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The importance of ants in cave ecology, with new records and behavioral observations of ants in Arizona caves

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Abstract: The importance of ants as elements in cave ecology has been mostly unrecognized. A global list of ant species recorded from caves, compiled from a review of existing literature, is presented. This paper also reviews what is currently known about ants occurring in Arizona (USA) caves. The diversity and distribution represented in these records suggests ants are relatively common cave visitors (trogloxenes). A general utilization of caves by ants within both temperate and tropical latitudes may be inferred from this combined evidence. Observations of ant behavior in Arizona caves demonstrate a low level and sporadic, but persistent, use of these habitats and their contained resources by individual ant colonies. Documentation of Neivamyrmex sp. preying on cave-inhabiting arthropods is reported here for the first time. Observations of hypogeic army ants in caves suggests they may not penetrate to great vertical depth in search of prey, but can be persistent occupants in relatively shallow, horizontal sections of caves where they may prey on endemic cave animals. First cave records for ten ant species are reported from Arizona caves. These include two species of Neivamyrmex (N. nigrescens Cresson and Neivamyrmex sp.; Formicidae: Dorylinae), four myrmicines (Pheidole portalensis Wilson, Pheidole cf. porcula Wheeler, Solenopsis aurea Wheeler and Stenamma sp. Westwood), one dolichoderine (Forelius keiferi Wheeler) and three formicines (Lasius arizonicus Wheeler, L. sitiens Wilson, and Camponotus sp. Mayr).

Keywords: ants, cave ecology, Formicidae, trogloxene

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INTRODUCTION

The importance of ants as elements in the ecology of caves has been mostly unrecognized (Bellés, 1987; Tinaut & Lopez, 2001; Sendra et al., 2011) and historically, research has focused on determining if there are any truly troglobiotic ant species (Kempf, 1961; Wilson, 1962; Tinaut, 2001; Roncin & Deharveng, 2003). Due, in part, to the search for cave-adapted ants, the importance of individual ant colonies utilizing caves has not received adequate attention. The few researchers that have treated ants generally as potentially important elements in cave ecology include Peck, in his studies of caves in the Caribbean (Peck, 1974; 1981a; 1981b; 1982; 1992), Reddell & Cokendolpher (2001) and Cokendolpher et al. (2009) in similar studies in Belize, Mexico, and California and Texas in the USA, and Roncin & Deharveng (2003) in Laos. The great majority of ants occurring in caves probably function as trogloxenes, and it is in this capacity that their importance in cave ecology lies.

Arizona (USA) has a significant cave resource, with approximately 1,500 known caves, but our knowledge of arthropods in caves in the state is very limited (Pape, 2014; Pape & OConnor, 2014). This paper reviews what is currently known about ants occurring in Arizona caves. The first record of an ant in an Arizona cave was not reported until 1999, when Muchmore & Pape (1999) mentioned a Monomorium sp. Mayr from Arkenstone Cave near Vail, Arizona. This record is now known to actually be Pheidole cf. porcula Wheeler (this paper). Ten years passed before a second ant record appeared in Shear et al. (2009), for Camponotus ocreatus Emery from Cathedral Cave in northern Arizona. Wynne & Voyles (2014) reported four ant species from caves in the Grand Canyon-Parashant National Monument in Mohave County, Arizona. And most recently, studies in Kartchner Caverns at Kartchner Caverns State Park (KCSBP) near Benson, Arizona added an additional five species to the list of ants found in Arizona caves (Pape & OConnor, 2014).

This paper provides a literature summary of ant species recorded from caves around the World, with
new data from Arizona. Important elements of this data are the records and ecology of Neivamyrmex found in caves, including their predation on endemic cave arthropods. The sum of this data demonstrates the importance of individual ant colonies in the cave ecology, behavior that is probably global in extent.

MATERIALS AND METHODS

Literature review
A list of ant species recorded in caves was compiled from a review of existing literature, and is presented in Appendix A. Multiple cave records for species (such as the hundreds of records for Solenopsis invicta Buren from caves in Texas) have not all been included. Rather, the list was constructed with the intent of listing all genera and species reported from caves, and to assemble the known distribution of cave occurrences for each species. The cave ecological group assigned to these occurrences, when provided by the recording author(s), is included in Appendix A using their original terminology.

There is a long history of efforts to define the ecological associations of terrestrial cave animals, which have been summarized by several authors including Vandel (1965), Camacho (1992) and Sket (2008). To date the issue seems to not be satisfactorily resolved to the satisfaction of all (Romero, 2009, 2011; Lunghi et al., 2014; Pape, this paper), but the definitions proposed by Sket (2008) seem to be gaining greater acceptance among biospeleologists. I use the following definitions (modified after Sket, 2008) in the body of this paper.

A troglobiont is an obligate cave animal, which cannot live outside of the cave environment. A eutroglophile is a facultative cave animal that is capable of completing its life cycle within caves, but may also do so in similar habitats. A subtroglophile is an animal that has a proclivity for regularly using caves to meet one or more of its ecological needs, such as food, water, shelter, etc., but must return to the epigean environment to meet some required life cycle need(s). A trogloxene is an animal that opportunistically uses cave resources that occur within its territory. The term incidental (rather than “accidental”) is reserved for animals that randomly enter a cave, and that derive no ecological benefit from their presence in these habitats.

Taxonomy follows ANTCAT (Bolton, 2014) and phylogenetic sequence follows Ward (2014).

Field studies
Field studies for this paper consisted of in-cave biological surveys conducted in 35 caves in Arizona spanning 25 years. Surveys were conducted to document all macrobiotic resources, and did not specifically target ants. Search effort varied considerably, ranging from a couple of hours during a single visit to an individual cave (as at Porcupine Cave) to more extensive efforts involving numerous visits to a single cave over many years (as at Arkenstone Cave, with approximately 500 in-cave hours during 124 visits over 14 years, and supplemented by observations at the cave entrance during related studies). Surveys consisted of searching human-accessible portions of caves, turning over floor debris including small rocks, broken cave formations, and organic debris, and searching dead animals, scat and bat guano deposits. No pitfall traps were employed. Baits, consisting of raw wood blocks, were used only during the recent two-year Kartchner Caverns study (Pape & O’Connor, 2014). Since field studies in most of the caves were concerned primarily with establishing baseline biological inventories, no quantitative measurements of available nutrients were performed.

Most caves in Arizona contain sensitive archaeological, paleontological, biological or mineralogical resources, or some combination of these constituents. Few of these sites have adequate protections in place to protect these resources. Due to the presence of sensitive resources and the limited value of the negative findings at 29 of the studied caves, many of which were only visited once, they are not named in this paper. Descriptions are provided below for the six caves where ants were found during the field studies and for one previous record (at Cathedral Cave), for which such information was available.

Cave descriptions

- Arkenstone Cave is a designated research site located within Colossal Cave Mountain Park 37 km southeast of Tucson, near Vail, Arizona. The cave is situated in Arizona Upland Subdivision Sonoran Desertscrub (Turner & Brown, 1982) at 1,112 m asl. The cave is formed in Paleozoic age (370-260 mya) limestone strata, and is approximately 1,000 m in length. The cave supports several endemic troglobiotic arthropods (Muchmore & Pape, 1999).
- Kartchner Caverns State Park, near Benson, Arizona, lies within the Basin and Range Province physiographic region of the western United States, and is situated in the Semidesert Grassland biotic community (Brown & Makings, 2014). Kartchner Caverns is contained within a group of small hills situated low on the east flank of the Whetstone Mountains, and is formed in the Mississippian age (359-318 mya) Escabrosa Limestone (Jagnow, 1999). The cave is 1,428 m above sea level (asl), and is approximately three kilometers in length (Fig. 1). Kartchner Caverns supports a maternity colony of the cave myotis (Myotis velifer Allen) and a large and diverse suite of invertebrates, including several endemic troglobionts (Welbourn, 1999; Pape & O’Connor, 2014).
- Hidden Cave is a small cave on the east side of the Santa Rita Mountains in Santa Cruz County, Arizona. The cave is situated in Madrean Evergreen Woodland (Brown, 1982a), at 1,613 m asl, and its entrance overlooks a small, ephemeral riparian drainage. The cave is developed in the Mississippian age Escabrosa Limestone.
- Porcupine Cave is situated in Petran Montane Conifer Forest (Pase & Brown, 1982) on the Mogollon Rim in central Arizona, at approximately 2,180 m asl. The cave is formed in Permian age (270 mya) Kaibab Limestone, and is 1,585 m long.
Ecology of ants using caves

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Fig. 1. Plan view map of Kartchner Caverns showing areas of the cave mentioned in this paper and locations where each ant species was documented. The records for the Throne Room (Pheidole sp.) and Rotunda Room (Pheidole rhea) were single individuals found along tour trails and are presumed to be vagrant occurrences. Arrows show known surface connections that are accessible to invertebrates.

Patagonia Bat Cave is situated in Madrean Evergreen Woodland (Brown, 1982a) at 1,590 m asl near Patagonia, Arizona. The cave is formed in rock of igneous origin, and because of this is rather small, consisting of only two contiguous moderate-sized rooms (Fig. 2). The rear room serves as a seasonal roost for the lesser long-nosed bat (*Leptonycteris yerbabuenae* Martinez & Villa-R), a species currently listed as endangered under the United States Endangered Species Act (ESA). A few cave myotis also occupy the cave.

PATAGONIA BAT CAVE

Fig. 2. Profile map of Patagonia Bat Cave near Patagonia, Arizona.
• Nugget Cave is a small cave situated in Semidesert Grassland (Brown & Makings, 2014) at an elevation of 1,500 m asl in the Santa Catalina Mountains north of Tucson, Arizona.
• Cathedral Cave is a limestone cave located near Ash Fork, Arizona in Great Basin Conifer Woodland (Brown, 1982b) at an elevation of 1,621 m asl (Shear et al., 2009).

