves were estimated for drift samples from the temporary and perennial springs and for the benthic samples from spring the mouth and the spring brook, respectively. The total species richness was calculated by using the equation Chao2 applied on the incidence-based data where the number of uniques and duplicates are considered (Chao, 1987). The calculations were computed using EstimateS (Version 8.2, R. K. Colwell, http://purl.oclc.org/estimates) (Colwell, 2009).

RESULTS

Physicochemical characteristics of the spring water
Water temperature was 6.7 ± 0.1 °C and conductivity 247 ± 63 µS cm⁻¹ (Table 1). pH ranged from 7.5 to 8.0, alkalinity from 2411 to 3050 µeq l⁻¹ of HCO₃⁻, and oxygen concentration from 10.4 to 11.8 mg l⁻¹. NO₃⁻ concentration was from 1.8 to 2.5 mg l⁻¹ while PO₄³⁻ was under a detection limit and not presented in Table 1. Ca²⁺ prevailed in the water (55.7 - 73.3 mg l⁻¹) in comparison to Mg²⁺ (2.8 - 4.4 mg l⁻¹) and mean concentrations of Na⁺, K⁺, and SO₄²⁻ ions were 1.4, 0.2, and 2.5 mg l⁻¹ respectively. The mean total nitrogen and mean total phosphorus in the spring water were 0.9 ± 0.2 mg l⁻¹ and 12.5 ± 7.9 µg l⁻¹, respectively.
Microcrustacean assemblages

A total of 24 species were collected in the Lipnik springs (Table 2). By filtering the water from the temporary and perennial springs, 8 species were collected in the temporary spring 1 (T1), 13 in the temporary spring 2 (T2) and 17 in the perennial spring, while the sampling of benthos resulted in 11 species for the spring mouth and 14 species for the spring brook. The most common species frequently occurring in all samples were *Bryocamptus dacicus*, *Elaphoidella phreatica*, *Mixtacandona* sp. A and *Mixtacandona* sp. B (Tables 2 and 3). Three species were collected only once in the drift from the temporary spring T2 (*Nitocrella* sp., *Speocyclops infernus*, *Lessinocamptus pivai*) and one species only once in the drift from the perennial spring (*Bryocamptus rhaeticus*). Several species that were abundant in the drift of the perennial spring occurred also in the benthos but were never collected in the drift from the temporary springs (*Attheyela wierzejski*, *Bryocamptus pygmaeus*, *Microcrustacean assemblages*

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Table 1. Main physical and chemical characteristics of the water from the perennial spring (P1). Concentrations of PO₄³⁻ were below detection limit and are not listed in the table.

**Table 2. List of species, their mean densities, and in the brackets the number of samples where species were collected. STY - stygobiont (i.e. hypogean species) as defined from the literature (Botosaneanu, 1986).**
Bryocamptus zschokkei, Moraria cf. alpina, Moraria radovnae). One species was found only in the spring mouth of the perennial spring (Elaphoidella sp. A) and three species only in the spring brook benthos (Paracyclops fimbriatus, Fabaeforiscandona breui, Fabaeforiscandona sp. A). The overall mean number of specimens in the drift samples was the highest in the drift from the temporary spring 2 (T2) and was 51 specimens per 1000 m³ of filtered water. In the benthos, mean densities were similar for both habitats (i.e. 6 specimens per 0.25 m²).

Drift densities varied over the time and were the highest in August and November 2009 in all springs (P, T1, T2) (Fig. 2). This is the period when the water from the temporary springs (T1, T2) discharged 60 meters downstream from the usual spring outlet at the same altitude as the perennial spring. In general, the densities were normally higher during the lower volume of the filtered water. The lowest drift densities were in the temporary spring 1 (T1) in June and September 2010 and the highest in the temporary spring 2 (T2) during August 2009.

Non-metric multidimensional scaling (NMDS) (2D, Stress 0.16) indicated a similarity of samples collected from the same sampling sites (Fig. 3). An exception were the samples from the temporary springs (T1, T2) taken in August 2009 and from the temporary spring T2 collected in November 2009 which were in close similarity with the samples from the perennial spring. The most distant were the benthic samples from the spring brook (SB), while the benthos from the spring mouth of the perennial spring and the drift from the same spring mouth were relatively close together. The differences between the sample groups were mostly due to D. zschokkei, E. phreatica, Mixtacandona sp. B, B. dacicus and B. zschokkei (SIMPER, Table 3). D. zschokkei was the indicative species for drift samples from the temporary springs, E. phreatica for drift samples from the perennial spring and spring mouth benthos, and B. zschokkei, C. subterranea, A. wierzejski for the spring brook benthos (Fig. 3). Accordingly, ANOSIM revealed statistically significant differences between all sample groups (p<0.05) but not between the temporary springs 1 and 2 (p=0.74).

The species accumulation curves (Mao Tau curves) had a higher slope in the case of drift samples than in the case of the benthic ones (Fig. 4). Mao Tau curves reached an asymptote in the case of the benthic species.
precipitation regime, development and structure of epikarst, infiltration rates, and underground runoff in the vadose zone and draining from the phreatic zone determine the hydrological characteristics of karstic outlets (Mangin, 1994). Vervier and Gibert (1991) demonstrated that in the case of the vadose zone, the species composition of the fauna collected by sampling of drift from karstic outlets depends greatly on the precipitation regime and hydrological conditions. After high precipitation rates a flush of water outwashes species that would not be drifted out at a normal hydrological condition (Gibert, 1986). Accordingly, the timing of the sampling can greatly affect the results obtained by collecting drift. In this study, drift densities were generally lower during a larger volume of filtered water which occurred during high-water level conditions.

The attempts to interpret the origin of drifting species (the epikarst and vadose zones versus the phreatic zone) and to describe the characteristics of “epikarst/vadose” and “phreatic” assemblages needs to be tentative due to the fact that an indirect sampling method (filtering of the spring water) was applied and that at certain hydrological periods water discharging from the karstic outlets at the interface of both zones can be a result of mixing water originating from both zones or even from epikarst (Doctor et al., 2006). For several species that were collected from the perennial and occasionally from the temporary springs there exist different explanations: either 1) the species actually inhabit both zones or 2) the lifting of groundwater table and mixing of water from the two zones together with possible active movements of animals from the phreatic zone upwards resulted in the occurrence in both springs. Most likely, the species that are constantly present in the drift from both types of outlets (temporary, perennial) inhabit both zones; whereas that species that just occasionally occur exclusively in the drift from the temporary springs originate in the epikarst zone. Background knowledge on the ecology and habitat preferences of some species can be helpful in identifying the origin of the water. For example, *Speocyclops infernus* is a typical epikarst species that was commonly collected samples from the spring mouth and spring brook and in the case of drift samples from the perennial spring. The estimated mean numbers of species using the Chao2 equation were 10.5 species for the spring mouth benthos, 15.6 species for the spring brook benthos, and 19.1 species for drift from the perennial spring and 16 and 37.5 species for drift from the temporary springs 1 and 2, respectively (Table 4).

**DISCUSSION**

**Habitat heterogeneity and species richness**

The spatial and temporal variation in drift densities and species composition was high indicating strong dependence of drift on actual water level conditions (low/high-water levels) and most probably diverse origin of the water discharging from the studied karstic outlets. Geomorphology and hydrology of karstic systems are known to be spatially and temporally highly complex (Kaufmann, 2003). The precipitation regime, development and structure of epikarst, infiltration rates, and underground runoff in the vadose zone and draining from the phreatic zone determine the hydrological characteristics of karstic outlets (Mangin, 1994). Vervier and Gibert (1991) demonstrated that in the case of the vadose zone, the species composition of the fauna collected by sampling of drift from karstic outlets depends greatly on the precipitation regime and hydrological conditions. After high precipitation rates a flush of water outwashes species that would not be drifted out at a normal hydrological condition (Gibert, 1986). Accordingly, the timing of the sampling can greatly affect the results obtained by collecting drift. In this study, drift densities were generally lower during a larger volume of filtered water which occurred during high-water level conditions.

The attempts to interpret the origin of drifting species (the epikarst and vadose zones versus the phreatic zone) and to describe the characteristics of “epikarst/vadose” and “phreatic” assemblages needs to be tentative due to the fact that an indirect sampling method (filtering of the spring water) was applied and that at certain hydrological periods water discharging from the karstic outlets at the interface of both zones can be a result of mixing water originating from both zones or even from epikarst (Doctor et al., 2006). For several species that were collected from the perennial and occasionally from the temporary springs there exist different explanations: either 1) the species actually inhabit both zones or 2) the lifting of groundwater table and mixing of water from the two zones together with possible active movements of animals from the phreatic zone upwards resulted in the occurrence in both springs. Most likely, the species that are constantly present in the drift from both types of outlets (temporary, perennial) inhabit both zones; whereas that species that just occasionally occur exclusively in the drift from the temporary springs originate in the epikarst zone. Background knowledge on the ecology and habitat preferences of some species can be helpful in identifying the origin of the water. For example, *Speocyclops infernus* is a typical epikarst species that was commonly collected from the spring mouth and spring brook and in the case of drift samples from the perennial spring. The estimated mean numbers of species using the Chao2 equation were 10.5 species for the spring mouth benthos, 15.6 species for the spring brook benthos, and 19.1 species for drift from the perennial spring and 16 and 37.5 species for drift from the temporary springs 1 and 2, respectively (Table 4).

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from dripping water in the caves (Brancelj, 2002; Papi and Pipan, 2011) and its presence in the sample confirms that the water flows from the epikarst.

Despite the abovementioned constraints, a distinction in drift densities and species richness and composition between the perennial and temporary springs can give us an insight into hydrological dynamics of an Alpine karstic aquifer and corresponding outlets as well as indicate the characteristics of the hypogean fauna from the two zones (epikarst/vadose zone; phreatic zone). The fauna collected by filtering the water from the perennial spring most likely represents the fauna from the phreatic zone which is characterized by typical species such as *Elaphoidella phreatica*. This species is known from previous studies to live in the karstic phreatic zone and also in the porous aquifer (Mori and Brancelj, 2008; Galassi et al., 2013). On the contrary, the samples from the temporary springs (with the exception of the sampling during the low flow when the temporary springs discharged at the same altitude as the perennial spring), were lower in densities and species numbers, the dominating species being *Lessinocamptus piva*. The latter is the typical species known to inhabit the epikarst and vadose zone such as *Lessinocamptus piva*. The latter is the typical species known to live in the epikarst and vadose zone (Galassi et al., 2013).

Close correspondence between habitat diversity and species richness and species diversity is one of the central tenets in ecology (Larned, 2012). Karstic aquifers are hydrologically and geomorphologically highly heterogeneous, but with a clear distinction between the vadose zone (including the uppermost layer of epikarst) and the phreatic zone (Gibert et al., 1994). The main differences between the epikarst and vadose zones and the phreatic zone are their physical structure, hydrological characteristics and strength of hydrological connectivity with surface waters and adjacent aquifers (Gibert et al., 1994). The epikarst/vadose zone is highly fractured with mostly vertical but also lateral channels, sometimes comprising also larger caves where water flows fast during precipitation and also quickly disappears, while the phreatic zone is less densely fractured and channels are perennially filled with water (Gibert et al., 1994). In the epikarst and vadose zones, a higher water flow in the conductive subsystem during high rain is most probably detrimental to fauna which avoids this subsystem and mostly occupies the adjacent capillary zone where water flow is lower and retention of organic matter higher (Galassi et al., 2009a). In contrast, the phreatic zone lacks harsh fast flowing conduits and drying/rewetting phases and therefore encompasses better conditions for the hypogean fauna which occurs here in higher abundances. Stoch (1995) pointed out the importance of environmental stability and predictability of habitats for differences in species richness. If some environmental parameters fluctuate in a regular and predictable way, organisms may evolve some degree of dependence and specialization on temporary patterns of resource availability, enhancing diversity. The phreatic zone is hydrologically much more stable than the epikarst and vadose zones with their drying/rewetting cycles. Consequently, habitat fragmentation and processes of speciation are more intensive in the latter (Galassi et al., 2009b). Di Lorenzo et al. (2005) suggested that the species occurrence may be explained by hydrological connections between hypogean and surface waters. This was confirmed also in this study where many of the species occurring in the spring benthos were present also in the drift of the perennial spring indicating constant migration of surface species into hypogean waters. In contrast, the benthic species rarely occurred in the temporary springs but the species abundantly present in the drift of the perennial spring were often collected also in the drift from the temporary springs, what is indicative of interconnectivity of the two zones. Galassi et al. (2009b) observed a higher regional distributional rarity of the species from the vadose zone compared to that in the phreatic zone, which was not the case in this study where only one site was investigated. Here the species richness was the highest in the drift from the perennial spring. However, the species richness estimator Chao2 indicated that total species richness would be more than twice higher than observed in the temporary springs that are presumably mainly fed from the epikarst and vadose zones.