Vertical subsurface depths reported for ant observations in the study caves were determined by surveying using Suunto brand hand held compass and clinometer, Leica DISTO™ lite5 laser meter and a 100-foot Keson fiberglass tape. Survey data was reduced using Fountian Computer Product’s 2003 version of Compass cave survey software.

Voucher specimens have been placed in the University of Arizona Insect Collection (UAIC), Tucson, Arizona.

Portions of these studies were conducted under an Arizona State Parks permit dated 10 September 2009 (Kartchner Caverns), and United States Forest Service Permits SUP0065, dated 5 April 1993, Extension dated 23 August 1995 and SUP0065-01, dated 30 April 2014.

RESULTS

Literature review

This paper is the first effort to consolidate records of ants from caves (Appendix A). The included data provides support for the discussion on the importance of these animals in cave ecology. Records of ants occurring in caves are numerous, dispersed among a variety of taxa, and include approximately 300 species in 80 genera (Wheeler, 1938; Wilson, 1962; Reddell & Mitchell, 1971a, b; Decu et al., 1998; Reddell & Cokendolpher, 2001; Framenau & Thomas, 2008). The records are distributed globally, with the majority occurring in tropical regions or adjacent low latitudes (Appendix A). The list likely includes the great majority of available records, but it is probable that a few have eluded my search efforts.

Over half (56%) of ant subfamilies and nearly one quarter (24.4%) of currently valid genera have been recorded from caves (Appendix A). This broad representation across the family is evidence for a persistent presence of these animals in caves. It also speaks to the ubiquitous presence of ants globally, where they opportunistically occupy most available habitats. Hypogaecic species would intuitively be anticipated to occur more commonly in caves, but this is not evident in the available records. None of the genera appearing in the records are represented disproportionately to their species richness, and thus no affinity for cave use by any group is readily apparent. Since many of the records are ancillary, and not the result of targeted surveys for ants, attaching significance to any apparent prominence of a taxon among this data is problematic. Such analysis would require a review of all records of each species recorded from caves and is beyond the scope of this paper. Additionally, existing records are almost certainly clustered due to variability of search efforts regionally. Some regions have received focused attention, while others remain almost completely unstudied. This complicates any analysis using current records, and much additional data from ant-focused research is needed before meaningful analyses can be performed.

Less than half of the records include an author-assigned ecological association (ecological group) for the ants with the caves where they were found. And, nearly half of the records providing an association are considered incidental (accidental) occurrences by the record author(s). Ants are thus seldom included in ecological discussions that accompany cave biological inventories. Since authors often fail to provide specific definitions to assigned ecological groups for reported occurrences (Sket, 2008), comparisons between records are problematic. A proliferation of classification systems for ecologically stratifying cave biota has, over the years, introduced considerable confusion into the biospeleological literature (Sket, 2008), and this further aggravates record comparisons. Thus, no effort has been made in this paper to adjust or correlate the record authors’ intent for ecological categories they assigned.

Field studies

Observed nutrient resources in the studied caves varied significantly in quantity. Six of the caves have a long history of use by bats, and contain significant, annually refreshed bat guano deposits (Hoffmeister, 1986; Cockrum 1991; Cockrum & Petryszyn, 1991; Mizutani et al. 1992; Buecher & Sidner 1999; USFWS, 1995; Pape, 2014; Pape & O’Connor, 2014). However, ants were found in only two of these caves, and only at Patagonia Bat Cave were ants observed actively foraging at a bat guano deposit. Dead vertebrate and invertebrate remains contributed to overall nutrient resources in several of the studied caves. Ringtail scats (Bassariscus astutus Lichtenstein) are important nutrient sources for many cave invertebrates (Muchmore & Pape, 1999; Pape, 2014; Pape & O’Connor, 2014), and were present in many of the caves in this study. Cave crickets and their guano are sometimes important nutrient sources in caves (Barr, 1967; Hubbell & Norton, 1978; Muchmore & Pape, 1999; Lavoie et al., 2007), particularly where cricket populations are significant, such as in Arkenstone Cave (Poulson, 1992; Muchmore & Pape, 1999; Lavoie et al., 2007).

Plant materials found in the caves were generally quite limited, particularly in lower elevation desert caves, and were almost always concentrated near surface connections where such debris cascades into cave entrances. Only Porcupine Cave contained significant plant material in the cave interior. This cave occasionally takes a significant quantity of water directly into the entrance from the adjacent drainage during large hydrologic events, resulting in organic materials being transported into the cave. None of the studied caves contain a perennial vadose stream that provides a regular source of allochthonous nutrient input. However, a few of
the caves occasionally receive small to moderate quantities of vadose flow that enters the caves through the alluvium of adjacent surface drainages during spring runoff or stochastic precipitation events. These waters are usually devoid of coarse organic materials, which are apparently filtered out during passage through the alluvium. The raw wood blocks used as bait in the Kartchner Caverns study did not attract any of the five ant species found in that cave.

Ants were recorded in six of the 35 caves studied. These new records increase the number of species recorded from Arizona caves from 10 to 20 species (Table 1). The distribution of these records is shown in Fig. 3. The following species accounts provide information on each of the ant records from Arizona caves, and assign an ecological group to the subject colony. The discussion section following the species accounts addresses how these findings support the importance of ants in cave ecology.

### Table 1. Summary of ant species recorded from caves in Arizona and the ecological associations for observed colonies.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cave Name</th>
<th>Cave Entrance Elevation (m)</th>
<th>Ecological Group (Source Author)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorylinae</td>
<td>Neivamyrmex graciellae</td>
<td>Kartchner Caverns</td>
<td>1,428</td>
<td>Subtroglophile*</td>
</tr>
<tr>
<td>Dorylinae</td>
<td>Neivamyrmex leonardi</td>
<td>Kartchner Caverns</td>
<td>1,428</td>
<td>Subtroglophile*</td>
</tr>
<tr>
<td>Dorylinae</td>
<td>Neivamyrmex nigrescens</td>
<td>Arkenstone Cave</td>
<td>1,112</td>
<td>Trogloxene</td>
</tr>
<tr>
<td>Dorylinae</td>
<td>Neivamyrmex sp.</td>
<td>Arkenstone Cave</td>
<td>1,112</td>
<td>Subtroglophile</td>
</tr>
<tr>
<td>Dolichoderinae</td>
<td>Forelius keiferi</td>
<td>Arkenstone Cave</td>
<td>1,112</td>
<td>Trogloxene</td>
</tr>
<tr>
<td>Formicinae</td>
<td>Lasius arizonicus</td>
<td>Hidden Cave</td>
<td>1,613</td>
<td>Subtroglophile</td>
</tr>
<tr>
<td>Formicinae</td>
<td>Lasius sitiens</td>
<td>Porcupine Cave</td>
<td>2,140</td>
<td>Subtroglophile?</td>
</tr>
<tr>
<td>Formicinae</td>
<td>Nylanderia cf. hystrix</td>
<td>PARA 1801</td>
<td>3,540</td>
<td>Incidental</td>
</tr>
<tr>
<td>Formicinae</td>
<td>Camponotus ocreatus</td>
<td>Cathedral Cave</td>
<td>1,621</td>
<td>Unknown</td>
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<tr>
<td>Formicinae</td>
<td>Camponotus sp.</td>
<td>Hidden Cave</td>
<td>1,613</td>
<td>Incidental</td>
</tr>
<tr>
<td>Myrmicinae</td>
<td>Pheidole cf. porcula</td>
<td>Arkenstone Cave</td>
<td>1,112</td>
<td>Trogloxene</td>
</tr>
<tr>
<td>Myrmicinae</td>
<td>Pheidole portalesis</td>
<td>Arkenstone Cave</td>
<td>1,112</td>
<td>Subtroglophile</td>
</tr>
<tr>
<td>Myrmicinae</td>
<td>Pheidole rhea</td>
<td>Kartchner Caverns</td>
<td>1,428</td>
<td>Trogloxene</td>
</tr>
<tr>
<td>Myrmicinae</td>
<td>Pheidole rhea</td>
<td>Patagonia Bat Cave</td>
<td>1,592</td>
<td>Trogloxene</td>
</tr>
<tr>
<td>Myrmicinae</td>
<td>Pheidole vistana</td>
<td>PARA 2602</td>
<td>736</td>
<td>Incidental</td>
</tr>
<tr>
<td>Myrmicinae</td>
<td>Pheidole sp.</td>
<td>Kartchner Caverns</td>
<td>1,428</td>
<td>Incidental*</td>
</tr>
<tr>
<td>Myrmicinae</td>
<td>Solenopsis aurea</td>
<td>Arkenstone Cave</td>
<td>1,112</td>
<td>Incidental</td>
</tr>
<tr>
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<td>Solenopsis xyloni</td>
<td>PARA 2602</td>
<td>736</td>
<td>Incidental</td>
</tr>
<tr>
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<td>Nugget Cave</td>
<td>1,518</td>
<td>Trogloxene?</td>
</tr>
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<td>Kartchner Caverns</td>
<td>1,428</td>
<td>Trogloxene</td>
</tr>
</tbody>
</table>

*Originally listed as a troglophile in Pape & O'Connor, 2014.
†Originally listed as a trogloxene in Pape & O'Connor, 2014.

### Species accounts

**Neivamyrmex graciellae**

*Mann was previously known only from the states of Jalisco and Oaxaca in central and southern Mexico, and from a single occurrence in southern Arizona at Florida Canyon, on the west flank of the Santa Rita Mountains (Snelling & Snelling, 2007). A recent study at Kartchner Caverns revealed a long-term presence of this species associated with that cave (Pape & O'Connor, 2014). N. graciellae has been observed active in the cave at depths ranging from 15 to 24 m below the surface.

During an earlier study at KCSP Welbourn (1999) reported the presence of what appeared to be old ant trails in the soil substrate (Fig. 4) near the entrance to the Red River Passage (Fig. 1). However, no ants were observed associated with the trails, or anywhere else in the cave during his study. Subsequent to Welbourn’s study and commercial development of the cave as a state park, KCSP personnel had observed and sampled ants in the cave on several occasions. My review of these materials revealed the presence of...
two army ant species; *N. graciellae* and *Neivamyrmex leonardi* Wheeler.

During the recent study we found contemporary evidence of ant trail building in the same area of the cave that Welbourn mentioned (Figs. 5 & 6). One of the trails (Fig. 5A) entered an access turret (Fig. 5B) atop a mud-covered calcite bridge that spans the Red River Passage in the cave. It is presumed that this natural bridge structure contained a bivouac of *N. graciellae* at one time. There are very few records of ant trails found in caves. The ant trails in Kartchner Caverns are similar to those of *Labidus coecus* Latreille, which have rarely been reported in caves in Texas and Mexico (Fig. 8 in Reddell & Cokendolpher, 2001). There are currently no Arizona records for *L. coecus*. *Nomamyrmex* (*N. esenbecki wilsoni* Santschi), a deep subterranean doryline (Schneirla, 1971), has been recorded in the United States only from south central Texas (Watkins, 1985), and has so far not been recorded in caves.

A raiding column of *N. graciellae* was observed in the Red River Passage on 3 October 2013. Hundreds of ants, consisting of both minor and major workers, were moving in both directions along the approximately 11 m of their trail that was visible. The ants were transporting prey, including pupae and workers of *Crematogaster opuntiae* Buren (Fig. 7). They entered the old bivouac site via the turret but emerged from the far side of the bridge and continued to the northeast. The old bivouac site was apparently not actively being used at the time. Two meters beyond the turret the column went beneath the east wall of the passage at the contact of the bedrock with the floor soil fill. The southwest arm of their trail entered a humanly inaccessible area of breakdown blocks on the cave floor. Small numbers of *N. graciellae* are occasionally observed in other areas of the cave by park staff or researchers.

An old waste midden consisting of hundreds of undigestible cuticular elements of *C. opuntiae* (Fig. 8) was found immediately adjacent to the raiding column trail, four meters southwest of the calcite bridge access turret. The midden is presumed to be the remains of an old *N. graciellae* bivouac site, where the undigestible parts were dropped out of the bivouac. The only other army ant bivouac records from caves are of *L. coecus*, found in at least three caves in Texas, including: Beck Crevice Cave, Beck Sewer Cave and Testudo Tube (Reddell & Cokendolpher, 2001).
It is not known whether *N. graciellae* feeds exclusively on ants, or may opportunistically prey on other arthropods in the cave, including those associated with the bat guano deposits. The absence of ant trails in the vicinity of the guano deposits suggests that *N. graciellae* does not forage at those sites.

Welbourn (1999) reported that some of the ant trails he observed were constructed over human footprints in the cave. Since the cave was not discovered until 1974, those trails would have been no older than 17 years in age at the time of his study (1989-1991). The old trails are still visible today (Fig. 4), and are now between 27 and 42 years in age. The old trails are approximately 1.5 m above the current hydrologic flow level of the ephemeral stream in the adjacent Red River Passage, and are unlikely to have been affected by flowing or pooled water. The degeneration of the trails probably results from a gradual equalization slumping of the clay soil substrate due to repeated changes in soil moisture content. Repeated expansion and contraction of the soil over long periods of time would gradually obliterate the trails. Entrenched ant trails have so far been found only in the vicinity of the Red River Passage.

*C. opuntiae*, has not been found in the cave, and is considered unlikely to occur there. *N. graciellae* probably leaves the cave to forage, and likely also during alate dispersal. Neither active bivouacs nor reproductives of *N. graciellae* have been observed in the cave, but the presence of the old bivouac access turret, the waste midden, active prey transport and multiple trails of varying age are ample evidence supporting a long-term use of the cave by this species. Based on this evidence *N. graciellae* is considered a subtroglophile in Kartchner Caverns.

**Neivamyrmex nigrescens**

While performing field observations at the entrance to Arkenstone Cave on 12 November 2000, I observed a small leader column of several *N. nigrescens* exiting the cave beginning at 10:26 hrs. About two-dozen ants were in the column, going upslope a distance of about 20 cm. They remained in the shade beneath a shallow overhang of the bedrock fault along which the cave is developed, and did not venture beyond the point where the bedrock was visibly damp. The ants appeared to want to continue further from the cave, but were apparently discouraged by daylight and/or moisture conditions (surface dryness) they encountered, and periodically returned to the cave.

The rock surface temperature was 8°C in the shade of the cave entrance sink at the time. The area the ants were traversing was approximately 0.9 m from the sun-shade interface at the north side of the cave entrance sink. The ants were intermittently active here between 10:26 and 14:10, a total of just over three hours. No prey was observed being transported along this short, exploratory column. Based on current knowledge, this colony being eutroglophilic is reviewed in the discussion section.

**Neivamyrmex sp.**

During a visit to Arkenstone Cave on 23 October 1999 a raiding column of *Neivamyrmex* sp. was observed actively foraging in the cave. The ants were first observed in the First Antechamber, which is just within the cave aphytic zone (Fig. 9). This location is 33 m from the cave entrance and 36 meters vertically below the surface of the cave hill.

The leading edge of the foraging column was somewhat dispersed. About two meters closer to the front of the cave, where the ants were more numerous, they had scavenged a tachinid fly and were transporting it back along the column in two pieces consisting of its left wing and the co-joined thorax and abdomen. The ant column was traced back towards the front of the cave where they were observed entering the cave directly from the epikarst into a breakdown-choked area approximately seven
meters east of the gated human entrance. From this point the ants had a single column, which was observed to divide only once. The left branch headed up a parallel passage a distance of at least two meters. The main foraging column extended for a distance of 24 m through the Register Room and into the adjacent First Antechamber (Fig. 9, red line). The linear extent of the column observed within the cave was approximately 34 m. The foraging ants averaged about 60 per decimeter along the column, resulting in an estimated 20,000 observed individuals.

Along the length of the column several invertebrates had been attacked by the ants, including two adult cave cricket (*Ceuthophilus* cf. *pinalensis* Hubbell: *Rhaphidophoridae*) (Fig. 10). Due to their large size, the crickets had not been moved along the column, but were being processed by the ants into smaller pieces for easier transport. The crickets had evidently been taken down where they stood when they were overcome by the advancing ant column. Several cave millipedes *Colactis utorum* Chamberlin (Diplopoda: Dorypetalidae) had also been subdued by the ants (Fig. 11). The millipedes are not highly mobile and were easily overcome by the advancing ant column.

This occurrence of *Neivamyrmex* sp. in Arkenstone Cave was the only time that the species was observed during more than 130 visits (including the 124 visits that had a biological survey component) to the cave between October 1987 and September of 2002. This is the first instance reporting an army ant colony preying on cave animals. *Neivamyrmex* sp. is a subtroglophile in the cave.

*Forelius keiferi*

*Forelius keiferi* Wheeler was observed entering and leaving Arkenstone Cave on 16 April 2013. Five ants were seen removing a small, round piece of debris from the cave, which may have been a piece of a ringtail scat. The species is a trogloxene that forages in the cave entrance area.
Lasius arizonicus

During a visit to Hidden Cave on 11 January 2013 a single ant nest access soil turret and an adjacent tunnel surface breach were observed on the cave floor 30 m from the cave entrance. No ants were present at the time of this visit. Several subsequent visits to the cave revealed additional digging activity of the ants in the front part of the cave, but the ants (Lasius arizonicus Wheeler) were not found and sampled until 27 June 2015. During this visit two colonies of L. arizonicus were found active in the cave. Approximately a dozen ants were roaming over the cave floor and beneath debris in proximity to their nest in the front part of the cave, in the area where their diggings had previously been observed. An abundance of white fungus is present on fine plant rootlets in the soil substrate where the ants occur in this area. It is not known if the fungus is significant in the ecology of the species. A second colony was found at the very back of the cave. Here, a small cluster of approximately 20 individuals were assembled in a concentrated group on the floor of the cave. A nest entrance was not evident in the soft floor sediments at this location. Due to the nesting activity and persistent use of Hidden Cave by L. arizonicus, the colonies occurring in the cave are assigned the status of a subtroglophile.

Lasius sitiens

A few workers of Lasius sitiens Wilson were found in the front part of Porcupine Cave in northern Arizona on 15 May 1999, approximately 6 m in from the cave entrance. The association of this colony with the cave was not readily apparent, but Wilson (1955) made the following comments regarding this species: “The light coloration, small eyes, and shortened appendages of sitiens constitute a remarkable morphological convergence to the species of the subgenus Cautolasius, and specifically to the primitive species L. (C) alienoflavus Bingham. There can be little question that the characters shared by these two species are the mark of a subterranean mode of life.” The cave is subject to occasional flooding during significant precipitation events. The presence of organic materials adhered to the ceiling in the interior of the cave indicates that the cave totally fills with water on occasion. The colony of L. sitiens in Porcupine Cave may be subtroglophile.

Nylanderia (Paratrechina) cf. hystrix

There is a single record of this species from a cave (PARA 1801) in the Grand Canyon-Parashant National Monument in northern Arizona. The cave is located at an elevation of 3,540 m. The occurrence was considered incidental (accidental) by the authors. Ants for all the PARA cave records included in this paper were taken in baited pitfall traps in the cave entrances (Wynne & Voyles, 2014). There is only one previous record of N. hystrix from Arizona, collected in 2003 from beneath a stone in a desert wash at Matkatamiba Canyon in Grand Canyon National Park (AntWeb). The Matkatamiba Canyon specimens are archived in the collection at the California Academy of Sciences (CASENT 0056873).