Sampling effort and sampling methodology can produce different species richness estimations (Hancock and Boulton, 2009). The latter authors demonstrated that a single sampling collected 30–87 % of the total species. In this study, the first sampling collected 54 % of total species in the perennial spring and 45 % and 43 % of all species in the two temporary springs (T1, T2), respectively. The species accumulation curves did not reach asymptote in the drift of the temporary springs indicating that additional sampling would result in higher species richness. The fauna in the epikarst and vadose zones is much scarcer than in the phreatic zone and hence an intensive repeated sampling is needed here to obtain total species numbers. A comparison of species composition in the samples revealed some similarity between the samples from drift of the perennial spring and the benthos of the spring mouth, but still only 41 % of species were in common. A similar fact was shown in France where sampling in caves resulted in few species of copepods, while filtering of the water from a karstic outlet on several occasions gave 21 species of harpacticoids, with 3–200 specimens per m³ (Rouch, 1984). The sampling of the spring mouth benthos does not seem to be an accurate method for estimating the species richness and community composition in the vadose zone, but it can be a proxy for it in the phreatic zone.

### Table 4. Mean Chao2 with 95 % confidence intervals (lower and upper bounds) for five sample groups.

<table>
<thead>
<tr>
<th></th>
<th>Chao2 Lower Bound</th>
<th>Chao2 Mean</th>
<th>Chao2 Upper Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benthos</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring mouth</td>
<td>10.0</td>
<td>10.5</td>
<td>15.8</td>
</tr>
<tr>
<td>Spring brook</td>
<td>14.2</td>
<td>15.6</td>
<td>25.7</td>
</tr>
<tr>
<td>Drift</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perennial spring</td>
<td>17.3</td>
<td>19.1</td>
<td>31.6</td>
</tr>
<tr>
<td>Temporary spring 1</td>
<td>9.0</td>
<td>16.0</td>
<td>72.8</td>
</tr>
<tr>
<td>Temporary spring 2</td>
<td>16.6</td>
<td>37.5</td>
<td>180.2</td>
</tr>
</tbody>
</table>

International Journal of Speleology, 42 (3), 257-266. Tampa, FL (USA) September 2013
Characterization of microcrustacean assemblages

The sampling of drift from karstic outlets at the interface of the vadose and phreatic zones of an alpine karstic aquifer revealed high species richness of microcrustaceans with altogether 20 species recorded in the drift, 16 being copepods. This is surprisingly high since alpine karst has been known previously as species poor due to glaciations and low water temperatures. For example, the study from the northern Italy resulted in 19 microcrustacean species that were collected in 16 high altitude caves in Trentino (Stoch, 2000) and Pipan and Pipan (2011) collected 2 copepod species in the epikarst of an alpine cave over 7 sampling occasions.

In this study, 14 species were collected from two temporary springs and 13 from the perennial one. One of the reasons for high species numbers is repeated samplings (5 to 6 times in two years). Repeated sampling is a must for a good assessment of hypogean species richness (Rouch and Danielopol, 1997; Schneider and Culver, 2004). The other reason is the fact that the sampling in this study was carried out at the contact zone between the vadose and the phreatic zone which is in contrast to other alpine studies where epikarst and caves located within the vadose zone were investigated. The former two studies were focused on the alpine epikarst and consequently resulted in less species than this study where the alpine temporary spring most probably fed from the epikarst and vadose zones and occasionally also from the phreatic zone were sampled. The number of copepod species from this study is similar to that in other studies from the Dinaric region where 12 copepods were collected in the epikarst of a non-alpine (Dinaric) cave on eight sampling occasions (Brancelj, 2002), and 11 to 14 copepods in the caves of the Postojnska jama cave system (Slovenia) (Pipan and Brancelj, 2004; Pipan, 2005; Pipan and Culver, 2007a, b). To summarize, the high species richness comparable to that in the Dinaric areas in the studied aquifer is firstly due to the mode of sampling (repeated filtering of water in karstic outlets), and secondly due to a highly developed underground system of fissures (availability of space) and close connection with the phreatic zone and further on with the porous aquifer of the Radovna river (interconnectivity).

The microcrustacean assemblages were composed of several ecological groups: 1) abundant species frequently occurring in all samples; 2) abundant species frequently occurring in all samples, but not in the drift from temporary springs; 3) rare species occurring exclusively in the drift from the temporary springs; 4) rare species most probably restricted to the benthos of the spring mouth; and 5) species exclusively from the spring brook benthos (Table 5). In general, a typical epikarst microcrustacean assemblage is comprised of harpacticoid Lessinocamptus, Paramorariopsis, Porastenocaris genus and the cyclopoid species Speocyclops infernus (Brancelj, 2002; Pipan, 2005; Brancelj, 2009; Galassi et al., 2009b). Previously, Speocyclops infernus was shown to be widespread over broader geographical areas in the epikarst and vadose zones (Pipan, 2005; Brancelj, 2009; Pap and Pipan, 2011), while Lessinocamptus pivai was reported to be collected only from one karstic site (the vadose zone) in the northern Italy (Stoch, 1997). E. phreatica, accompanied by Attheyella wierzejski, Bryocamptus rhaeticus and Moraria radovnae, was frequently found in alpine springs (Bottazzi et al., 2008; Mori and Brancelj, 2008) and alluvial habitats (Pesce and Galassi, 1986; Malard et al., 2003). The species is considered common in southern and central Europe, inhabiting predominantly porous aquifers (as indicated by its name phreatica) (Illies, 1978). On the other hand, little is known about ecology and distribution of the ostracod genus Mixtacandona in Slovenia, the third most abundant species in the Lipnik springs. Mixtacandona is exclusively hypogean genus with an array of species inhabiting karstic as well as alluvial aquifers (Rogulj and Danielopol, 1993; Mori and Meisch, 2012). In terms of biogeography, the species differ from those inhabiting Dinaric karst. Most probably the species that are widely distributed in the Alps, such as Elaphoidella phreatica are post-glacial colonizers, while the species with a restricted distribution mostly in the epikarst colonized the area during Pliocene and they survived glaciations in deep aquifers (Stoch, 2000; Sambugar et al., 2008).

**CONCLUSIONS**

The study demonstrated that alpine karstic groundwaters previously assumed as species poor environments can be rich in the hypogean fauna. The study of drift from the temporary and perennial springs brings new insights into its temporal variability due to variability in hydrology (low/high water levels). There is an indication that during high water levels drift densities are lower and species typical for epikarst/vadose zone occur in the drift, while during low water levels mainly fauna characteristics for phreatic zone occur in the drift. A comparison of the samples from the drift of the perennial spring and the samples from the spring mouth benthos revealed a distinct species composition indicating that filtering of the spring water is most likely a more accurate method for the assessment of the hypogean species richness and characterization of its species composition. However, this hypothesis needs to be tested on a larger scale and needs to include also the sampling in the caves and boreholes.

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Table 5. Classification of the species on the basis of their occurrence across habitats (drift of temporary and perennial springs, benthos in the spring mouth and spring brook).

<table>
<thead>
<tr>
<th>Species</th>
<th>Drift of temporary springs</th>
<th>Drift of perennial springs</th>
<th>Benthos in spring mouth</th>
<th>Benthos in spring brook</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bryocampus dacicus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elaphoidella phreatica</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixtacandona sp. B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bryocampus zschokkei</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moraria radovnae</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cavernocypris subterranea</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixtacandona sp. A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Speocyclops infernus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lessinocamptus pivai</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moraria alpina</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitocrella sp.</td>
<td></td>
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<td></td>
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<tr>
<td>Paracyclops fimbriatus</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Fabaeformiscandona breulli</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Fabaeformiscandona sp. A</td>
<td></td>
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</tbody>
</table>
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The touristic and religious use of caves is frequent in Brazil. The religious use generally involves the construction of chapels, shrines and even churches at the cave entrances (Souza-Silva, 2008). However, cave visitation may have serious impact on the hypogean system, such as: 1) enrichment of the environment by organic and inorganic matter left by visitors, 2) change of pristine climate (e.g. temperature, concentration of carbon dioxide, humidity), 3) stepping on fauna and microbiota, 4) compacting of soil (Pulido-Bosch et al., 1997; Barton, 2006; Barton & Northup, 2007) and 5) altering cave biodiversity distribution and composition.

INTRODUCTION

Caves are stable environments with characteristics favoring the development of microorganisms. The allochthonous input of organic matter and microbes into the warm Neotropical caves may favor the development of filamentous fungi, including pathogenic species. Histoplasma capsulatum is a pathogenic species commonly found in caves and associated with bat and bird guano. Many Brazilian caves have been historically visited due to scenic and religious tourism. The objective of this study was to perform a microbiology study for a management plan of a show cave in Brazil, focusing on the presence and distribution of pathogenic and opportunistic fungi in the cave. Statistic analysis was used to verify the influence of touristic activity on airborne fungi spore load. Fungi were isolated from air and guano in Lapa Nova Cave. Samples were obtained through serial dilution, direct and settle plate techniques. For H. capsulatum, samples were incubated in specific media and conditions. Airborne fungal spore load was compared prior and during visitation and statistically analyzed. A total of 2,575 isolates from the genera Aspergillus, Calcarisporium, Chaetomium, Cladosporium, Curvularia, Emericella, Eurotium, Fusarium, Geotrichum, Gliocladium, Mucor, Purpureocillium, Paecilomyces, Penicillium, Rhizopus and Trichoderma were identified. Significant (p<0.05) variations on fungal richness in the air occurred due to cave visitation. Areas of potential microbiologic risks were indicated and management actions suggested. The results suggest a diverse community inhabiting the cave. Possible opportunistic species should be monitored in show caves and microbiota should always be included in the elaboration of cave management plans. This is the first detailed microbiologic study for a management plan of a show cave in the country. It provides relevant information for future management plans.

Keywords: cave; pathogenic fungi; microbiology; management plan; cave tourism

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Touristic activities may favor the dispersion and import of new microbes (Barton, 2006). This could lead to fungal outbreaks including potential pathogenic species and causing tourists innumerous health problems (Cury et al., 2001).

Caves harbor a diverse array of fungi which may include pathogenic and opportunistic species. Opportunistic fungal infections have become more and more frequent in past decades, mainly due to the increasing number of immunosuppressing therapies and emerging diseases (Armstrong, 1989; Pfaller & Diekema, 2004). The number of fungi reported to cause opportunistic infections has been increasing in the past decades. The genera Aspergillus, Penicillium, Paecilomyces, Purpureocillium, Fusarium and Candida have been frequently reported as causative agents (Armstrong, 1989; Pfaller & Diekema, 2004; Trabulsi & Alterthum, 2004). Some opportunistic species are even related to fatal opportunistic infections in immunosuppressed people.

Concerning the true fungal pathogens (fungi capable of causing disease in immunocompetent organisms) inhabiting subterranean environments, special attention has been given to the thermo-dimorphic fungus Histoplasma capsulatum. This species causes histoplasmosis, which is a serious respiratory and systemic infection (Gompertz et al., 2004; Guimarães et al., 2006). The symptoms range from mild sickness (often mistaken with a more serious flu) to disseminated infection and death. It may affect both immunocompromised and immunocompetent individuals, with severity usually related to personal immune response (Woods, 2002; Gompertz et al., 2004; Guimarães et al., 2006).

Histoplasma capsulatum is commonly found in its filamentous form in the environment. It grows in locations rich in nitrogen (guano and soil) and pH higher than 5 (Mahvi, 1970; Carvajal-Zamora, 1977; Trabulsi & Alterthum, 2004) and produces macro- and microconidia. Infection occurs by inhalation or contact with microconidia. Cave visitation may cause conidial suspension and expose visitors to possible infection (Ashford et al., 1999; Lyon et al., 2004). Cases of histoplasmosis have been linked with cave visitation in Brazil (Cury et al., 2001). However, many cases are commonly misdiagnosed and treated as bacterial pneumonia, flu or allergy.

The environmental legislation concerning touristic and economic use of caves has been changing in Brazil in the past decade (CECAV/IBAMA, 2009). Management plans are now required by the government prior to touristic use of any caves in national territory.

The National Center of Study Protection and Management of the Caves (CECAV) created a “term of reference” in 2008 (CECAV/IBAMA, 2008) which is the basis for development of management plans aimed at touristic use of caves in the country. This document requires an array of studies in different areas. The main focus is on cave fauna and ecology with little attention paid to microbiological studies. All management plans should include an inventory of pathogenic fungi (especially the species H. capsulatum) and their distribution within caves. Baseline data on the presence and distribution of subterranean microbiota is needed to delimit touristic paths in caves and guide monitoring actions.

Vanderwolf and colleagues (2013) bring an interesting review on distribution of fungal species isolated from caves worldwide. As observed in their work there is a gap concerning microbial diversity in Brazilian caves. The authors also highlight the importance of performing studies about fungal distribution, fungal communities and their relation with the environment. We bring in this study a pioneer assay trying to understand the effects of tourism in fungal dispersion as well as testing methods to be used in mycological studies aimed at management plans of show caves.

The general objective of the present study was to perform a more detailed mycological study for the creation of a management plan for a touristic cave in Brazil, and to suggest monitoring actions. This work was requested by government agencies responsible for the monitoring and licensing of cave tourism in Brazil. We aimed to identify the presence of potential pathogenic species, isolate H. capsulatum, and detect areas of microbiological risk. We also investigated the effect of touristic activity on air spore-load and air-borne fungi diversity. In the end, we indicate areas of microbiologic risk as a baseline for the creation of a safer touristic route. We also suggest some management actions to minimize the risks of pathogenic fungal outbreaks.

**METHODOLOGY**

**Study area**

Lapa Nova Cave (“Gruta Lapa Nova”) is a 4.55km dolomitic cave with two levels and three known entrances. It is located in the municipality of Vazante, Northwestern Minas Gerais state (Brazil), in the environmentally protected area known as APE Lapa Nova (Área de Proteção Estadual Lapa Nova-APE Lapa Nova). This cave is surrounded by areas of urban development, zinc mining, agriculture, and pasture (Auler, 2001).

The environment in Lapa Nova is characterized by an average temperature of 17°C - 22°C and humidity between 92-100%. Deeper zones of the cave present a constant humidity of 99-100% and average temperature of 20°C. Although food resource seems to be scarce in many sites there are many small bat guano patches (fresh and old) distributed along the cave. Lapa Nova is still inhabited by hundreds of bats from at least three species, including hematophagous (Desmodus rotundus and Diphyllyma ecaudata) and frugivorous (Pityrrhinus sp.) groups. There is a fourth colony, which was not identified due to its location. The cave has many guano patches left by frugivorous, insectivorous and hematophagous bats. Active colonies were mainly observed in a deep area of the cave where there is also a large deposit of mixed bat guano. External vegetal debris is also imported into the cave through two skylights (Fig. 1).

**Tourism in Lapa Nova Cave**

Lapa Nova has been visited for at least 140 years (Auler, 2001). Currently, visitation is accessed by the
main entrance and limited to part of the first level. There are four touristic circuits: the cave entrance, the entrance chamber (accessed by stairs), touristic circuit on the right and touristic circuit on the left side from the entrance chamber (Fig. 1).

Tourism in this cave is mainly represented by a single intense touristic event honoring Virgin Mary. It is known as “Festa da Virgem da Lapa”. This event lasts a few days between late April and early May. During this period, the cave is visited by thousands of people drawn mainly by religious beliefs. Throughout the year, visitation in Lapa Nova cave is almost insignificant. The touristic path is quite rudimentary and solely delimited by zebra tapes. A small number of guides are responsible for monitoring visitation.

Sampling stations

A total of eight sampling stations were selected for the microbiologic inventory and analysis of air spore-load. Four stations were selected in touristic zones (S1, S2, S3 and S6), and the other four in non-touristic zones (S4, S5, S7 and S8). Bat guano samples were also collected to register the presence of any possible pathogenic species and other species of filamentous fungi. Special attention was given to guano presenting visible fungal growth.

The air samples were collected before the intense touristic event (March, 2009) and during the intense touristic event (last day of the religious festival - May, 2009). This procedure aimed to verify the variation on the abundance (total colony forming units - CFU) and richness (number of species) of air-borne fungi between these two periods. The presence of possible pathogenic species was also noted. The number of total visitors was registered during the touristic event in the touristic circuits to compare representativeness of each circuit.

Microbiologic sampling of airborne fungi

The isolates were obtained through the settle plate technique. Two Petri dishes containing Dichloram Rose Bengal Cloramphenicol Agar (DRBC, Acumedia Laboratories, Lansing, MI USA) and Sabouraud Dextrose Agar (SDA, Difco Laboratories, Detroit, MI USA) with cloramphenicol (0.1%) were placed in each sampling station and exposed for twenty minutes. After exposure, they were sealed and incubated at 25°C for seven days as standardized for filamentous fungi and considering the cave temperature (17-22°C). Colony forming units (CFU) were the quantitative parameter considered and used for comparison. The final abundance was determined by the sum of CFUs per sampling station.

Isolates were purified on Malt Extract Agar (MEA, Difco) and incubated at room temperature (25°C) for at least seven days. The purified colonies were identified to genus and then incubated at specific media for morphological identification (macroscopic and microscopic) of each genus using specific identification keys (Pitt, 2000; Klich, 2002; Samson & Frisvad, 2004; Domsch et al., 2007). The media used were Neutral Creatine Sucrose Agar (CNS) (according to Samson & Frisvad, 2004), CYA (Labsynth, Diadema, SP BRAZIL), MEA (Difco) PDA (Acumedia Laboratories, Lansing, MI USA) and YES (according to Samson and Frisvad, 2004) for the genera Aspergillus, Calcarisporum, Cladosporium, Chaetomium, Curvularia, and Emericella.
**Guano sampling**

A total of two large (≥ 25m length) and eight smaller (≤ 5m length) guano patches were sampled in touristic and non-touristic areas (Fig. 1). Each guano deposit presented different characteristics concerning composition (type of guano and other allochthonous material) and time of deposition (according to visual analysis of color, moisture and texture) (Table 1).

One sample of each bat guano of different ages (fresh and old) were collected with sterilized scoops from a delimited area (15 cm x 10 cm) of the chosen guano patch. These samples were stored in sterilized vials, sealed in situ and refrigerated at 4°C until their processing (not more than 5 days after collection). These samples were processed in laboratory and the isolates were obtained through a 10-fold dilution technique. Guano samples were first diluted in sterilized distilled water (1:10) and thoroughly mixed for homogenization.

This solution was then diluted (10⁻¹ to 10⁻⁵) in sterilized water (1:10) and thoroughly mixed for homogenization. These samples were stocked in sterilized material from a delimited area (15 cm x 10 cm) of the chosen site with active bat colony entrance set to analyze the composition (type of guano and other allochtonous material) and time of deposition (according to visual analysis of color, moisture and texture) (Table 1).

**Presence of *H. capsulatum* and other possible pathogenic species**

There has been much research attempting to validate a golden standard for isolation of *H. capsulatum* from the environment (Guimarães et al., 2006; Léon et al., 2012). We attempted to isolate *H. capsulatum* from air and guano using three different methods: 1) direct plating of suspicious material, 2) serial dilution (10⁻¹ - 10⁻⁵) in distilled water enriched with peptone (0.1%) (triplicates were performed for each sample), and 3) settle plate method (20 minutes).

The media used were SDA (25°C) and Brain Heart Infusion Agar (BHA, Acumedia) (37°C) (Carvajal-Zamora, 1977; Gompertz et al., 2004; Guimarães et al., 2006) with chloramphenicol (1%) to inhibit bacteriological growth. All dishes were observed from five to forty-two days after inoculation. Daily observations were performed in the first 21 days and three times a week afterwards.

Concerning other possible pathogenic species, the first pathogenic feature considered for triage was growth at 37°C on SDA and BHA. All colonies growing at this temperature were identified and compared to known medical cases of opportunistic human fungal infections. These species were then classified as potentially pathogenic species and locations with large concentrations were considered areas of risk and marked for microbiological monitoring. We categorized an area as 'large concentration' when the possible pathogenic species represented more than 30% of the total CFU isolated from the sampling station.

**Statistical analysis**

The non-parametric Kruskal-Wallis test was used to verify the visitation effect on the abundance and richness of air-borne fungi per sampling station. For that we compared results obtained before the touristic event with those obtained on the last day of the festival (touristic event). To standardize sampling effort, we placed the Petri dishes (duplicate) in the exact same place during both events. The media, Petri dish size and time of exposure were also standard in both sampling periods. The program Statistica 9.0 was used for statistical analysis.

**RESULTS**

**Abundance, richness and diversity of filamentous fungi**

A total of 2,575 filamentous fungi were isolated from the guano (1,739) and from the air (836). These isolates included 47 species distributed among 15 genera (Table 2).

Sampled air-borne fungi were composed of 25 species and 10 genera: *Aspergillus* (33.3%), *Penicillium* (29.1%), *Cladosporium* (8.3%), *Fusarium* (8.3%), *Calcarisporium* (4.1%), *Chaetomium* (4.1%), *Curvularia* (4.1%), *Emericella* (4.1%), *Geotrichum* (4.1%), and *Trichoderma* (4.1%). The most diverse genera were *Aspergillus* and *Penicillium* with eight
and seven of the total species isolated from the air respectively. The highest abundance (86 CFU) registered among the air samples, was registered in the sampling station S3, during the religious festival (Fig. 2). The highest richness registered from the air samplings before the Festival was obtained in S2 and S8 (S=16). However, the highest richness registered during the Festival was obtained in S3 (S=16). The results for the other sampling stations may be observed in Figs. 2 and 3.

A total of 2,039 CFUs were counted from the guano samples including yeasts (300) and filamentous fungi (1,739). The guano patch with the highest counting was G1, which is a fresh mixed large guano deposit (Table 1). Fungi isolated from guano was represented by at least 43 species of 12 genera of filamentous fungi: Aspergillus (34.8%), Penicillium (34.8%), Cladosporium (8.3%), Eurotium (4.6%), Fusarium (4.6%), Mucor (4.6%), Emericella (2.3%), Gliocladium (2.3%), Paecilomyces (2.3%), Purpureocillium (2.3%), Rhizopus (2.3%) and Trichoderma (2.3%). Once more, the most diverse genera were Aspergillus and Penicillium, with 15 species each. They were also the most abundant with 806 and 359 isolates respectively. The genera Trichoderma and Purpureocillium also presented a high number of isolates (259 and 226 respectively).

The highest abundance (914 CFU) of filamentous fungi isolated from guano was obtained in the sampling station G1, followed by G5 (305 CFU) and G8 (257 CFU) (Table 1).

Potential pathogenic and allergic species

Histoplasma capsulatum was not isolated from Lapa Nova cave. However, various isolates from eleven species were considered possible pathogenic species: Aspergillus flavus, Aspergillus japonicus, Aspergillus niger, Aspergillus versicolor, Aspergillus ustus, Fusarium oxysporum, Fusarium solani, Mucor sp., Purpureocillium lilacinum, Rhizopus sp. and Trichoderma viride. These species presented positive growth at 37°C and have already been associated with various opportunistic fungal infections in humans (Lacaz et al., 2002). Cladosporium cladosporioides is known for producing serious toxins and for being a strong allergic species (Tasic & Tasic, 2007). Since some isolates of C. cladosporioides were also able to grow at 37°C and due to its allergic potential, it was included in this list.

The species considered of medical importance were distributed along the cave in touristic and non-touristic stations (Table 3). However, the species A. niger, Mucor spp, P. lilacinum and R. solani were solely isolated from guano samples.

The occurrence of the possible pathogenic species isolated from the air varied before and during touristic visitation in some stations, as shown in Table 3. However, Aspergillus flavus, C. cladosporioides, F. solani, F. oxysporum and T. viride were constant in other areas (especially in S8). This last air sampling station was located close to the large guano patch. Fusarium solani was isolated from almost all air sampling stations. In most cases it presented low frequency (<10%). However, its frequency was high in S5 (27%), S8 (26.6%) and S7 (26.8%) as well as highly isolated from G1 and G2.

Three areas were considered of microbiologic risk since they had high concentration of possible pathogenic fungi (Fig. 1). Two of these areas were classified as highly risky due to elevated levels of airborne fungi, high frequency of possible pathogenic species, and presence of active bat colonies producing large guano patches. The other area was considered of mild risk since its only
Table 2. Fungi isolated from air and guano in Lapa Nova Cave and reference* of their occurrence in other caves indicating worldwide distribution in subterranean ecosystems.