Camponotus ocreatus

This species is recorded from Cathedral Cave in northern Yavapai County, Arizona (Shear et al., 2009). The Shear paper addressed new cave millipede species from the southwestern United States, and there was no ecological information specific to C. ocreatus included in the work, thus its ecological status is unknown.

Castanea sp.

During a visit to Hidden Cave on 7 January, 2015 a single Castanea sp. major was found approximately 30 m into the cave. This individual was alive, but mostly immobile, and barely able to stand. It is assumed that this individual is an isolated, vagrant from the surface that could not find its way out of the cave. Regular human traffic in the cave may easily have obliterated its chemical trail, causing the animal to become lost in the cave. This species is not C. ocreatus. This record is considered an incidental occurrence in the cave.

Pheidole cf. porcula

This small, uniformly brownish-yellow ant has a fairly regular presence within Arkenstone Cave, where they forage on nutrients in the twilight zone. Unfortunately only minors have ever been found. This species was incorrectly reported as Monomorium sp. in Muchmore and Pape (1999). This ant seems most similar to Pheidole porcula Wheeler, but may be an undescribed species. The species has been found no deeper than 27 m from the entrance, and they rarely enter the aphotic zone. They have been observed in the cave during most months of the year, with seldom more than a couple dozen foraging individuals present at any given time.

They have been observed scavenging a variety of dead invertebrates from the cave, including a lithobiid centipede, an epigean grasshopper (Phrynotettix tshiuavensis Haldeman) and cave crickets (C. cf. pinalensis). They dismember larger carcasses, and cooperatively remove pieces from the cave to their nest site. This species was incorrectly reported as Monomorium sp. major was found out of the cave. Regular human traffic in the cave may have vagrant from the surface that could not find its way in. This species is a trogloxene in Arkenstone cave.

Pheidole portalenis

Pheidole portalenis Wilson was recorded from Arkenstone Cave on 16 November 2003, when at least ten ants were seen foraging from the cave entrance. One ant was carrying the abdomen of a small beetle (Hyporhagus sp.; Zopheridae) into the cave. The ants did not go any further than about 30 cm outside the cave, and it is presumed that their nest was in the front portion, and not deep within the cave. According to Stefan Cover (as cited in Wilson, 2003), P. portalenis regularly nests in cracks in rock walls, from which they forage. In this instance apparently, the ants selected a cave for their nest site. This colony is assigned a subtroglophile status based on the presence of their nest within the cave.
Fig. 12. Several Pheidole cf. porcula inside Arkenstone Cave transporting a leg of Ceuthophilus cf. pinalensis out of the cave, July 19, 1992.

Pheidole rhea

*P. rhea* was occasionally found in Kartchner Caverns during the recently completed two-year study (Pape & O'Connor, 2014). They were usually found in the Jackrabbit Shaft and the adjacent portion of the Jackrabbit Gallery (Fig. 1). There is one additional record of the species taken from a tour trail curb deeper in the cave, in the Rotunda Room. This latter record likely represents a vagrant ant, which may have been transported deep into the cave on a tour visitor. *P. rhea* is relatively common on the hill above the cave.

There is evidence that *P. rhea* in Kartchner Caverns is occasionally preyed upon by the endemic thread-legged bug *Phasmatocoris labyrinthicus* Pape (Pape, 2013). Three ants, including one major, were found lying dead on the cave floor in the Jackrabbit Shaft door below where the predatory thread-legged bugs have been found on several occasions. We do not know why this ant species comes into the cave. Their presence could be a simple case of proximity to a nest in the soil horizon near the top of the Jackrabbit Shaft. A variety of invertebrates occur in this area of the cave, and *P. rhea* could possibly be preying on some of these species, although this has not been observed. Due to its somewhat regular presence and a suspected nexus with the ecology of the cave, the *P. rhea* colony at Kartchner Caverns is presumed to be a trogloxene.

A second *P. rhea* colony was observed at Patagonia Bat Cave in southern Arizona on 17 August 1996. The ants accessed the cave through the lower entrance, and a steady column of minor workers was busily removing arthropods and other materials from the main bat guano deposit at the rear of the cave (Fig. 2). Among the prey were larvae of a dermestid beetle (*Dermestes carnivorus* Fabricius; Fig. 13; arrow 1) and two species of bat flies (Diptera: Streblidae); *Trichobius major* Coquillett (Fig. 13; arrow 2) and *T. sphaeronotus* Jobling. Live bat flies are highly unlikely to occur on the guano deposit on the floor of the cave. Possibly the ants were retrieving flies that had died of natural causes, or that may have been injured or killed by grooming bats. Flies dispatched in this manner would fall to the guano deposit where they could be scavenged by the ants. Because *Pheidole* commonly forage on the ground, and seldom climb very high in vegetation even when harvesting seed (Wilson, 2003), it seems improbable that the ants would capture live flies since they would have to forage on the cave ceiling where the flies typically occur in the bat roost.

*Leptonycteris* primarily feed on nectar and pollen of cacti and agaves during nocturnal foraging forays, and return to the roost to digest their meal and groom. Pollen in their guano, combined with remnants groomed from their faces, accumulates below the roost into a thin, consolidated yellow cake. The ants were seen removing rather large pieces of this material from the cave. The ants were also removing cactus fruit seeds from the bat guano deposit. These were identified as seeds of the organpipe cactus (*Stenocereus thurberi* Engelmann) by W.D. Peachey (personal communication, 24 October 2013). The site was revisited on 30 August 2014 but no ants were found foraging in the cave. The nearest *Pheidole* nest located during this second visit was approximately 235 m south of the cave. The *P. rhea* colony documented foraging in the cave is assigned the status of a trogloxene.

Pheidole vistana

Three *P. vistana* were sampled in the entrance area of a cave (PARA 2602) in the Grand Canyon-Parashant National Monument in northern Arizona by Wynne & Voyles (2014). The cave is located at an elevation of 736 m. This occurrence of *P. vistana* in the cave was considered incidental (accidental) by the authors.

Fig. 13. Foraging *Pheidole rhea* in Patagonia Bat Cave, 16 August 1996. Prey include larvae of the dermestid beetle *Dermestes carnivorus* (arrow 1) and the streblid fly *Trichobius major* (arrow 2). Faint, yellow pollen guano splatters of the lesser long-nosed bats (*Leptonycteris yerbabuena*) that occupy the cave can be seen on the rock in the upper left portion of the photograph.
Phidole sp.
A single minor of this species was found on 27 May, 2007 in the Throne Room, deep within Kartchner Caverns. However, because there is only this single record, and the sample location was on the tour trail, this individual is most likely an incidental that entered into the cave with one of the commercial tours.

Phidole sp.
A single unidentifiable minor worker of this species was taken in the entrance to PARA cave 2204 (Wynne & Voyles, 2014). This species was considered an incidental (accidental) by the authors.

Solenopsis aurea
At least one species of Solenopsis (the invasive S. invicta) is known to forage within caves (Elliott, 1992, 2000; Longacre, 2000; Taylor et al., 2005; Cokendolpher et al., 2009). A single worker of the desert fire ant (Solenopsis aurea Wheeler) was found just inside the entrance to Arkenstone Cave on 14 January 2012. The ants could potentially prey on invertebrates that occupy the cave. The association of this species with the cave is not known, but it is a presumed trogloxene.

Solenopsis xyloni
Five S. xyloni were taken in the entrance of PARA cave 2602 (Wynne & Voyles, 2014). The ants were considered incidentals (accidentals) by the authors. As the numbers of an ant species found in an individual cave increase, either at one time, or cumulatively during separate events, there is an implied greater potential that their presence has some ecological significance. Normally, the presence of five ants would suggest foraging behavior within the cave. However, since the ants were taken in baited traps this confounds the question, since the ants may have been attracted to the bait from outside of the cave. This record should continue to be considered incidental until further evidence suggests otherwise.

Stenamma sp.
Three Stenamma were sampled at Nugget Cave in the Santa Catalina Mountains north of Tucson on 21 July 1993. Unfortunately the specimens have been lost, and had not been identified to species. An attempt to relocate this species at Nugget Cave was made on 17 August 2014, but the ants were not present at that time. The association of this colony with the cave is suspected to be that of a trogloxene.

Trachymyrmex arizonensis
T. arizonensis was recorded from Kartchner Caverns during the recent two-year macro-invertebrate study of the cave (Pape & O’Connor, 2014). Most observations of the species were in the Jackrabbit Shaft (Jackrabbit Gallery area; Fig. 1). The ants were observed moving chaff from their fungus garden to a refuse midden near the top of the shaft. The chaff had overflowed this repository and had cascaded down into the shaft. The fungus garden was not visible, but was likely present immediately adjacent to the shaft in a fracture in the pediment, or in the adjacent soil profile.

During a visit to the cave on 31 May 2012 the species was observed excavating a nest in a crack in the bedrock wall about 2.5 m. below the top of the shaft. There were about two dozen ants actively working the hole and removing soil particles. A soil spoils pile had accumulated on part of the metal framework for the shaft ladder structure. This nest was still active two years later, on 19 October 2014.

Army ants (Neivamyrmex spp.), many of which are primarily ant predators, have been recorded preying on T. arizonensis in southern Arizona (LaPolla et al., 2002; Rabeling et al., 2007). Neivamyrmex have not been found in proximity to the Jackrabbit Shaft. If N. gracillae does prey on T. arizonensis, this likely occurs outside the cave, in the epikarst or the overlying soil profile. N. leonardi is probably too small a species to successfully predate Trachymyrmex spp., and the single record for the species is deep within the cave, far from where T. arizonensis has been found.

The presence of T. arizonensis in the cave is opportunistic in that they have incorporated this small portion of the cave as a part of their occupied habitat. Since their nest is not technically within the cave and the ants forage outside the cave, this colony of T. arizonensis is assigned the status of a trogloxene.