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Distribution in Lapa Nova</th>
<th>Other cave occurrence (*)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ascomycota:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alseptoglium caespitosum Raper &amp; Thom 1944</td>
<td>A, G</td>
<td>9</td>
</tr>
<tr>
<td>A. candidus Link 1809</td>
<td>A, G</td>
<td>7</td>
</tr>
<tr>
<td>A. clavatus Desmazieres 1834</td>
<td>G</td>
<td>7</td>
</tr>
<tr>
<td>A. flavus Link 1809</td>
<td>A, G</td>
<td>1, 3, 4, 6, 7</td>
</tr>
<tr>
<td>A. japonicus Saito 1906</td>
<td>A, G</td>
<td>9</td>
</tr>
<tr>
<td>A. nigri Tieghem 1867</td>
<td>G</td>
<td>1, 2, 3, 4, 6, 7, 9</td>
</tr>
<tr>
<td>A. niveus Blochwitz 1929</td>
<td>G</td>
<td>9</td>
</tr>
<tr>
<td>A. ochraceus Wilhelm 1877</td>
<td>A, G</td>
<td>3, 9</td>
</tr>
<tr>
<td>A. restrictus Smith 1931</td>
<td>G</td>
<td>9</td>
</tr>
<tr>
<td>A. soleratorum Hubber 1933</td>
<td>G</td>
<td>9</td>
</tr>
<tr>
<td>A. sydowii (Bainier &amp; Sartho) Thom &amp; Church 1926</td>
<td>A, G</td>
<td>9</td>
</tr>
<tr>
<td>A. ustus (Bainier) Thom &amp; Church 1926</td>
<td>A, G</td>
<td>7, 9</td>
</tr>
<tr>
<td>A. versicolor (Vellumien) Tiraboschi 1908</td>
<td>A, G</td>
<td>5, 7, 9</td>
</tr>
<tr>
<td>A. weissi Wehmer 1886</td>
<td>G</td>
<td>3, 9</td>
</tr>
<tr>
<td><strong>Aspergillus</strong> sp.</td>
<td>G</td>
<td>-</td>
</tr>
<tr>
<td>Calanioporum sp.</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td>Chaetomium sp.</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td>Cladosporium cladosporioides (Fresenius) Wies 1952</td>
<td>A, G</td>
<td>1, 4, 6, 7, 9</td>
</tr>
<tr>
<td>C. herbarum (Persoon) Link 1816</td>
<td>A, G</td>
<td>1, 4, 7, 9</td>
</tr>
<tr>
<td>Cuvuluria sp.</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td>Emericella rugulosa (Thom &amp; Raper) Benjamin 1955</td>
<td>A, G</td>
<td>9</td>
</tr>
<tr>
<td>Eurotium amstelodami Margin 1909</td>
<td>G</td>
<td>9</td>
</tr>
<tr>
<td>Fusarium oxysporum Schlechtendal 1824</td>
<td>A, G</td>
<td>1, 4, 6, 9</td>
</tr>
<tr>
<td>F. solani (Martius) Saccardo 1881</td>
<td>A, G</td>
<td>4, 7, 8, 9</td>
</tr>
<tr>
<td>Geotrichum sp</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td>Gibberdium roosei Bainer 1907</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>Pyrenopezicium Macinorn (Thom) Luangsa-ant, Hoobranken, Hywel-Jones &amp; Samson 2011</td>
<td>G</td>
<td>1, 6, 7, 9</td>
</tr>
<tr>
<td>Plectomycies varioli Bainer 1907</td>
<td>G</td>
<td>7, 9</td>
</tr>
<tr>
<td>P. brevicompactum Diercks 1901</td>
<td>A, G</td>
<td>9</td>
</tr>
<tr>
<td>P. chrysogenum Thom 1910</td>
<td>A, G</td>
<td>1, 4, 6, 7, 9</td>
</tr>
<tr>
<td>P. citrinum Thom 1910</td>
<td>A, G</td>
<td>7, 9</td>
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<tr>
<td>P. decumbens Thom 1910</td>
<td>G</td>
<td>7, 9</td>
</tr>
<tr>
<td>P. expansum Link 1809</td>
<td>A, G</td>
<td>7, 9</td>
</tr>
<tr>
<td>P. glabratus (Wehmer) Westling 1911</td>
<td>A</td>
<td>7, 9</td>
</tr>
<tr>
<td>P. graeoseutum Diercks 1901</td>
<td>A, G</td>
<td>9</td>
</tr>
<tr>
<td>P. islandicum Sopp 1904</td>
<td>G</td>
<td>-</td>
</tr>
<tr>
<td>P. oxalicum Curtie &amp; Thom 1915</td>
<td>G</td>
<td>9</td>
</tr>
<tr>
<td>P. pinophilum Hedgcock 1910</td>
<td>G</td>
<td>9</td>
</tr>
<tr>
<td>P. purpureogenum Stoll 1904</td>
<td>G</td>
<td>7, 9</td>
</tr>
<tr>
<td>P. simplicissimum (Oudemans) Thom 1930</td>
<td>A</td>
<td>9</td>
</tr>
<tr>
<td>P. solisum Westling 1911</td>
<td>G</td>
<td>7</td>
</tr>
<tr>
<td>P. thomii Maire 1917</td>
<td>G</td>
<td>7, 9</td>
</tr>
<tr>
<td>P. variable Sopp 1912</td>
<td>A, G</td>
<td>1, 4, 7, 9</td>
</tr>
<tr>
<td>Trichoderma viride Persoon 1794</td>
<td>A, G</td>
<td>9</td>
</tr>
<tr>
<td><strong>Zygomycota:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucor sp.1</td>
<td>G</td>
<td>-</td>
</tr>
<tr>
<td>Mucor sp.2</td>
<td>G</td>
<td>-</td>
</tr>
<tr>
<td>Rhizopus sp.</td>
<td>G</td>
<td>-</td>
</tr>
</tbody>
</table>

*Only species citation was considered;
A = isolated from air; G = isolated from guano;

S (1-8) = sampling stations; * = touristic pathway sampling stations; 0 = absent; + = present. Fungi species represented by: (A) Aspergillus flavus, (Aj) Aspergillus japonicus, (Au) Aspergillus ustus, (Av) Aspergillus versicolor, (Cc) Cladosporium cladosporioides, (Ch) Cladosporium herbarum, (Fo) Fusarium oxysporum, (Fs) Fusarium solani, and (T) Trichoderma viride.

Table 3. Distribution of potential pathogenic species (filamentous fungi) isolated from the air before (B) and during (D) the touristic event along the air sampling stations (S).

<table>
<thead>
<tr>
<th>Stations</th>
<th>Af</th>
<th>Aj</th>
<th>Av</th>
<th>Au</th>
<th>Co</th>
<th>Ch</th>
<th>Fo</th>
<th>Fs</th>
<th>T</th>
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</thead>
<tbody>
<tr>
<td>S1 B*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
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<tr>
<td>S1 D*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
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<td>+</td>
</tr>
<tr>
<td>S2 B*</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S2 D*</td>
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<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>S3 B*</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>S3 D*</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>S4 B</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>S5 B</td>
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<td>0</td>
<td>0</td>
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<td>S5 D</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>S6 B*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
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<td>S6 D*</td>
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<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
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</tr>
<tr>
<td>S8 B</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S8 D</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
</tbody>
</table>

feature was presence of high frequency of *F. solani* and moderate to high airborne spore-load.

**Variation of conidial air dispersion during touristic activity**

Data provided by Carste (2009) showed that a total of 3,202 tourists visited the cave entrance chamber during the religious festival in 2009. From this total, 1,438 tourists visited the touristic circuit TC1 and 1,023 tourists visited the touristic circuit TC2.

From the 836 airborne isolates obtained in both sampling events, 366 (43.77%) were obtained before the religious festival and 470 (56.23%) during the event. The abundance (CFU) of air-borne filamentous fungi increased from before and during intense visitation in 62.5% at sampling stations (S2, S3, S6, S7, and S8) and decreased in 37.5% (S1, S4 and S5) (Fig. 2). However, no statistically significant relation was observed.

Concerning richness of air-borne filamentous fungi, a statistically significant increase occurred in almost all stations (except for S7) during the intense touristic activity (Fig. 3). The Kruskall-Wallis analysis showed a statistically significant (p<0.05) relation between the richness of air-borne filamentous fungi and the intense touristic activity in touristic sampling stations along Lapa Nova Cave. However, this relation was not significant (p>0.05) for the sampling stations located in non-touristic areas (Fig. 4).

**DISCUSSION**

**Fungal assemblages in Lapa Nova cave**

Lapa Nova Cave holds a variety of types of guano with different times of deposition. This provides a mosaic of microhabitats for fungal colonization and
growth. Nieves-Rivera et al. (2009) discussed that fresh guano (rich in nutrient) combined with the stable environmental conditions of cave systems may provide excellent conditions for fungal growth. Ferreira and colleagues (2000) have already registered significant fungal diversity associated with bat guano in a cave in Brazil.

The high fungal abundance observed in the largest guano patch (G1) could be related to time of exposure and heterogeneous composition. It is widely known that more heterogeneous habitats may provide more diverse communities (Ricklefs & Schluter, 1993; McClain & Barry, 2010). Difference of guano age (old and fresh) and type (frugivorous, hematophagous, and insectivorous) observed in this site could be providing a more heterogeneous habitat for fungal growth, promoting high abundance. In addition, the active bat colony may also be contributing with constant import of microorganisms and organic matter.

Previous studies have already shown that touristic activities influence microbial distribution in a cave (Barton & Northup, 2007; Wang et al., 2010, Shapiro & Pringle, 2010; Fernández-Cortés et al., 2011). This relation could easily be associated with conidial suspension, microbial import and environmental changes caused by visitation (Vanderwolf et al., 2013). Fernández-Cortés and colleagues (2011) monitored fungal and bacterial variations associated with touristic activity and found that fungal dispersion inside the cave was related with opening of the entrance door. This does not seem to be occurring in Lapa Nova, as there are no doors controlling tourist entrance. It is possible that visitation is influencing spore suspension and causing variations on fungal dispersion and species occurrence. The significant relation between visitation and fungal richness observed in touristic areas sustains this hypothesis and points to possible enrichment of these areas through fungal import by tourists, as previously discussed by Barton & Northup (2007). Visitation may also cause conidial transfer from different sites in the cave or even allochtonous import from the epigean system.

Shapiro & Pringle (2010) observed that fungal diversity decreased in highly trafficked sites. Contrary to their findings, we observed that the straightest passage with higher human traffic (S3) presented the highest variation on spore-load and fungal richness during visitation. However, this data was not sufficient to provide with statistically significant results. Finally, after analyzing the data obtained in this study, it is plausible to state that fungal dispersion in Lapa Nova cave is influenced by tourism in visited areas, since a significant relation between visitation and fungal richness was only observed in touristic sites.

Aspergillus and Penicillium were the most common genera isolated from Lapa Nova Cave. This result is in accordance with data presented by Vanderwolf and colleagues (2013) in their review on fungal communities in caves worldwide. Both genera are the most commonly isolated from many different environments (Pitt, 2000; Klich, 2002). Similar results have been observed in other caves in Brazil (Casirillión et al., 1976) and worldwide (Koilraj et al., 1999; Nieves-Rivera, 2003; Ulloa et al., 2006; Nieves-Rivera et al., 2009; Novaková, 2009; Vanderwolf et al., 2013), showing that these are the most common filamentous fungi isolated from subterranean environments.

Comparisons with other studies on cave mycobiota in Brazil are difficult since studies on cave microbiota are scarce and focus on different topics (consequently using different media and methods). The few published studies mainly focus on identification of Basidiomycetes (Pedro & Bononi, 2007), isolation of dermatophytes (Casirillión et al., 1976; Silveira, 1985), fungal response to antifungal drugs (Resende-Stoianoff et al., 2012) and fungi association with other invertebrates (McCarthy et al., 2011). However, some similarities have been observed, such as that Fusarium solani has already been isolated from guano in a Brazilian cave (Resende-Stoianoff et al., 2012) and the fact that Aspergillus and Penicillium have been isolated in the same proportion by Casirillión and colleagues (1976). These similarities and the lack of studies highlight the importance of performing more studies on cave fungi in the country for further comparison.

Concerning pathogenic species, H. capsulatum was not isolated from Lapa Nova cave. This may represent its true absence or the result of a low spore concentration in the environment. It may also be related with the efficiency of current isolation methods (Carvajal-Zamora, 1977; Guimarães et al., 2006; Léon et al., 2012). Léon and colleagues make a comparison and discuss about the efficiency of H. capsulatum isolation methods (direct isolation, molecular identification and mice inoculation) indicating possible deficiencies in diagnosis. Thus, epidemiological studies are also supposed to be considered when working with the microbiological risks during management plan of show caves. It is important to highlight that although...
histoplasmosis has already been recorded in Brazil (Capone et al., 1999; Unis et al., 2005; Guimarães et al., 2006), with cases related with cave visitation (Cury et al., 2001) or contact with bat guano (Oliveira et al., 2006), there are no records of histoplasmosis associated with the study cave or region.

The guano patches G1 and G2 should be classified as high risk sites, since they feature conditions favorable for sudden fungal outbreaks. Their trophic status, fungal abundance, presence of possible pathogenic species, risk of introduction of new species by active bat colony (G1) and cave entrance (G2) are factors that enhance the risk of a sudden fungal outbreak.

Lapa Nova cave harbors opportunistic fungal pathogens from the genera Aspergillus, Cladosporium, Mucor, Rhizopus, Purpureocibium and Fusarium. Boutati and Anaissie (1997) reported many medical cases of opportunistic infections caused by Fusarium spp. in immunocompromised patients. Nucci & Anaissie (2002) presented medical cases relating Fusarium to cutaneous infection in both immunocompromised and healthy patients. However, no studies have been conducted associating opportunistic infections with cave visitation in Brazil. The scarce existing studies generally focus on histoplasmosis associated with cave visiting (Cury et al., 2001) and isolation of dermatophytes (Casirillon et al., 1976; Silveira, 1985).

We have collected A. flavus (an important infective agent causing pulmonary aspergillosis in immunocompromised patients), F. solani and F. oxysporum from the air and the soil in many of the studied sites (Table 3). These species were most frequent in the two large guano piles (G1 and G2), which could be a reason to classify these sites as dangerous for touristic use.

Other species isolated during this study are known to be associated with opportunistic fungal infections, such as onycomycosis (A. versicolor, A. ustus, F. solani, and F. oxysporum) (Veraldi et al., 2010), human otitis (A. flavus, A. japonicus) (Agudo et al., 2011), cutaneous mycosis (P. lilacinum) (Takayasu et al., 1977), pulmonary aspergillosis (A. flavus) (Kousha et al., 2011) and other opportunistic fungal infections (F. solani and F. oxysporum) (Nucci & Anaissie, 2002). We have also isolated well known allergenic species (C. cladosporioides and C. herbarum) (Zhang et al., 1996; Chou et al., 2008). Although they are not considered fungal pathogens, both species produce extremely allergenic compounds and could cause respiratory problems to cave visitors (Zhang et al., 1996). Interestingly, these species were added to three of the four touristic airborne sampling stations during visitation. Since these fungi are common indoors mold found in human households, their dispersion in these sites could be human related or merely dispersion between cave sites due to human visitation. Furthermore, we have also observed that other opportunistic species (Fusarium oxysporum and F. solani) were more frequently isolated from the air and more distributed among the sites during visitation.

These species are ubiquitous (commonly isolated from air, soil, plants and animals) and commonly found associated with humans. This could be another evidence of fungal dispersion or import due to human visitation.

The possible pathogenic (A. flavus, A. japonicus, A. ustus, A. versicolor, F. oxysporum, F. solani and Trichoderma sp.) and allergenic (C. cladosporioides and C. herbarum) species have already been registered in touristic and non-touristic caves in other countries (Koilraj et al., 1999; Hsu & Agoramooorthy, 2001; Nieves-Rivera, 2003; Nieves-Rivera et al., 2009; Novakova, 2009, Vanderwolf et al., 2013). Although these potential pathogenic species were isolated in Lapa Nova Cave, they are commonly found in external environments. However, caves are enclosed systems with environmental conditions that could favor fungal growth. Thus, the spore levels of these species in the air should be constantly monitored to minimize the exposure of tourists to heavy microbial loads in the air.

Finally, monitoring (air, sediment and guano) should be a common practice in all show caves in order to detect sudden fungal outbreaks, such as the histoplasmosis outbreak registered by Lyon and colleagues in a show cave in Costa Rica (2004).

Final considerations for the touristic use of Lapa Nova Cave

The large number of show caves in the country, the lack of information on microbial communities, presence of pathogenic and opportunistic species and absence of a microbiological monitoring protocol for management plans in show caves are alarming. We agree with Vanderwolf et al. (2013) on the importance of performing more studies on microbial communities in subterranean environments, particularly their interactions and relation with touristic activities. We considered the lack of basic information among tourists and the possible risks posed by visiting show caves and selected the following actions as highly commendable.

All visitors should be advised about: 1) the microbiologic risks, 2) avoiding contact with contaminated substrates (e.g., cave floor, walls, water, vegetal debris, guano and speleothems), 3) wear proper clothing covering exposed body parts which could be in contact with contaminated substrate (e.g., feet and legs), 4) avoid eating or drinking in the cave, and 5) not to litter (to avoid the introduction of organic matter that may serve as substrate for the development of cave microorganisms). This information could be provided through flyers, a visitors center with introductory presentation or even guidance by trained monitors.

More specifically, investigation of presence of H. capsulatum and other possible pathogenic species should be performed at least once every trimester (since it is a slow growing species), and especially before this intense touristic event (“Festa da Virgem da Lapa”). Guides must be advised about the symptoms and risks of histoplasmosis and medical check-ups.
should be periodically provided by the managers responsible for the show cave.

Any material introduced into the system, either for structural or maintenance reasons should first be evaluated and sterilized to avoid introduction of new microorganisms or organic matter for the development of fungal colonies. Thus, the risk of any fungal outbreak may be diminished and the trophic pristine conditions preserved. Since the soil is extremely compacted in touristic areas, it does not seem urgent the construction of pathways. However, this could be confirmed with long-term microbiologic monitoring.

**CONCLUSION**

The present study showed that caves hold an array of filamentous fungi which also include some possible opportunistic species. The distribution of cave mycobiota of Lapa Nova Cave is a dynamic process that should be considered when using these systems for touristic purposes. The impacts that touristic activities may have on fungi and the risk these microorganisms may pose to the visitors show the importance of constantly advising tourists and guides. The dynamic distribution of fungi along a cave shows the importance of a periodic microbial monitoring, especially in bat inhabited touristic caves.

Microbial communities are present in different substrates, especially on organic matter deposits. Therefore, it is important to assess the microbial composition of a cave before starting touristic activities and introducing structures which may serve as food resources and possibly development of pathogenic organisms. Microbiologic studies of air, soil and other substrates should be performed before establishing a touristic route and building touristic structures. Efforts should be made to agree with the many areas involved in a management plan and approve building structures that will diminish the risk of soil disturbance and consequent aerosols and conidial dispersion, also aiming for a minimum impact.

More studies on methods and the development of a standard protocol to be used in management plans for show caves should be conducted. Future management plans should include more detailed microbiological studies, including more sampling events, to better understand relationships between cave microbiota and the environment. It is also clear that different methods should be used in the microbiologic studies. For future studies, molecular and soil washing methods should be included for a better result. More studies concerning isolation of *H. capsulatum* should also be encouraged. Local governmental representatives should invest in training and informing local health agents (e.g., health caretakers) in order to aid in the diagnosis and epidemiologic reports of more serious fungal infections that might be related with cave visitation, such as histoplasmosis.

**ACKNOWLEDGEMENTS**

Part of the information contained in this study aided in the creation of the management plan of Lapa Nova cave, which was the first plan containing a more detailed microbiologic study to be approved by CECAV/IBAMA (National Center of Study Protection and Management of the Caves) in Brazil. The authors are thankful to the companies CAPES, VOTARANTIM and CARSTE CONSULTORES for the financial support; the cave guides Severino and Rivaldo for help in the field and information provided; Edson Luís Resende and Professor Ludwig H. Pfennig from Laboratório de Sistêmática e Ecologia de Fungos da UFLA (Laboratory of Fungal Systematics and Ecology at the Federal University of Lavras - UFLA), Dr. Daiani Silva, Misc. Fabiana Passamani, Professor Luis Roberto Batista and all the friends from NETAX (*Aspergillus* and *Penicillium* Taxonomic Nucleus) for the help on the identification of the isolates; and Professors Lucas Del Bianco Faria and Patricia Gomes for all the suggestions in the manuscript.

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Mycological study of a neotropical show cave


Concentration and stable carbon isotopic composition of CO₂ in cave air of Postojnska jama, Slovenia

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Abstract: Partial pressure of CO₂ (pCO₂) and its isotopic composition (δ¹³CairCO₂) were measured in Postojnska jama, Slovenia, at 10 locations inside the cave and outside the cave during a one-year period. At all interior locations the pCO₂ was higher and δ¹³CairCO₂ lower than in the outside atmosphere. Strong seasonal fluctuations in both parameters were observed at locations deeper in the cave, which are isolated from the cave air circulation. By using a binary mixing model of two sources of CO₂, one of them being the atmospheric CO₂, we show that the excess of CO₂ in the cave air has a δ¹³C value of -23.3 ± 0.7 ‰, in reasonable agreement with the previously measured soil-CO₂ δ¹³C values. The stable isotope data suggest that soil CO₂ is brought to the cave by drip water.

Keywords: karst; cave; CO₂ concentration; ¹³C of cave-air CO₂; Postojnska jama

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INTRODUCTION

Speleothem formation in karst caves can be controlled by various parameters such as drip rate, drip water temperature, soil temperature and moisture, CO₂ partial pressure (pCO₂) in soil and in cave air, calcium and bicarbonate concentration in drip water (Baldini, 2010 and references therein). The spatial and temporal behaviour of many of these parameters is fairly well-constrained by numerous studies. During the last two decades several studies on pCO₂ in cave air showed the importance of seasonal variations of pCO₂ and the origin of CO₂ for speleothem formation (Bourges et al., 2006; Genty, 2008; Faimon et al., 2012). Intra- and inter-annual variability in stalagmite growth rate can be ascribed to rapid and seasonal shifts in cave air pCO₂ (Spötl et al., 2005; Baldini et al., 2008; Mattey et al., 2008; Genty, 2008). In addition, visitor activity (number of visitors, door opening and closing) can also influence cave CO₂ concentrations (Faimon et al., 2012; Šebela et al., 2013). Although cave air pCO₂ does not directly affect δ¹⁸O of stalagmites, it may affect the seasonality of calcite deposition, and therefore the annual mean of δ¹⁸O in stalagmites can be biased toward the δ¹⁸O of the season with the fastest deposition and in such cases δ¹⁸O of speleothems should not be used for paleotemperature studies (Baldini, 2010; Mattey et al., 2010).

Postojnska jama (jama means cave in Slovenian) (Fig. 1) is world’s one of the largest and most famous karst cave. It has also been a site of extensive (scientific) investigations of cave environmental conditions and speleothem formation mechanisms (Urbanc et al., 1985, 1987; Gospodarič, 1988; Gams & Kogovšek, 1998; Genty et al., 1998; Vokal, 1999; Horvatinčič et al., 2003; Zupan Hajna et al., 2008; Šebela, 2010).

Studies of cave environmental conditions and their dependence on external temperature included studies of CO₂ concentration and cave air circulation (Gams, 1974a), radon concentration (Vaupotič & Kobal, 2004; Gregorič et al., 2011; Bezek et al., 2012), relation between number of tourists and the cave climate (Prelovšek et al., 2011; Šebela et al., 2013),...
and microclimatic characteristics (cave air pressure and temperature) (Sebela & Turk, 2011).

The first measurements of CO₂ concentration within the Postojna jama (Gams, 1974a) revealed seasonal fluctuations (lower concentration in winter and spring, higher in summer and autumn) and a spatial distribution along the passages, as well as different types of air circulation in the colder and in the warmer period of the year. No influence of the large number of visitors on the cave CO₂ concentration was observed. However, the origin of excess CO₂ concentration in the cave was not explained. CO₂ concentration (pCO₂) in the cave air has been recently monitored using a data logger at one location in the cave (Gabrovšek, unpublished).

The carbon isotopic composition (δ¹³C/¹²C and ¹⁴C) of the cave air CO₂ in Postojnska jama has not been studied systematically. The only available isotope data, δ¹³C and ¹⁴C activity (a¹⁴C), of CO₂ in the atmosphere outside and at a single location in the cave showed that δ¹³C and a¹⁴C were lower inside the cave (-9.3 ± 0.2 ‰ and 102.6 ± 1.6 pMC¹, respectively) than outside (-8.0 ± 0.2 ‰ and 109.5 ± 1.1 pMC), and this difference was explained by contribution of soil CO₂ brought to the cave by drip water (Vokal, 1999; Horvatiničić et al., 1998). However, the CO₂ concentration was not measured so no further quantification was possible. The stable carbon isotopic composition of both soil CO₂ and soil organic matter (SOM) were measured at two locations above the Postojnska jama. The δ¹³C of SOM was -26.7 ‰ at both locations indicating the dominant vegetation of C₃ type, while the δ¹³C values of CO₂ in the soil atmosphere were -21.7 ‰ and -20.0 ‰ (Vokal, 1999).

Measuring cave air composition, both pCO₂ and the carbon isotope composition δ¹³C, for at least one year could greatly assist geochemical and isotopic studies of speleothems (Baldini, 2010) and help identify the cave ventilation patterns. Therefore, as a part of the comprehensive monitoring of Postojnska jama (Mandić et al., 2012; Mandić, 2013), we measured concentration of CO₂ in cave air and its stable carbon composition (δ¹³C in CO₂) during a one-year period at several sampling points inside the cave and in the outside atmosphere. The aim of this study was to determine spatial and temporal distribution of pCO₂ and δ¹³C of the cave air, and to determine how soil CO₂ and cave ventilation influence the cave air.

**SITE DESCRIPTION**

Postojna karst area is located on NW part of the Dinaric Karst (Fig. 1). It is composed mostly of Cretaceous carbonate rocks, while Triassic and Jurassic dolomites appear on the northern and north-eastern side. On the western and south-western side this karst region is in contact with non-carbonate Eocene flysch rocks from where some rivers are flowing and sinking into the Postojna karst area in which more than 60 caves are known. The largest cave system is Postojnska jama, 20.5 km long, consisting of several caves separated by sumps among which Postojnska jama (10,399 m, Fig. 2) is the largest (Sebela, 1998).

The area is positioned between the sub-Mediterranean climate of the North Adriatic Sea and the continental climate of central Slovenia. Annual precipitation in the period from 1971 to 2000 is 1587 mm and mean temperature is 8.7 °C (data from meteo.si). In the period 1982 – 2011, the mean temperature was higher (9.2 °C) and precipitation slightly lower (1528 mm) than in the previous period. The annual amount of precipitation has not significantly changed in this period, but deviations from the mean increased in the last decade, e.g., 2010 was the year with the highest (1940 mm), and 2011 with the lowest (1078 mm) amount of precipitation in the last 30-year period. Average temperature in the six-year period 2006 – 2011 at the meteorological station Postojna was 9.9 °C. The mean temperature showed an increase of about 0.05 °C/yr in 1982 – 2011. A similar increase was observed also for Zagreb, Croatia (0.06 °C/yr, 1983-2007, Barešić, 2009) and Nontron, France (0.09 °C/yr, 1984 – 2007, Genty, 2008).

Postojnska jama was formed by the sinking Pivka River. There are two main levels of cave passages (Fig. 3): the upper, which is mostly dry and the lower where the river flows. The entrance to the upper level of the cave is at an altitude of 529 m a.s.l., which is 18 m above the actual Pivka River sinkhole. The entrance leads to dry upper cave passages and larger halls which are also connected with the lower river passage. The surface above the cave is covered by numerous dolines and is about 50-100 m above the cave. This vertical development creates enough gradient for strong air circulation.

The relative humidity in the cave is high (>94%) and constant (Gams, 1974a; Vaupotić & Kobal, 2004; Bezek et al., 2012). Such a high relative humidity minimizes evaporation of cave drip water and the deposition of secondary calcite occurs by degassing of CO₂ from carbonate saturated drip waters (McDermott, 2004).

**SAMPLING AND MEASUREMENT**

Nine locations (Figs. 2 and 3), at different distances from the entrance, were chosen as monitoring stations. Because of the importance of the Pivka River for the evolution of the Postojnska jama and its influence on environmental conditions two locations on the Pivka River were chosen for observation. One is outside of the cave near the sinkhole of the river (location 11). This represents the external atmospheric conditions. The other sampling site on the Pivka River is about 2.5 km downstream inside the cave (location 10). Field work was carried out in March, June, August, September and November 2010, and in February 2011.