DISCUSSION

There are currently over 13,000 recognized species of ants (Formicidae) in the World, possibly representing only about one half to one third of the actual number of extant species (Bolton, 2014; Ward, 2014). Considering their impressive species diversity, global distribution, and integration into nearly every terrestrial ecotope, it seems intuitive that ants should have a significant presence in the ecology of caves.

Obstacles to recognition of the role that ants play in cave ecology have included: the apparently low level or relative infrequency of their presence in these environments; the fact that many cave biological studies are limited in extent; and an apparent misalignment of the presence of observers with the peak activity periods of ants in caves. That is, researchers commonly visit caves during daylight hours, due to convenience, and may miss crepuscular or nocturnal ant activity, particularly some of the subterranean species, such as Neivamyrmex. Additionally, perceptions of what constitutes “typical” behavior for a given ant species should not preclude recognition of individual colony behavior associated with use of cave nutrients.

The number of ant species recorded in individual caves was to a great extent a function of search effort expended. Six ant species were recorded from Arkenstone Cave, where cumulative search time was approximately 500 hours over 14 years. The five species of ants found in Kartchner Caverns were documented during 210 hours of searching during a two-year study (Pape & O’Connor, 2014). Search efforts in the remaining caves were considerably less intense, often involving only a few hours during
a single visit. Only one of the caves (Hidden Cave) yielded more than a single ant species (two), one of which was considered an incidental occurrence. No ants were found in 29 of the study caves. Since ants seem to have a low level and sporadic, but persistent presence in caves, their detection may require extensive searching. Thus the negative findings from the low-effort sites are inconclusive, and the apparent absence of ants in these caves in no way precludes their use of those sites.

Observations of cave invertebrates in Arizona have revealed a low level and sporadic, but persistent use of cave habitats and their contained resources by ants. That is, an ant species may be present in a cave irregularly or occasionally (sporadic), and yet is persistent over time, with repeated occurrences separated by months or years. The importance of ants in cave ecology is supported, in part, by observations of colonies of 13 ant species exploiting resources in Arizona caves (this paper). The majority of these observations are from two caves in southeast Arizona, Kartchner Caverns and Arkenstone Cave, which have been extensively studied. It is assumed that similar use of cave resources by ants occurs elsewhere, particularly in the lowland humid tropics where ants have a more pronounced presence, and where nutrients are generally more available in both epigean and hypogean environments (Romero, 2009, 2011). This is supported by the numerous records of ants reported from caves in these regions (Appendix A).

**Subterranean terrestrial habitats**

Subterranean terrestrial habitats have in common their humid, aphotic environments and spatial proximity. Subterranean profiles in karst regions typically include the following habitats, from top to bottom: soil, epikarst, bedrock fissures, and caves (Juberthie, 1980; Camacho, 1992; Juberthie, 2000; Culver & Pipan, 2014). Each of these habitats supports its own unique biota (Howarth, 1983; Camacho, 1992; Culver & Pipan, 2009a, 2014), but due to proximity and microclimatic similarity there is much biotic movement across their boundaries (Howarth, 1983; Culver & Pipan, 2014; Pape, personal observation). Cave entrances serve as movement corridors that are readily accessible to animals occupying both epigean and subterranean habitats (Howarth, 1983; Romero, 2009; Prous et al., 2015; Pape, personal observation). Thus, caves are often intimately connected with habitats occupied by edaphic ant species, and nutrients present in caves are commonly within the foraging range of their colonies.

**Nutrients in caves**

Caves have long been recognized as comparatively oligotrophic environments, with most nutrients being transported into caves from photic epigean ecosystems by hydrologic movement, gravity or animal transport (Camacho, 1992; Polis et al., 1997; Culver & Pipan, 2009a; Romero, 2009; Pape & O’Connor, 2014; Prous et al., 2015). Bacteria and fungi are important decomposers of organic materials in caves, and the role of bacteria and archaea in chemolithoautotrophic primary production in these environments is only beginning to be understood and appreciated (Cunningham et al., 1995; Ortiz et al., 2013). Nutrient sources commonly found in caves include plant debris, small vertebrates, macro-invertebrates, animal carcasses, and feces (Howarth, 1983; Ferreira & Martins, 1999; Hüppop, 2000; Culver & Pipan, 2009; Trajano & Bichuette, 2009; Prous et al., 2015; Pape, personal observation). Bat and bird guano deposits in caves are occasionally extensive, and often support their own diverse invertebrate fauna (Ferreira & Martins, 1999; Dehaveng & Bedos, 2000; Moulds, 2004; 2006; Ferreira et al., 2007; Pape, 2014; Pape & O’Connor, 2014). Animals, including ants, forage at these guano deposits, where they scavenge materials and/or prey on invertebrates integral to the guano food web. The guano of cave-roosting frugivorous bats commonly includes the seeds of fruits on which they feed, and may be gathered by seed harvesting ants, such as Messor, Monomorium, Pheidole, Pogonomyrmex, and others.

So it is not only scavenging and predatory ants that forage at cave guano deposits, but seed harvesting species may also be present. The Oil Bird (Steatornis caripensis Humboldt), or Guacharo, nests in caves in northern South America and the Caribbean region. Oil Birds are also frugivorous and regurgitate fruit parts, including seeds, from their diet in their nesting caves (Polis et al., 1997; Holland et al., 2009; Romero, 2009). The seeds of fruits consumed by Oil Birds are too large to be scavenged directly by ants, but fruit parts or arthropods associated with these deposits may provide nutrients for ants foraging in these caves.

**Cave foraging ants**

Cave foraging ants are either epigean species, which may include adventitious foraging in caves among their primarily epigean provisioning activities, or hypogeous species that live in the soil profile or epikarst, but that include caves that are integrated into their occupied habitat. The use of caves by ant colonies is primarily in the role of a trogloxene, where the ants include foraging in caves only as a portion of their overall provisioning behavior. This was recognized early on by Kempf (1961) and Wilson (1962). These colonies return their cave-garnered provisions to their nests located outside caves. Obviously, cave nutrients must be within the foraging range of the colony. Because of this, use of a given cave by ants may vary from year to year depending on the areal distribution of the colonies over time. This was evident for the *P. rhea* colony at Patagonia Bat Cave discussed previously.

Some of the more important ant genera using caves, such as *Labidus, Neivamyrmex*, and (probably) *Nomamyrmex*, already possess an innate hypogeous life style. Living in the soil profile or epikarst, these ants occupy thermally moderated, humid, aphotic habitats that are very similar to the cave environment. The New World tropical *Chelioniymyx*, which are distinctly subterranean (Schneirla, 1971), have so far not been recorded from caves. This may be due to these ants being generally uncommon (Holdoüber & Wilson, 1990) or the lack of a proactive search effort,
rather than an indication of their absence from caves.

The genus *Neivamyrmex* currently contains 131 described species (Kronauer, 2009; HOL), making it the largest group among the New World Dorylinae. There are currently 20 *Neivamyrmex* species known to occur in Arizona (AntWeb), four of which have been documented from caves in the state (Pape & O'Connor, 2014; this paper). As a group *Neivamyrmex* is predominantly hypogeic, with their bivouacs and predatory hunting activities mostly confined to subterranean habitats (Schneirla, 1971; Ryder Wilkie et al., 2007; Pacheco & Vasconcelos, 2012). Surface activity levels vary among *Neivamyrmex* species, and are limited primarily to predatory raids, migrations and reproductive flights conducted at night or on overcast days (Smith, 1942; Rettenmeyer, 1963). *Neivamyrmex* are primarily predators of other ant species or termites, but some species also take other arthropod prey including both soft and hard-bodied forms (Rettenmeyer, 1963; Schneirla, 1971; Mirenda et al., 1980; Holldobler & Wilson, 1990; this paper).

Due to their nomadic, subterranean life style, *Neivamyrmex* may commonly intersect caves as they migrate through the soil and epikarst in karst terrains, and are probably more common in these environments than existing records might suggest. The earliest cave record for *Neivamyrmex* (*N. fallax* Borgmeier) was from Cotterell Cave in Travis County, Texas, where its occurrence was presumed to be incidental (accidental) (Reddell & Cokendolpher, 2001). Adding to the *N. fallax* record, two additional *Neivamyrmex* (*N. graciellae* Mann, *N. leonardi* Wheeler) were recorded at Kartchner Caverns (Pape & O'Connor, 2014), and two more (*N. nigrescens* and *Neivamyrmex* sp.) in Arkenstone Cave (this paper). These last four species records are the first evidence demonstrating the association of *Neivamyrmex* with the ecology of caves.

The ant that appears most prominently in cave records, the red imported fire ant (*S. invicta*), is considered one of the 14 worst invasive alien insect species in the World (Lowe et al., 2004). *S. invicta* has been documented preying on a variety of arthropods in Texas caves, and is a significant threat to endemic cave animals (Elliott, 1992, 2000; Longacre, 2000; Taylor et al., 2005; Cokendolpher et al., 2009). *S. invicta* occurs in Texas as a non-native, invasive species, and has been recorded from well over 200 caves within a relatively small area in the central portion of the state (Reddell & Cokendolpher, 2001). The large number of cave records for the species is mostly the result of extensive surveys in caves that are known to (or potentially) support more than a dozen endemic cave invertebrates that are listed as endangered under the ESA. While the Texas records for *S. invicta* are a special circumstance, in that they resulted from an extensive and concerted search effort, they demonstrate the potential for ants to affect the ecology of caves, in this case in a negative manner.