Prior to each field trip 10 mL borosilicate vials were flushed with He gas 6.0 and capped by butyl rubber septa. At the test site the sample vial was opened for a couple of minutes and closed again. The concentration
of air CO₂ and air temperature were measured in situ by the hand-held detector Vaisala Carbocap GM 70 and a GMP 222 probe. At each location the drip rate, i.e., the number of drips per minute, was measured using a stopwatch, and the mean value of ten 1-minute measurements was recorded.

δ¹³C analyses were carried out using an on-line continuous-flow system (Gasbench II) linked to a Thermo Fisher Scientific DELTA¹⁺XL isotope ratio mass spectrometer. The experimental design of the measurements was comparable to the experimental setup described by Spötl (2004). The samples, an in-house calcite standard and two international reference materials (NBS_19 and NBS_18) were simultaneously analyzed by using the phosphoric acid method. The measured values are reported according to the VPDB scale. For replicate measurements the overall analytical uncertainty (1σ) for δ¹³C_airCO₂ is 0.15 ‰.

RESULTS

Temperature
Cave-air temperature at all locations in the studied period March 2010 – February 2011 is shown in Fig. 4a, while the mean values are presented in Table 1. The mean cave temperature reflects the average outside temperature of the last several years (9.9 ºC) but shows much smaller variation at all sampling sites. In a poorly ventilated cave the cave-air temperature remains essentially constant throughout the year, typically with variations of ±1 ºC (McDermott, 2004). Most of our locations have similar variations of less than ±1.6 ºC. Somewhat larger variations are observed for sampling sites close to the entrance (e.g., location 01) where the exchange with the open atmosphere is expected, at location 10 where the Pivka River influences the cave temperature, and at the outside location 11. The mean temperature at this location is somewhat higher than the mean temperature in Postojna because our measurements were single-point measurements usually taken during the warmer part of the day.

Drip rate
Drip rate showed a large variability among different locations (Table 1), e.g., at location 02 the drip rate was constantly low and showed no dependence on the precipitation amount, while at locations 07 and 08 the drip rate was higher and showed large variability, being the highest about 2 months after major

<table>
<thead>
<tr>
<th>Location number and name</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>01 - Slonova glava</td>
<td>■</td>
</tr>
<tr>
<td>02 - Biospeleološka postaja</td>
<td>○</td>
</tr>
<tr>
<td>03 - Vodopad</td>
<td>▲</td>
</tr>
<tr>
<td>04 - Kongresna dvorana</td>
<td>△</td>
</tr>
<tr>
<td>05 - Podrti kapnik</td>
<td>△</td>
</tr>
<tr>
<td>06 - Stebrišče</td>
<td>△</td>
</tr>
<tr>
<td>07 - Čarobni vrt</td>
<td>⚫</td>
</tr>
<tr>
<td>08 - Vrh Velike Gore</td>
<td>⬤</td>
</tr>
<tr>
<td>09 - Zgornji Tartar</td>
<td>★</td>
</tr>
<tr>
<td>10 - Pivka River inside</td>
<td>✗</td>
</tr>
<tr>
<td>11 - Pivka River outside</td>
<td>+</td>
</tr>
</tbody>
</table>

Fig. 2. Map of the Postojnska jama Cave with sampling locations.

Fig. 3. Schematic plot of the longitudinal profile of the Postojnska jama Cave with altitudes of the sampling sites. Symbols are the same as in Fig. 2. Linear distances are not in scale.
**CO₂ concentration**

Seasonal variations of CO₂ concentration (pCO₂) are shown in Fig. 5. The mean pCO₂ at sampling site 11, (398 ± 46) ppmv (Table 1), corresponds to the open atmosphere. This value can be compared to the mean yearly atmospheric CO₂ concentrations at the global reference site on Mauna Loa (Hawaii) in 2010 and 2011 (389.8 ppmv and 391.6 ppmv, respectively, average 390.7 ppmv) (http://www.esrl.noaa.gov/gmd/ccgg/trends). The pCO₂ in cave air is always higher than the outside atmospheric pCO₂. The lowest mean CO₂ concentration in the cave is observed at sampling locations 01 – 04 that are close to the entrance (770 to 840 ppmv), while locations 05 – 08 deeper in the cave show mean values >1000 ppmv. Locations 09 and 10 can be considered as a separate group with similar mean pCO₂ as well as similar seasonal variations, which are determined by the possible air circulation between these two locations (Fig. 3). The highest mean pCO₂ (1578 ppmv) and also the highest mean pCO₂ (1578 ppmv) and also the highest pCO₂ was measured in September (1300 ppmv).

**Carbon isotope (δ¹³C) composition of cave air**

Seasonal variations of δ¹³C_airCO₂ in cave air are shown in Fig. 6. δ¹³C_airCO₂ of the outside air (location 11) with the mean value -7.8 ± 3.9 ‰ VPDB is in agreement with global δ¹³C values of atmospheric CO₂ (Verburg, 2007). Mean values of δ¹³C_airCO₂ in cave-air CO₂ are lower than in the outside atmosphere. At locations 01 - 04 mean δ¹³C_airCO₂ varies from -12.6 to -13.9 ‰, while at other locations it has more negative values, the lowest being -18.0 ‰ at location 07 - “Čarobni vrt” where the highest mean pCO₂ was measured (Fig. 6, Table 1).

All locations inside Postojnska jama show similar seasonal variations, i.e., the lowest δ¹³C_airCO₂ values were measured in September 2010 and the highest in February 2011 (Fig. 6). A good correlation between individual pCO₂ and δ¹³C_airCO₂ data was observed at each location (R² > 0.7, p < 0.1): the higher the pCO₂, the lower the δ¹³C_airCO₂. No correlation was observed between the individual drip rate and δ¹³C_airCO₂ data. Exceptionally low δ¹³C was measured in September 2010 at the outside location 11 (-15.5 ‰), and this was probably caused by strong ventilation from the cave. The general cave air circulation in Postojnska jama is from the interior towards the exit in the warmer part of the year (May – October). When the cave temperature is higher than the outside temperature (November – April), cave air is released from the cave into the open atmosphere due to air draught caused by the “chimney effect” (Bezek et al., 2012; Šebela et al., 2013). The two main types of air circulation in Postojnska jama are schematically summarized in Fig. 7.

**DISCUSSION**

Fig. 8 presents mean values of both pCO₂ and δ¹³C_airCO₂ at each location as functions of the distance from the main entrance and the grouping of locations is obvious. Locations 01 - 04, which are relatively close to the entrance, have lower pCO₂ and higher δ¹³C_airCO₂ than locations 05 - 08 deeper in the cave. Locations 09 and 10 have similar both pCO₂ and δ¹³C_airCO₂ values, in between the two main groups of locations, and can be treated as the separate group.

Comparison of data presented in Figs. 5, 6 and 8 shows that the carbon isotopic composition of cave-air CO₂ varies inversely with pCO₂. The relation

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**Table 1.** Mean air temperature (T_air), mean CO₂ concentration in air (pCO₂) and mean δ¹³C isotope composition of the air (δ¹³C_airCO₂) at 11 sampling locations, and mean drip rate at nine sampling locations in the cave.

<table>
<thead>
<tr>
<th>Location</th>
<th>T_air (°C)</th>
<th>pCO₂ (ppmv)</th>
<th>δ¹³C_airCO₂ (‰ VPDB)</th>
<th>drip rate (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01 - Slonova glava</td>
<td>9.8 ± 2.2</td>
<td>782 ± 334</td>
<td>-13.1 ± 4.3</td>
<td>53 ± 53</td>
</tr>
<tr>
<td>02 – Biopspeolška postaja</td>
<td>9.9 ± 1.0</td>
<td>840 ± 335</td>
<td>-13.9 ± 4.5</td>
<td>14 ± 9</td>
</tr>
<tr>
<td>03 - Vodopad</td>
<td>9.7 ± 1.6</td>
<td>770 ± 320</td>
<td>-13.2 ± 4.8</td>
<td>60 ± 79</td>
</tr>
<tr>
<td>04 – Kongresna dvorana</td>
<td>10.3 ± 1.4</td>
<td>783 ± 300</td>
<td>-12.6 ± 4.5</td>
<td>50 ± 25</td>
</tr>
<tr>
<td>05 – Podrški kapič</td>
<td>11.1 ± 0.4</td>
<td>1162 ± 463</td>
<td>-15.0 ± 3.7</td>
<td>56 ± 69</td>
</tr>
<tr>
<td>06 – Stebrisče</td>
<td>11.3 ± 1.2</td>
<td>1038 ± 399</td>
<td>-16.3 ± 3.7</td>
<td>108 ± 48</td>
</tr>
<tr>
<td>07 – Čarobni vrt</td>
<td>10.7 ± 0.8</td>
<td>1578 ± 385</td>
<td>-18.0 ± 2.1</td>
<td>144 ± 140</td>
</tr>
<tr>
<td>08 – Vrh Velike gore</td>
<td>11.6 ± 0.7</td>
<td>1187 ± 470</td>
<td>-17.5 ± 2.6</td>
<td>118 ± 173</td>
</tr>
<tr>
<td>09 – Zgornji Tartar</td>
<td>10.7 ± 0.9</td>
<td>937 ± 325</td>
<td>-16.1 ± 3.2</td>
<td>80 ± 103</td>
</tr>
<tr>
<td>10 – Pivka River inside</td>
<td>10.9 ± 4.1</td>
<td>995 ± 348</td>
<td>-14.6 ± 4.9</td>
<td>118 ± 140</td>
</tr>
<tr>
<td>11 – Pivka River outside</td>
<td>12.1 ± 9.8</td>
<td>398 ± 46</td>
<td>-7.8 ± 3.9</td>
<td>144 ± 140</td>
</tr>
</tbody>
</table>
Fig. 4. a) Measured cave-air temperature at different locations (symbols) and mean monthly temperature (step line) at the meteorological station Postojna (−). b) Drip rate (symbols) and monthly precipitation (solid line) at meteorological station Postojna.
between $p_{\text{CO}_2}$ and $\delta^{13}\text{C}_{\text{airCO}_2}$ is shown as Keeling plot in Fig. 9. A good correlation between $1/p_{\text{CO}_2}$ and its $\delta^{13}\text{C}_{\text{airCO}_2}$ value has been obtained:

$$\delta^{13}\text{C}_{\text{airCO}_2} = (6.2 \pm 0.4) \frac{1000}{p_{\text{CO}_2}} + (-23.3 \pm 0.7), \text{N} = 49, R^2 = 0.79$$

(1)

The $\delta^{13}\text{C}$ value of -23.3 ‰ (intercept in eq. 1) is close to the previously determined carbon isotopic composition of soil CO$_2$ (-21.7 and -20.5 ‰, Vokal, 1999) in the area.

Based on the observed correlation between $p_{\text{CO}_2}$ and $\delta^{13}\text{C}_{\text{airCO}_2}$ (Fig. 8) we propose a simple mixing model of CO$_2$ with two sources contributing to the cave air CO$_2$. We assume the atmospheric CO$_2$ with $\delta^{13}\text{C} = -7.8$ ‰ and a concentration of 398 ppmv as one source, as determined here for location 11 (Table 1). The other source is $^{13}\text{C}$-depleted CO$_2$, and we assume it is the CO$_2$ generated by the decay of soil organic matter and root respiration.

Mathematically, the mixing model can be described as

$$p_{\text{atm}} \times \delta^{13}\text{C}_{\text{atm}} + (p_{\text{meas}} - p_{\text{atm}}) \times \delta^{13}\text{C}_{x} = p_{\text{meas}} \times \delta^{13}\text{C}_{\text{airCO}_2}$$

(2)

where $p_{\text{atm}}$ and $\delta^{13}\text{C}_{\text{atm}}$ are the concentration and stable isotope composition of atmospheric CO$_2$, respectively, (location 11, Table 1), and $p_{\text{meas}}$ and $\delta^{13}\text{C}_{\text{airCO}_2}$ are the corresponding measured values for each location 01 to 09, respectively. $\delta^{13}\text{C}_{x}$ is the carbon isotopic composition of the unknown end member, source of additional CO$_2$ inside the cave, which in our model contributes to the difference between the atmospheric $p_{\text{CO}_2}$ and the measured $p_{\text{CO}_2}$. Equation (2) has been applied to all individual measurements and the obtained $\delta^{13}\text{C}_{x}$ values range mostly between -22 and -26 ‰ (Fig. 10), in reasonable agreement with the $\delta^{13}\text{C}$ of soil CO$_2$ (Vokal, 1999) and the intercept of the Keeling plot (eq. 1). Therefore we conclude that the soil CO$_2$ brought to the cave by drip water contributes to the CO$_2$ concentration in the cave. To corroborate such a conclusion the relations between mean values of drip rate and $p_{\text{CO}_2}$ as well as between drip rate and $\delta^{13}\text{C}_{\text{airCO}_2}$ are shown in Fig. 11: the higher the mean drip rate, the higher the mean $p_{\text{CO}_2}$ ($R^2 = 0.6, p = 0.01$) and the lower the mean $\delta^{13}\text{C}_{\text{airCO}_2}$ ($R^2 = 0.7, p = 0.003$). It should be noted here that although there were not observed...
correlations between individual instantaneous values of the drip rate and pCO$_2$ as well as between the drip rate and the $\delta^{13}$C$_{\text{airCO}_2}$ at any of the studied locations, the statistically significant correlations have been obtained for the corresponding mean values (Fig. 11).