**Hypogeic ants and foraging depth in caves**

Subterranean army ants are a potentially significant threat to cave animals, particularly invertebrates, as documented for *Neivamyrmex* sp. at Arkenstone Cave (Figs. 10 & 11). Army ant attacks on endemic cave invertebrates that have small populations or a limited distribution within a cave could result in adverse population-level impacts or extirpation of these animals. Since hypogeic army ants are at home in the subterranean environment, it would seem reasonable that they are capable of penetrating deeply into caves. The limited evidence we have so far, however, suggests that while they may travel substantial horizontal distances within caves, their movements seem to be vertically constrained.

Many army ant species prey primarily on other ant species, or on termites (Schneirla, 1971; Holldobler & Wilson, 1990). Deep substrate penetration is used by termites to obtain water or clay in areas where these resources are scarce. Termites have been found as deep as 70 m, and are suggested to possibly seek water sources as deep as 100 m (Yakushev, 1968; Lee & Wood, 1971; Cloud et al., 1980). However, the bulk of individuals within termite colonies typically occur in the top couple of meters of the soil horizon, where food resources are most abundant (Lee & Wood, 1971; Matsumoto, 1976; Sheikh & Kayani, 1982), and their numbers decrease significantly with depth (Yakushev, 1968). Ant nest depth varies among species and is, to a degree, a function of colony size (Buhl et al., 2004; Mikeyev & Tschinkel, 2004; Tschinkel, 2004). Schneirla (1958) describes his excavation of a *N. nigrescens* bivouac within one of the galleries of a fungus ant in southern Arizona, which did not exceed one meter in depth. The larger, more permanent foraging trunk trails of the hypogeic *Dor峪lus laevigatus* Smith (Dorylinae) were found to typically occur at depths between 8 and 12 cm in the soil horizon in Malaysia (Berghoff et al., 2002). Other *Dor峪lus* nest at depths between 1 and 4 m (Gotwald, 1995). Other species, which are more hypogeous by nature, may excavate to somewhat greater depths. Nests of some larger ant colonies (e.g. *Atta texana* Buckley) have been recorded reaching as deep as 7.6 m within the soil horizon (Moser, 2006). These huge nest structures, which occur in several genera, are supported by colonies that may contain several million individuals (Holldobler & Wilson, 1990). A majority of ant colonies are considerably smaller in scale and are typically concentrated much closer to the surface, usually within the upper three meters or so of the soil horizon (Antoniali & Giannotti, 2001; Berghoff et al., 2002; Tschinkel, 2004, 2005, 2009; Bollazzi et al., 2008).

Foraging behavior of the more hypogeous army ant species likely co-evolved over time in concert with their mostly subterranean prey, and the habitats that these prey occupy. Since army ant prey abundance likely attenuates with depth in the soil horizon and epikarst, the evolved predatory foraging behavior of army ants may have become depth constrained to the approximate limits normally occupied by their prey.

Army ants periodically occupy prey nests between foraging episodes (Schneirla, 1958, 1971; Gotwald, 1995). While prey nest sites initially provide food for army ants, they also provide shelter, and minimize the need for them to construct nests. It is not known if,
or to what extent, army ants may modify prey nests. Since such bivouacs are temporary for these nomadic animals, it seems likely that they would not make extensive modifications to these structures. This suggests that army ants may not excavate beyond the pre-existing limits of prey nests.

Another factor that may have molded army ant behavior over time, and may affect the vertical depth they will pursue prey, is the air chemistry within prey nests. The percentage of \( \text{O}_2 \) and \( \text{CO}_2 \) in the air within ant nests is normally in balance. Some ants have been observed to partition their age classes vertically within nests based on atmospheric concentrations of \( \text{CO}_2 \), which increases significantly with depth in the nest, and furthest from surface connections where air exchange occurs (Kleineidam & Roces, 2000; Tschinkel, 2004). When large numbers of marauding army ants enter their prey nest, activity of both the invaders and prey is increased to a high level of vigor. Oxygen is consumed at higher rates and carbon dioxide is increased in the atmosphere through respiration. The likely significant increase in \( \text{CO}_2 \) concentration resulting from these struggles may make conditions in the deeper reaches of the nest marginally suitable during the brief times the invaders are present. The presence of inhospitable atmospheric conditions in the deeper reaches of prey nests, along with diminishing numbers of prey with vertical depth, may inhibit downward vertical predatory foraging in hypogean ants.

Cave entrances allow animal movements into and out of caves, and are thus avenues of nutrient input to caves. These cave-surface interfaces commonly support a greater species richness and animal abundance than normally occur in cave interiors (Prous et al., 2004; Culver & Pipan, 2014; Pape & O'Connor, 2014; Prous et al., 2015). One would expect ant colonies that occupy networks of cave passages to concentrate their activities near surface connections, where nutrients are likely to be more abundant. This appears to be how \( N. \text{graciellae} \) is distributed within Kartchner Caverns (Fig. 1). Because of this, cave systems with multiple, closely-spaced surface connections should be a potentially ideal environment for cave-inhabiting army ant colonies.

\( N. \text{graciellae} \) has been observed active at depths ranging from 15 to 24 m below the surface at Kartchner Caverns. Measured depths include 16 m in the Tarantula Room area (C in Fig. 14), 24 m in the Red River Passage (B in Fig. 14), and 15 m in the Anticipation Room. \( N. \text{graciellae} \) is currently known to prey only on other ant species (Watkins & Coody, Pape, 1986; this paper). The presence of this predator at depths well below where its prey is likely to occur, within the top couple of meters of the surface, would seem to be ineffectual behavior. However, an analysis of the structure of the cave relative to ant activities reveals that these in-cave depths are functionally artificial, and that the ants are not accessing the cave in a precipitous, vertical descent. \( N. \text{graciellae} \) probably primarily occupies the late Pleistocene alluvial deposits that overlie the lower portions of the bedrock pediment of the cave hill. Their activities within the cave are associated with sub-horizontal access zones that are discontinuously situated at the periphery of the cave (Figs. 1 & 14; access zone). These access zones are comprised of the shallow surface alluvium and the contiguous consolidated soil sediments that were emplaced by gravity and autogenic meteoric waters in collapse, fault and fracture structures, and cave passages. Activities of \( N. \text{graciellae} \) in the cave seem to be concentrated in the vicinity of the Red River Passage, which is approximately 40 m from the surface of the cave hill along the presumed access zone shown in Fig. 14. This is the shortest distance from the cave that would allow the ants to reach the zone where their prey is presumed to occupy the alluvium overlying the cave. This suggests that army ants, even in a karst situation, do not normally penetrate
to great vertical depth in search of prey, and that their activities may be mostly confined to horizontal underground movements.

*N. leonardi* is a small, delicate, pale and blind species, which probably has small colonies. The species is certainly less mobile than the larger, longer-legged *N. graciellae*, and probably has a smaller foraging range. *N. leonardi* was found deep within Kartchner Caverns, 28 m vertically and 130 m horizontally from the surface of the cave hill (Fig. 14). The combination of a presumed low mobility of this species, and its location deep within the cave suggests some potential exists that the colony may be eutroglophilic. A combination of small body size along with small colony size in ants occurring in caves was suggested by Tinaut & Lopez (2001) as a possible evolutionary solution that might compensate for the large deme requirement in social insects that is usually unsupportable within oligotrophic cave habitats.

**Ecological groups, troglomorphy, and troglobiotic ants**

The ecological group assigned to ant colonies using caves should be a function of the nature and level of their association with these habitats, and not contingent on the presence of troglomorphy. Eutroglophiles and troglobionts do not always exhibit a readily apparent adaptive morphology (Romero, 2009, 2011; Pipan & Culver, 2012; Pape, 2013). This is particularly true for those that have only recently adopted a cavernicolous existence (Pape, 2013). Isolation of animal populations in caves, which may eventually result in troglomorphy, can occur due to a variety of causes, including vicariance resulting from geophysical or hydrologic changes, changes in climate, or partitioning due to resource availability, competition, or predation (Coineau & Boutin, 1992; Holsinger, 2000; Culver & Pipan, 2009, 2014). Advanced troglomorphy is more evident in some other cave-occupying terrestrial arthropod taxa (Scorpionida, Pseudoscorpiones, Araneae, Opiliones) but, based on existing evidence, appears to be generally not well expressed in ants.

The reduced eyes and shortened appendages in primitive ants such as *Amblyopone* spp. suggest adaptation to aphytic environments early in ant evolution (Hollodbler & Wilson, 1990; Wilson & Hollodbler, 2005). However, few ant species exhibit significant loss of pigmentation, and even fewer show significant attenuation of the body, limbs or sensory structures present in many troglobionts. No known ant species exhibits extreme troglomorphy, as occurs in troglobions in other taxonomic groups, where these morphologies co-evolved.

The fascination with troglomorphic, troglobiotic species gathers much attention from cave biologists (Pipan & Culver, 2012; Prous et al., 2015), and the search for troglobiotic ant species has a long history. Very few ant species have been suggested as true cavernicoles, particularly where the association implies a troglobiotic life style. Most that have were eventually found to also occur in epigean habitats (Roncin & Deharveng, 2003), and thus at most have occasional subtroglophilic colonies. Some of these are: *Aphaenogaster cardenai* Espadaler in Spain, *Carebara* (*Oligomyrmex*; *Ereboruma*; *Spelaeomyrmex*) *urichi* Wheeler in Trinidad, and *Hypoponera ragusai* Santschi in Granada (Wilson, 1962; Tinaut, 2001; Tinaut & Lopez, 2001; Roncin & Deharveng, 2003). More recently, Ortúñol et al. (2014) presented information regarding *A. cardenai* as an inhabitant of the mesovoid shallow substratum. They precluded *A. cardenai* as a eutroglophil (troglophile) based on its epigean reproductive dispersal behavior. This is likely a limiting factor for most ant colonies residing in caves, which could otherwise be considered eutroglophiles. Reddell suggested that *Nylanderia* (*Paratrechina*) *pearsei* Wheeler might be a troglobiotic species. This pale yellow, microphthalmic species is poorly known and has been recorded only from cave aphytic zones, typically found on moist flowstone or rock surfaces, or associated with drip pools (Reddell, 1977). Wheeler (1938), in his description of the species, referred to it as belonging to a small group of cavernicolous species. There have been no ecological studies of this species and its status is thus not currently established. The species may have subtroglophilic colonies.

Ant nesting activities have rarely been reported occurring in caves. Tinaut & Lopez (2001) report three records of ants nesting in caves from the literature: *Carebara urichi* in Trinidad (Wilson, 1962); *Lasius umbratus* Nylander in Spain (Lopez Gomez, 1988); and *Hypoponera ragusai* in southern Spain (Tinaut, 2001). The bivouac records for *L. coecus* in Texas caves (Reddell & Cokendolpher, 2001) could possibly also include statary phase bivouacs, which are the reproductive phase nests of army ants.

Wilson (1962) proposed that limitations are imposed on the evolution of troglobiotic forms in social insects by their need to form very large demes to support genetic viability and stability comparable with that found in non-social insects. He suggested that large demes would seldom be sustainable in cave environments due to the compounding factors of limitations on available nutrients and physical space (suitable habitat) present in these ecotopes. This likely explains why the preponderance of observations of ants found in caves has, until recently, not revealed any potentially troglobiotic forms.

The only ant species currently known that exhibits both a complex troglomorphy and an apparently strict cavernicolous habit is *Leptogenys khmouanensis* Roncin & Deharveng, which lives deep within large caves in Laos (Roncin & Deharveng, 2003). The species has reduced eyes with 15-20 ommatidia, the body color is a pale, orange-yellow, and the entire body form, including the legs and antennae, is moderately attenuated. *L. khmouanensis* is larger than other *Leptogenys* species, some of which have a morphology that suggests a subterranean evolution, but not necessarily a strict cavernicolous, or troglobiotic existence (Roncin & Deharveng, 2003). Both dwarfism and gigantism occur occasionally in troglobiotic species (Vandel, 1965; Culver & Pipan, 2009b; Trontelj et al., 2012), and the larger size of

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L. khammouanensis, compared with its congeners, may be additional evidence of troglobromy. While the ecology of this species has not been studied, it is possible that it may be the first truly troglobiotic ant species that has been found.

SUMMARY

The literature review revealed a diversity of ant subfamilies and genera occurring in caves globally, demonstrating their persistent presence in these habitats. The nature of the existing records, consisting of a high percentage of ancillary data, and likely skewed by an unintended emphasis of studies regionally, currently precludes meaningful analyses regarding any affinity for caves by discrete ant taxa, or regional prominence for the use of caves by ants.

This paper consolidates the 10 previously reported records of ants from five Arizona caves and adds 11 new records, for ten additional species, from five other Arizona caves. The new records are elements from a larger data set of biological surveys of macrobiont fauna in 35 Arizona caves over a 25 year period. Ants were found in only six of the 35 caves studied. Survey efforts ranged from a single visit (Porcupine Cave) to 124 visits from a single cave over a 25-year period (Arkenstone Cave). Considering that the known cave resource in Arizona includes over 1,500 caves, the 35 caves visited represent a very small data set. Yet, based on this limited information it is apparent that ants play an important role in the ecology of some caves. It seems reasonable that similar behavior probably occurs on a global scale. This is particularly anticipated for the tropics, where ants are more diverse and there are greater available nutrient resources to support the animals in both epigean and cave environments. While the presence of ants in caves seems to generally occur at low levels, and may be sporadic, they are persistent in their presence in these habitats over time. The significance of cave habitats in providing both a suitable environment and nutrient sources for epedaphic ant colonies is no longer in question. Observations of ant behavior in caves in Arizona ranged from simple foraging by scavenging species, such as Pheidole, to the large-scale marauding by Neivamyrmex sp. observed at Arkenstone Cave. Behavioral analysis of N. graciellae in Kartchner Caverns and Neivamyrmex sp. in Arkenstone Cave suggests that hypogean army ants may not penetrate to great depth to search for prey, but can be persistent occupants of relatively shallow, horizontal sections of caves, where they may prey on endemic cave animals (Neivamyrmex sp.). Information contained within this paper will hopefully encourage researchers to reconsider the role of ants in cave ecosystems.

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ATP luminescence assay as a bioburden estimator of biomass accumulation in caves

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Abstract: A commercially available adenosine triphosphate (ATP) detection system (Hygiena, USA), supported by cultivable microbial indicators, was used to estimate bioburden in different habitats in and outside show caves: air, water and solid surfaces. A strong positive correlation between ATP concentration expressed as Relative Light Units (RLU) and Colony-Forming-Units (CFU) was observed for swab samples from cave surfaces. In terms of ATP units, surfaces in a single cave system (Postojna Cave) varied considerably (240-1,258,800 RLU/20 cm²) and commonly exceeded the bioburden level of analogues on the surface (0-114,390 RLU/20 cm²). Cave sub-habitats were colonized by physiologically distinct microbial communities in terms of their nutrient demands, temperature requirements and r/K growth strategy. The highest ATP biomass indicator (1,258,800 RLU/20 cm²) for the speleothem that had been touched but accompanied with comparable concentration of CFU (~10⁶ CFU/20 cm²) for other cave sub-habitats, can be related to the presence of deposited human epithelium skin cells. Show cave infrastructures containing heavy metals, e.g. copper used in safety fences, reduce the viability of microbiota. Mass cave visitation and the presence of allochthonous organic matter result in high levels of airborne and total biomass. Once such material becomes airborne, the location of its settling depends upon natural and human-induced air movements. Underground habitats play an important role in the preservation and concentration of microbial biomass using air and water as transport mechanisms.

Keywords: underground, biomass, swab, air, water, microorganisms

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INTRODUCTION

Microbes face stressful environmental conditions during transfer through the atmosphere (Morris et al., 2011) and also within water (Balkwill et al., 1998). Having successfully overcome stressors while in transit and reached the new destination, preservation of their viability is further challenged by different factors. Once the microbes are in contact with the surface, their fate depends upon their ecophysiological capabilities (Guerrero et al., 2002; Schimel et al., 2007), substrate characteristics (Warscheid & Braams, 2000), environmental conditions, e.g. UV (Wynn-Williams & Edwards, 2002), desiccation (Barnard et al., 2013), and interactions with any (eventual) pre-existing microbiota (Friman et al., 2014). An example of constant transport of microbes passing different barriers is provided by karst caves, which commonly show good connectivity with the surface through many fissures and voids (Ford & Williams, 2007). Caves can thus serve as models for the transport of particulate material, and for microbial interactions, because some natural stressors, such as UV and desiccation, are absent (Summers Engel & Northup, 2008; Hauer et al., 2015). Accurate sampling and determination of microbial biomass in these habitats is normally one of the prerequisites that direct downstream analyses.

Direct microscopic fluorescent counts using nucleic-acids-staining dyes represent a good start in evaluating the microbial abundance rather than biomass (Norland, 1993; Senjarini et al., 2013). Different protocols based on fluorochrome-stained cells have been developed for distinct samples and applications (Cragg and Parkes, 2014). Techniques based on specific monoclonal antibodies represent another powerful tool to study microbial populations from natural environments (Hamasaki et al., 2016). Furthermore, nowadays, nucleic acids probing coupled with specific techniques offers powerful insights into individual constituents of the natural occurring microbial community, for example Fluorescence In Situ Hybridization (FISH) and its combinations with...
microautoradiography, Raman spectroscopy and secondary ion mass spectroscopy (Musat et al., 2012). Flow cytometry is not used solely to estimate microbial biomass, but serves also for rapid microbial community fingerprinting (De Roy et al., 2012) and for tracking the changes in microbial subpopulations or on a single-cell level (Sgier et al., 2016).

Quantitative measurements of microbial cellular components give a reliable estimation of the biomass (White et al., 1997) and of community structure and functioning (Röling & van Bodegom, 2014). For example, lipid phosphatase or phospholipid ester-linked fatty acids provide a quantitative measure for microbes with intact cellular membrane (Gottschalk, 2012), and lipopolysaccharides (LPS) as essential life molecules for the Gram-negative bacteria (Botos et al., 2016) are used specifically to estimate their presence in the environment (Parker et al., 1982). Adenosine triphosphate (ATP) is a universal measure of metabolizing cells (Karl, 1993). Levels of environmental microbial ATP correlate strongly with the results of aerobic plate counts (Chen & Godwin, 2006). ATP-based methods have previously been used in environmental microbiology, for example, to measure microbial activity in aquatic environments (Hammes et al., 2010), in marine oil spills (Röling & van Bodegom, 2014), in mineral leach liquors (Okibe & Johnson, 2011) and in an orthoquartzite (quartz-cemented sandstone) cave (Barton et al., 2014). The surfaces from this cave contained a high level of microbial biomass determined by an ATP-based luminescence assay when compared to other (carbonate) cave systems (Barton et al., 2014).

Even though cultivable microbes represent only a very small part of the community (Stewart, 2012), cultivation is still widely used as a routine laboratory procedure to quantify environmental indicators, estimate biomass and, particularly, in efforts to isolate new, biotechnologically important microorganisms (Bull et al., 2000; Giovannoni & Stingl, 2007). Data on bacterial growth dynamics on a nonselective agar medium can be used to work out their growth strategy. As a community develops, fast growing opportunistic species (r-strategists) are gradually replaced by slow-growing equilibrium species (K-strategists). The ratio of r- vs. K-strategists is a measure for a succession state in a microbial community (Kristufek et al., 2005; Andrews & Harris, 2013).