Up to 500,000 visitors yearly visit Postojnska jama per year (Šebela et al., 2013). Human breath contains about 40 000 ppmv CO$_2$ (Faimon et al., 2012), considerably higher than the atmospheric pCO$_2$ (380 – 400 ppmv) and the cave air pCO$_2$ (up to 3000 ppm, Fig. 5). $\delta^{13}$C of exhaled anthropogenic CO$_2$ reflects the $\delta^{13}$C of the source organic matter used as food and cannot be distinguished from the $\delta^{13}$C of soil CO$_2$ providing that C3 plants prevail in the region and that the human diet is based on C3 plants (Epstein & Zeiri, 1988). Human breath can thus significantly alter cave CO$_2$ concentrations while its influence on $\delta^{13}$C$_{\text{airCO}_2}$ cannot be simply determined. A recent study of pCO$_2$ in Postojnska jama during and immediately after a large number (2000 – 4000) of visitor per day over the course of several holiday periods showed that during the visit pCO$_2$ significantly increases, e.g., the observed increase in pCO$_2$ was between 450 and 1750 ppmv, but it returned to background levels within 1 – 10 days (Šebela et al., 2013). Our measurements were performed on days when only few visitors were present in the cave, and the highest pCO$_2$ and lowest $\delta^{13}$C$_{\text{airCO}_2}$ were observed at locations closed for tourists, so we believe that we measured the background pCO$_2$ levels in the cave, i.e. that the excess pCO$_2$ (above the atmospheric level) was brought to the cave by drip water.

**CONCLUSION**

The monitoring of pCO$_2$ in cave air and its $\delta^{13}$C value at several locations in Postojnska jama revealed their seasonal and spatial variations. The seasonal variations in both pCO$_2$ and $\delta^{13}$C$_{\text{airCO}_2}$ are...
more pronounced in deeper and closed parts of the cave, while some locations close to the entrance are prone to ventilation. Ventilation is observed in both directions, from the open atmosphere to the cave in winter and vice-versa in summer. Production of CO₂ in the soil zone and its transport by percolating water into the cave is the main source of CO₂ in the cave atmosphere. The excess of CO₂ in the cave air has a δ¹³C value of -23.3 ± 0.7 ‰. Postojnska jama can be described as well-ventilated cave with relatively low maximum and mean pCO₂ values, although not all locations show the same degree of ventilation.

ACKNOWLEDGEMENTS

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Epilithic and aerophilic diatoms in the artificial environment of Kungsträdgården metro station, Stockholm, Sweden

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Abstract: The Kungsträdgården metro station is an artificial and urban subsurface environment illuminated with artificial light. Its ecosystem is almost completely unknown and as a first step to better understand the biology and rock wall habitats the diatom flora was investigated. A total of 12 species were found growing on the rock walls of Kungsträdgården metro station. The results show the diatom flora in Kungsträdgården to be dominated by e.g. Diadesmis contenta, Diadesmis perpusilla, Pinnularia appendiculata, Nitzschia amphibia, Nitzschia sinuata and Diploneis ovalis. One species, Caloneis cf. aerophila, has never been reported from Sweden before. Significant differences in the species composition between the sampling sites indicate Kungsträdgården metro station to be a heterogeneous habitat that provides different microhabitats.

Keywords: epilithic diatoms; lampenflora; metro station

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INTRODUCTION

Urban underground facilities like utility tunnels (e.g. drainage, sewers, water, electrical power, gas, central heating) and tunnels for transportation is a crucial part of the urban infrastructure. Some urban subsurface areas, like metro systems, are constructed to daily hold and transport large quantities of people. An increased human presence in such systems urges the need to increase our understanding and knowledge of these environments. From an infrastructural or engineering point of view this means that the need to increase our understanding and knowledge of these environments. From an infrastructural point of view these environments are well known but other areas of the urban subsurface realm, like its biosphere, are still less known. This opens up a whole new niche for ecological research.

So far, there is a gap in our knowledge about the presence of diatoms in subsurface urban settings. In caves, on the other hand, diatoms have been studied at several locations (e.g. St. Clair and Rushforth, 1976; Garbacki et al., 1999; Lauriol et al., 2006; Pouličková and Hašler, 2007; Mulec et al., 2008), typically in karst caves. Kashima et al. (1987), however, studied diatoms of the genus Melosira and their contribution in the production of coralloid speleothems in a cave developed in deposits of quaternary pyroclastic flows.

Caves are considered to be a difficult environment for autotrophic organisms to inhabit since they need light for the process of photosynthesis. Diatoms seem to be able to grow to a point where light conditions are about 54-127 lx (Abdullin, 2011), but the weak light affects both the morphology and physiology of diatom cells. Cell volume, chloroplast size and number of thylakoids increases with decreasing light intensities. Light quality affects the diatom cells; blue light seems to induce the highest rate of photosynthesis (Jørgensen, 1977). However, artificial light can also be utilized and the term Lampenflora is used collectively for all photosynthetic organisms that live in caves with artificial illumination (Roldán and Hernández-Maríné, 2009; Mulec, 2012). Diatoms as Lampenflora have been studied in show caves e.g. in Slovenia (Mulec et al., 2008), the Czech Republic (Pouličková and Hašler, 2007) and in Kentucky, USA (Smith and Olson, 2007) and in catacombs (Albertano, 1993, Albertano and Urzi, 1999, Llop et al., 2012). A characteristic of Lampenflora is that its species diversity is low compared to that of other habitats, e.g. cave entrances (Mulec, 2012).
Here we report a study of diatoms, conducted in Kungsträdgården metro station, which is an environment illuminated with artificial light. It is the first study of diatoms in this type of urban environment. The aim of the study was to describe the aerophytic diatom community of Kungsträdgården metro station and increase the understanding of the wall habitats as well as how the diatoms dispersed into this system.

Epilithic and aerophilic diatoms
Diatoms are single-celled algae, which can be found in almost every aquatic habitat, often in high abundances and species richness (Julius and Theriot, 2010). Due to the presence of silica in their cell walls (the frustules), diatom morphology remains preserved during sedimentation and they are known from fossil assemblages from the Jurassic and onwards (Round et al., 1990). Their high abundances and often high species richness together with the fact that the species composition is very sensitive to factors in the environment, such as pH, salinity, nutrient availability and temperature, make the diatoms excellent as paleoenvironmental indicators (Smol and Stoermer, 2010). Diatoms are also used for monitoring water quality and classifications thereof (Stevenson et al., 2010).

Diatoms are divided into planktonic taxa and benthic taxa, based on the ecological region in which they can be found. The ecology of benthic diatoms is less understood than that of planktonic diatoms (Round et al., 1990). Benthic diatom communities can be categorized by their preferred substrates, e.g. epipsammon (growing on sand grains), epiphyton (growing on submerged macrophytes or large microalgae), epizoon (growing on animals) or epilithon (growing on hard substrata, e.g. on rock) (Pouličková et al., 2008). Many classification systems of aerial habitats have been proposed (e.g. Kolbe, 1932; Petersen, 1935; Ettl and Gärtner, 1995). Patrick (1977) argues for the recognition of two groups: moist aerial habitats and dry aerial habitats.

An aerial habitat is a more stressful environment than an aquatic habitat (Patrick, 1977; Johansen, 2010). Johansen (2010) even describes these types of habitats as harsh and limiting in several respects. One important factor in aerial habitats is moisture availability and how desiccation resistant the taxa are. In a study from Subantarctica, Van de Vijver and Beyens (1999) show that species richness is closely related to the degree of moisture; dry habitats will contain fewer species than a wet habitat. Van de Vijver and Beyens (1997) further describe how the size of *Pinnularia borealis* Ehrenberg diminishes in dryer habitats. Temperature extremes are another important limiting factor in aerial habitats (Johansen, 2010), and water conductivity and pH affect diatom species composition (Fránková et al., 2009). Type of substrate influences the diversity of diatom communities. For example, plain rock faces or rock faces with mosses will be inhabited by different diatom flora. Additionally, many aerial diatoms only tolerate moderate levels of nitrogen (Johansen, 2010).

**MATERIAL AND METHODS**

Diatom preparation and analysis
Three samples from Kungsträdgården metro station were collected on 5 November 2012 (Fig. 2). A spoon...
An XL30 environmental scanning electron microscope (ESEM) with a field emission gun (XL30 ESEM-FEG) was used to analyse the minerals and the diatom ooze. The ESEM was equipped with an Oxford x-act energy dispersive spectrometer (EDS), backscatter electron detector (BSE) and a secondary electron detector (SE). The acceleration voltage was 20 or 15 kV depending on the nature of the sample and the instrument was calibrated with a cobalt standard. Peak and element analyses were made using INCA Suite 4.11 software.

RESULTS

The diatom flora in Kungsträdgården metro station

Altogether 12 species are documented from Kungsträdgården metro station (Table 1). Relevant synonyms are mentioned in Table 1 and in the discussion; for more detailed nomenclatural information, see the webpage http://www.algaebase.org. All identified taxa are pennate diatoms and have at least one raphe. Size and morphology of reported diatoms conform to ranges and descriptions in Krammer and Lange-Bertalot (1986-1991), unless otherwise is stated below.

Sample K1

Sample K1 consisted of a “calcareous diatom ooze” (Fig. 3) that occur in depressions (~1-2 cm) of coralloid speleothems. The composition of the speleothems were analysed by ESEM/EDS and consists of CaCO3 (calcite or aragonite) and the diatom ooze of a calcareous mix with traces of Si, Mg, and S (Table 2; Fig. 4). Eight species were identified in this sample. Diadesmis contenta (relative abundance, ra=44.2%) (Fig. 5d), Pinnularia appendiculata (ra=20.5%) (Fig. 5h), Nitzschia amphibia (ra=19.8%) and Grunowia sinuata (ra=12.7%) (Fig. 5j) were dominating in the sample. Species present at a lower abundance in this sample are Cymbella laevis (ra=1.9%) (Fig. 5g), Diadesmis perpusilla (ra=0.5%) (Fig. 5e-f), Halamphora normanii (ra=0.2%) (Fig. 5i) and Caloneis cf. aerophila (ra=0.2%) (Fig. 5b).

For Scanning Electron Microscopy (SEM), cleaned samples were air-dried on carbon tape, placed on stubs. The samples were coated with a thin layer of gold (20 seconds) using an Agar high resolution sputter coater. The SEM investigations were carried out using the microscope Oxford Instruments model 6853, and the software Pc-SEM. Both the light microscope images and the SEM images were used to aid species identification.
Sample K7

Sample K7 did not consist of calcareous material, but of a dark brown diatom algae mat. Six species were identified. Grunowia sinuata dominated in the sample (ra=86.6%) (Fig. 5j), together with Nitzschia amphibia (ra=9.6%). Caloneis cf. aerophila (ra=1.5%) (Fig. 5b), Halamphora normannii (ra=1.1%) (Fig. 5i), Pinnularia appendiculata (ra=0.7%) (Fig. 5h) were also documented from sample K7. Further, an unusually small and unidentified Caloneis species (ra=0.4%) (Fig. 5c) was found in this sample. It is approximately 5 µm long and 2.5 µm wide. Striae are 5 in 2 µm, which gives 25 in 10 µm.

Sample K7

Like sample K1, sample K7 consisted of a “calcareous diatom ooze” (Fig. 3) that occur in depressions (~1-2 cm) of coralloid speleothems. Ten species were identified in sample K7. Diadesmis contenta was dominating (ra=37.7%) (Fig. 5d), together with Nitzschia amphibia (ra=33.8%), Caloneis cf. aerophila (ra=11.4%) (Fig. 5b) and Diploneis ovalis (ra=8.9%) (Fig. 5k). Species found in less abundance in this sample were Cymbella laevis (ra=3.6%) (Fig. 5g), Grunowia sinuata (ra=2.2%) (Fig. 5j), the small unidentified Caloneis species (ra=1.4%) (Fig. 5c), Caloneis cf. bacillum (ra=0.5%) (Fig. 5a), Pinnularia appendiculata (ra=0.2%) (Fig. 5h), and Navicula sp. (ra=0.2%).

DISCUSSION

The diatom flora of Kungsträdgården in comparison with other cave environments

Cave entrances are known to be inhabited by a flora of cyanobacteria, algae (e.g. chlorophyta, chrysophyta and diatoms), mosses, ferns and angiosperms (Lundberg, 2011; Mulec, 2012). Algae mats often cover large parts of cave walls (Roldán and Hernández-Maríné, 2009). The moist cave environment, in which temperature typically is relatively stable, facilitates diatom growth (Palmer, 2007), and while decreasing light eventually will become a limiting growth factor in a cave, it is typically available in sufficient intensity near the cave entrance. The diatom species richness in Kungsträdgården was low compared to most cave inventories (e.g. St. Clair and Rushforth, 1976; St.

Table 1. Taxa found in Kungsträdgården and their relative abundance (%).

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Notes</th>
<th>K1</th>
<th>K3</th>
<th>K7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caloneis cf. aerophila Bock</td>
<td></td>
<td>0.2</td>
<td>1.5</td>
<td>11.4</td>
</tr>
<tr>
<td>Caloneis cf. bacillum (Grunow) Cleve</td>
<td></td>
<td>---</td>
<td>---</td>
<td>0.5</td>
</tr>
<tr>
<td>Caloneis sp.</td>
<td>Specimens notably smaller than the normally reported size range</td>
<td>---</td>
<td>0.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Cymbella laevis Nägeli</td>
<td></td>
<td>1.9</td>
<td>---</td>
<td>3.6</td>
</tr>
<tr>
<td>Diadesmis contenta (Grunow ex Van Heurck) D.G. Mann</td>
<td>Synonym: Navicula contenta Grunow ex Van Heurck</td>
<td>44.2</td>
<td>---</td>
<td>37.7</td>
</tr>
<tr>
<td>Diadesmis perpusilla (Grunow) D.G. Mann</td>
<td>Synonyms: Navicula perpusilla Grunow, Navicula gallica var. perpusilla (Grunow) Lange-Bertalot, Diadesmis gallica var. perpusilla (Grunow) Lange-Bertalot</td>
<td>0.5</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Diploneis ovalis (Hilse) Cleve</td>
<td></td>
<td>---</td>
<td>---</td>
<td>8.9</td>
</tr>
<tr>
<td>Grunowia sinuata (Thwaites) Rabenhorst</td>
<td></td>
<td>12.7</td>
<td>86.6</td>
<td>2.2</td>
</tr>
<tr>
<td>Halamphora normannii (Rabenhorst) Levkov</td>
<td>Synonym: Amphora normanii Rabenhorst</td>
<td>0.2</td>
<td>1.1</td>
<td>---</td>
</tr>
<tr>
<td>Navicula sp.</td>
<td></td>
<td>---</td>
<td>---</td>
<td>0.2</td>
</tr>
<tr>
<td>Nitzschia amphibia Grunow</td>
<td></td>
<td>19.8</td>
<td>9.6</td>
<td>33.8</td>
</tr>
<tr>
<td>Pinnularia appendiculata (C. Arardh) Cleve</td>
<td></td>
<td>20.5</td>
<td>0.7</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Table 2. EDS analysis of speleothems and the associated calcareous ooze.

<table>
<thead>
<tr>
<th></th>
<th>CaCO3</th>
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<th>Ooze</th>
<th>Ooze</th>
</tr>
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<tbody>
<tr>
<td>C</td>
<td>11.40</td>
<td>14.73</td>
<td>16.90</td>
<td>12.80</td>
</tr>
<tr>
<td>O</td>
<td>50.76</td>
<td>54.15</td>
<td>57.90</td>
<td>43.27</td>
</tr>
<tr>
<td>Mg</td>
<td>0.50</td>
<td>0.58</td>
<td>0.58</td>
<td>0.58</td>
</tr>
<tr>
<td>Si</td>
<td>0.39</td>
<td>0.76</td>
<td>0.58</td>
<td>0.58</td>
</tr>
<tr>
<td>Fe</td>
<td>0.43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>37.85</td>
<td>29.80</td>
<td>24.44</td>
<td>42.77</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Fig. 3. Local occurrence of “calcareous diatom ooze” on coralloid speleothems found in Kungsträdgården metro station.

Fig. 4. ESEM image of the calcareous ooze.
Fig. 5. Species found in Kungsträdgården samples. a) *Caloneis cf. bacillum* (scale bar = 10 µm); b) *Caloneis cf. aerophila* (scale bar = 10 µm); c) *Caloneis* species (scale bar = 5 µm); d) *Diadesmis contenta* (scale bar = 5 µm); e-f) *Diadesmis perpusilla* (scale bar = 5 µm); g) *Cymbella laevis* (scale bar = 10 µm); h) *Pinnularia appendiculata* (scale bar = 10 µm); i) *Halamphora normanii* (scale bar = 10 µm); j) *Grunowia sinuata* (scale bar = 10 µm); k) *Diploneis ovalis* (scale bar = 10 µm); l-m) SEM images of *Caloneis cf. aerophila* (scale bar = 5 µm in l and 10 µm in m).
This finding fits the commonly observed pattern that species diversity of Lampenfloras is poor in comparison with cave entrances characterized by sun light (Mulec et al., 2008; Llop et al., 2012; Mulec, 2012). However, the Lampenflora in Kungsträdgården is represented by 12 diatom species, and this is a higher species richness than in most other studies of Lampenfloras (e.g. Poulíčková and Hašler, 2007; Smith and Olson, 2007; Mulec et al., 2008; Abdullin, 2011). Conceivably, this can be explained by the urban environment, from which the Kungsträdgården samples were taken. Compared to other cave environments, Kungsträdgården is more frequently visited by humans and that may contribute to dispersal. The metro station is further illuminated 24 hours a day which is distinctly more than a show cave that might be illuminated a few hours a day or a few days a week.

**Ecology of selected taxa**

*Diadesmis perpusilla* (Grunow) D.G. Mann. Synonyms: *Navicula perpusilla* Grunow, *Navicula gallica* var. *perpusilla* (Grunow) Lange-Bertalot, *Diadesmis gallica* var. *perpusilla* (Grunow) Lange-Bertalot. Fig. 5e-f.

*Diadesmis perpusilla* is only found in sample K1, and at a low relative abundance. According to Krammer and Lange-Bertalot (1986) it is often found on moist rocks, mosses and in soils. All species of *Diadesmis* are considered to be aerophilic (Spaulding and Edlund, 2009). *Diadesmis perpusilla* is characteristic of environments with considerable reduction in light intensity, for example in caves (Krammer and Lange-Bertalot, 1986). Species of the genus *Diadesmis* can also produce alternative frustules where the raphe slits are filled with silica. Due to this, these specimens can be confused with *e.g.* taxa of the fragilarioid genera (Round et al., 1990). *Diadesmis perpusilla* is reported from caves in e.g. Canada (Lauriol et al., 2006), United States (St. Clair and Rushforth, 1976; St. Clair et al., 1981), Belgium (Garbacki et al., 1999) and Slovenia (Mulec et al., 2008).

*Diadesmis contenta* (Grunow ex Van Heurck) D.G. Mann. Synonym: *Navicula contenta* Grunow ex Van Heurck. Fig. 5d.

*Diadesmis contenta* occurs in high abundance in the calcareous ooze samples (K1 and K7) of Kungsträdgården. This species is often found on rocks and mosses where water is seeping (Krammer and Lange-Bertalot, 1986). *Diadesmis contenta* is also one of few diatom species, which can live on dry rocks (Patrick, 1977). Like *D. perpusilla*, *D. contenta* is often found in environments with reduced light intensity (Krammer and Lange-Bertalot, 1986) and has previously been reported from caves in e.g. Canada (Lauriol et al., 2006), United States (St. Clair and Rushforth, 1976; St. Clair et al., 1981; Dayner and Johansen, 1991), Czech Republic (Poulíčková and Hašler, 2007), Belgium (Garbacki et al., 1999), Slovenia (Mulec et al., 2008) and as Lampenflora in Kentucky, United States (Smith and Olson, 2007), Russia (Abdullin, 2011) and Malta (Llop et al., 2012).

**Pinnularia appendiculata** (C. Agardh) Cleve Fig. 5h.

*Pinnularia appendiculata* is present in all three samples, but is only abundant in sample K1 where it represents more than a fifth of the diatom community. It is an aerophilic species and can be found for example on wet mosses and rocks (Krammer and Lange-Bertalot, 1986). This species has been documented from a cave in Arizona, United States (Johansen et al., 1981).

**Diploneis ovalis** (Hilse) Cleve Fig. 5k.

*Diploneis ovalis* is only found in sample K7 where it comprises about 1/10 of the diversity. It is a common epipelic diatom (Krammer and Lange-Bertalot, 1986), previously reported from a cave in Spain (Roldán et al., 2004).

**Cymbella laevis** Nägeli Fig. 5g.

*Cymbella laevis* is found in the calcareous samples (K1 and K7). The species is associated with oligotrophic calcareous waters, for example in karst landscapes, particularly on mosses and rocks (Krammer and Lange-Bertalot, 1986).

**Caloneis cf. aerophila** Bock Fig. 5b.

*Caloneis aerophila* is a rare species, associated with wet rocks (Krammer and Lange-Bertalot, 1986). Nevertheless, it makes up 11% of the taxa in sample K7, and is also found in the other samples. Species identification in this genus is problematic and we have only tentatively assigned certain diatoms to *C. aerophila*. Characters overlap between *C. aerophila* and *C. bacillum*, a problem that exists for several *Caloneis* species (Krammer and Lange-Bertalot, 1986). To our knowledge *C. aerophila* has not been reported from Sweden before, but it was not described until 1963 and it may have been identified as another species, for example *C. bacillum*, by early diatomologists.

**Caloneis sp.** Fig. 5c.

The unusually small *Caloneis* specimens found in samples K7 and K3 could be small forms of *C. aerophila* or *C. bacillum*. If so, our findings would expand the known size range of these species (but see Kupe et al. 2010, who reported a small *Caloneis* species in a karst lake in Albania). Diatoms successively diminish in size as a natural consequence of vegetative reproduction and in cultivation, minute, round or misshaped valves are sometimes observed when cells fail to undergo the subsequent phase of auxospore formation (normally associated with sexual reproduction). However, such small or misshaped valves are rarely seen in natural diatom communities (Round et al., 1990), and the small specimens found here could instead represent a new, not yet described species of *Caloneis*. Both these scenarios are plausible, but a more expanded and detailed study (preferably with SEM) of this taxon is needed.

**Halamphora normanii** (Rabenhorst) Levkov. Synonym: *Amphora normanii* Rabenhorst. Fig. 5i.

*Halamphora normanii* is found in samples K1 and K3 at low relative abundances. This is an aerophilic
species, mainly reported from wet mosses and on rocks where water is seeping (Krammer and Lange-Bertalot, 1986). The only previous report of this species in Sweden is from a paleolimnological study of small lakes, which were isolated from Lake Vänern during the Holocene (Risberg et al., 1996). It has also been reported from caves in Italy (Giordano et al., 2000).

**Microhabitat and distribution patterns in the metro station**

Dispersal of diatoms into Kungsträdgården metro station is most probably accomplished by air. Diatoms have been shown to be able to disperse over long distances by air (Spaulding et al., 2010), but are not known to migrate through micro-fractures in rock. The platform is not located under the Baltic Sea, which also contradict migration through cracks in the rock. It is also likely that the constant flow of people at the metro station might increase the chances and speed of introduction of new species to the station.

A spatial distribution of different communities of diatoms, characterized by different species composition, could be observed in Kungsträdgården metro station. This indicates a heterogeneous habitat providing different microhabitats, similar to some natural subterranean habitats (Culver and Pipan, 2009). Since no rapheless diatoms were found in any of the samples, motility seems to be important in the microhabitats of the calcareous ooze and the brown algae mat. However, there are no indications of an ability of the diatoms to move between the microhabitats on the wall; the distances between them are too great and the lack of coherent water seeping makes it impossible for the diatoms to migrate. Samples K1 and K7 are rather similar in their species composition, both dominated by *Diadesmis contenta* and *Nitzschia amphibia*. Both K1 and K7 are calcareous ooze samples and they have higher species richness than the brown algae mat of sample K3. This is in line with other studies of diatom species richness, which show a positive correlation with pH, calcium and conductivity (Pouličková et al., 2004, Fránková et al, 2009). There are however differences between K1 and K7, such as *Pinnularia appendiculata* being abundant in K1 and the presence of *Diploneis ovalis* in K7 but not in K1.

None of the few species of diatoms known to be obligatory heterotrophs were found in this study. The diatoms in Kungsträdgården are primary producers, utilizing the artificial light from the illumination. They form, together with e.g. cyanobacteria and the moss *Eucladium verticillatum* (Brid.) Bruch & Schimp, the primary foundation of the food web in the metro station.

**The speleothems – a result of biogenic speleogenesis?**

The formation of coralloid speleothems was studied in Togawa-Sakaidani-do Cave, Japan, by Kashima et al. (1987). This cave has developed in deposits of Quaternary pyroclastic flows and both the bedrock and the speleothems are rich in silica. Kashima et al. (1987) suggests that the growth of these speleothems is triggered by the activities of diatoms (*Melosira* spp.). Diatoms have also been shown to influence calcite precipitation in travertine crusts (Pentecost, 1998). This is done by depositing calcium carbonate crystals in the secreted mucilage (Pouličková and Hašler, 2007).

Nothing in the results of Kungsträdgården indicates that the diatoms trigger the growth of the speleothems found. However, the calcareous ooze (samples K1 and K7) are dominated by diatoms and it is likely that the diatoms are involved in the formation of this microhabitat. Diatoms are known to dissolve carbonates by excreting organic acids, thus, it is possible that they are actively involved in the persistence of the ooze (Brehm et al., 2005). Clearly, the calcareous ooze is a small and specialized niche in the ecosystem of the walls at Kungsträdgården metro station.

**CONCLUSIONS**

The Lampenflora of Kungsträdgården metro station is dominated by aerophilic taxa and is represented by 12 diatom species, a relatively high species richness compared to other studies of Lampenflora. All identified diatoms are autotrophic and pennate. Dispersal of diatoms into Kungsträdgården has probably occurred by air. It is likely that the diatoms are involved in the formation of the calcareous ooze found on the walls of Kungsträdgården metro station.

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