The objective of the study was to test the versatility of ATP biomass indicator (Hygiena, USA) in different natural environments: air, water, and substrate surfaces that included samples from underground karst and corresponding above-ground analogues. Particularly in karst caves, a continuous flow of organic material and biota is well displayed (Prónk et al., 2006). In parallel with standard cultivation techniques, the biological burden of different cave sub-habitats expressed in ATP biomass was used to estimate the level of naturally occurring and human-induced microbial biomass in underground situations. This relative simple and affordable method has a potential for a wider use in cave microbiology and cave management, as a monitoring tool in efforts to restrict the adverse human impact on a cave ecosystem.

MATERIALS AND METHODS

Caves

Different sites in two Slovenian caves were selected for the study. The Postojna Cave system (including Črna jama, Magdalena jama, Otoška jama, Pivka jama and Postojnska jama) formed in Cretaceous limestone (Šebela, 2012) is 24.1 km long, with the underground Pivka River, which sinks at 511 m a.s.l. (45°46'56.94"N, 14°12'12.10"E). The Postojna Cave system occupies 3,066,517 m² of underground space, with 1,231,716 m² of contact surfaces (Franjo Drole, personal communication). A 5.0 km section of Postojna Cave (Postojnska jama) is visited by more than 500,000 tourists each year (in the last two years approximately 640,000 per year), and includes a 3.2 km round trip by underground railway (Fig. 1). The extent of tourist footpaths is approximately 3,788 m². The railway lines lie on 5,300 wooden ties (contact surface of each tie is 0.82 m²). The tourist use of Postojna Cave is reflected in cave climate, crushed-sand and metal dust from beneath the train wheels, surface contamination, light eutrophication, lampionflora growth and the presence of ultrasonic smog (Šebela & Turk, 2011; Muri et al., 2013; Šebela et al., 2013; Mulec, 2014; Šebela & Turk, 2014; Šebela et al., 2015). Some 7 km towards the northwest, Predjama Cave (Predjama, 45°48′55.89″N, 14°7′35.56″E), which formed in Cretaceous limestone, Upper Triassic dolomite and Jurassic limestone and dolomite (Car & Šebela, 2001), is 13.1 km long with the Lokva River, which sinks at 462 m a.s.l. Some galleries host bat colonies (Presetnik et al., 2009: 2012).

![Fig. 1. Sampling sites of settled aerosols, swabs, and waters in Postojna Cave (see Table 1 and Table 2 for details), with bioburden ranges for the surface swabs (green: log_{10}[ATP] < 25% of measured values for cave swabs; yellow: log_{10}[ATP] < 75 and ≥ 25% of measured values for cave swabs; red: log_{10}[ATP] ≥ 75% of measured values for cave swabs). Ground plan modified after the Cave Cadastre of the Karst Research Institute at ZRC SAZU.](image-url)
Mulec et al., 2013), which is why many surfaces in this cave section, including the tourist footpath, are spattered with bat excrement. Around 6,000 tourists visit Predjama Cave annually. The long-term average precipitation level in the area is 1,578 mm (Nadbath, 2007).

Swab samples

Various surfaces that are subjected to microbial colonization were sampled: concrete, flowstone, limestone, metal, and wood (03 November 2015). Each underground sample had an analogue on the surface, except the surfaces subjected to dust contamination from the track of the underground railway, and the tectonically polished surface along an underground fault plane in Postojna Cave. Analogues to the cave samples, from the above-ground environment, were considered if they had a similar composition, e.g. concrete, and if they had suffered comparable environmental impact, e.g. tourist handling (Table 1).

Table 1. Characteristics and locations of sampled surfaces in Postojna and Predjama caves with analogues on the surface.

<table>
<thead>
<tr>
<th>Location</th>
<th>Substrate</th>
<th>Sample (No.)</th>
<th>Sample (No.)</th>
<th>Substrate</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postojna Cave</td>
<td>concrete</td>
<td>tourist footpath (5)</td>
<td>tourist footpath (12)</td>
<td>concrete</td>
<td>Postojna</td>
</tr>
<tr>
<td>Postojna Cave</td>
<td>flowstone</td>
<td>stalagmite (4)</td>
<td>limestone (21)</td>
<td>limestone</td>
<td>Predjama</td>
</tr>
<tr>
<td>Postojna Cave</td>
<td>flowstone</td>
<td>stalagmite (4)</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Postojna Cave</td>
<td>flowstone</td>
<td>stalagmite, touched (6)</td>
<td>monument, touched (13)</td>
<td>limestone</td>
<td>Postojna</td>
</tr>
<tr>
<td>Postojna Cave</td>
<td>limestone</td>
<td>flooding zone, dry (1)</td>
<td>flooding zone, dry (11)</td>
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<td>Postojna</td>
</tr>
<tr>
<td>Postojna Cave</td>
<td>limestone</td>
<td>cave biofilm (18)</td>
<td>subaerial biofilm (20)</td>
<td>limestone</td>
<td>Predjama</td>
</tr>
<tr>
<td>Postojna Cave</td>
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<td>protection fence (9)</td>
<td>door handle (14)</td>
<td>metal</td>
<td>Postojna</td>
</tr>
<tr>
<td>Postojna Cave</td>
<td>wood</td>
<td>dead wood (10)</td>
<td>dead wood (17)</td>
<td>wood</td>
<td>Postojna</td>
</tr>
<tr>
<td>Predjama Cave</td>
<td>wood</td>
<td>railroad tie (3)</td>
<td>railroad tie (15)</td>
<td>wood</td>
<td>Postojna</td>
</tr>
<tr>
<td>Predjama Cave</td>
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<td>bat guano (19)</td>
<td>pigeon guano (16)</td>
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</tr>
<tr>
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<td>flowstone</td>
<td>dust (2)</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Postojna Cave</td>
<td>limestone</td>
<td>tectonic slickenside (8)</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Surfaces in the show caves were selected to estimate the human impact (e.g. tourist footpaths, stalagmites stained with a brownish patina due to tourist handling, and copper within the safety fences) vs. pristine surfaces. The location sampled at the safety fence is designated as an assembly point for tourist groups, and hence a high human impact (touching) was expected at this site. Surfaces that can provide nutrients and enhance microbial growth (dead wood, wooden railroad ties) were sampled too, as well as those that represent considerable microbial inoculum and biomass for the underground: a surface subjected to regular floods, a rock surface colonized by natural biofilm and exposed to bat droppings, a dusty rock surface along the underground railway, and a tectonically polished surface on an active fault (Sèbela et al., 2010) 58 m beneath the land surface (Table 1). It was demonstrated in a previous study (Sèbela & Mulec, 2011) that heterotrophic aerobic bacteria (cultivated at 37°C) were detected four months after sterilization of this tectonic slickenside on a fault plane (Sèbela & Mulec, 2011). Locations of sampling sites in Postojna Cave are shown in Figure 1.

Surface swab analogues were sampled in Predjama village close to the entrance of Predjama Cave (limestone cliff with subaerial biofilm) and in Postojna (limestone monument in the town centre, door handle at an apartment block, dead wood at the edge of the forest, railroad tie close to Postojna Railway Station and a concrete footpath spattered with pigeon guano). Surfaces spattered with pigeon guano are common in urban environments and, in a similar way to bat guano in caves, they represent a significant source of organic material. Sampling close to the entrance of Postojna Cave included: a footpath near where tourists enter the cave, and limestone rocks in the flooding zone where the Pivka River sinks into the cave (Table 1). To reduce transmission of microbes and organic matter by tourists, a disinfection barrier was introduced at the entrance of Postojna Cave in 2011.

Settled aerosol samples

The gravity-settling method (Borda et al., 2014) was used to sample airborne biomass in Postojna Cave. Sterilized limestone tablets with a diameter of 41 mm were exposed to the atmosphere for 34 days, starting on 30 September 2015. Stone tablets were cut from a limestone slab, taken from the homogenous upper Cretaceous Lipica Limestone (Gams, 1985; Mulec & Prelovsček, 2015). Tablets were placed in various parts of Postojna Cave to observe differences related to the surrounding environmental conditions: presence of sediments (sample No. 22 in Rov starih podpisov), underground train transportation (No. 23 in Stara jama), restricted visitation (No. 24 in Pisani rov, with less than 50 visitors during the study period), and mass tourism (No. 25 in Lepe jame where 55,000 tourists passed by during that period, Fig. 1). A reference tablet (No. 26) was exposed to the external atmosphere in Postojna town centre. After incubation in the cave the tablets were swabbed as described below (Fig. 2).

Water samples

Three distinctive types of sample were taken in Postojna Cave (03 November 2015): Pivka River after the ponor (No. 100), percolation water from active drips (No. 101 and No. 102) and percolation water captured in a pool with cave pearls (No. 103, Fig. 1). The cave ceiling is 80 to 115 m thick above the sampling sites with dripping water (Franjo Drole,
Fig. 2. Swabbing a limestone tablet with a flocked swab after exposure in Postojna Cave.

To compare biomass input in the underground karst of the Pivka River, the Lokva River was sampled at the ponor in Predjama Cave (No. 104). pH, temperature, specific electrical conductivity (SEC) and oxygen were measured using a WTW Multi Line P4 (Germany) and a Multi 3420 (Germany), respectively.

Swabbing procedure

After a surface with minimum irregularities was selected, it was further delimited by a template for bioburden control (5 × 4 cm, Copan). Up to three adjacent surfaces (60 cm$^2$) were swabbed (FLOQSwabs™, Copan) at sites with expected low biomass (Nos. 4, 7, 9, 11, 13, 14, 15, 17). Swabs were transferred in a tube with 1.0 ml of 0.9% physiological saline. In the laboratory, after vigorous vortexing, 0.8 ml of the saline solution was transferred into a new tube. Swabs in the original tubes were additionally centrifuged for 10 minutes at 4,000 RPM to release any remaining liquid (~0.1 ml). Samples were initially diluted 6-fold, and subsequently diluted serially up to 10$^{-3}$. Dilutions were used for ATP measurements and plating on microbiological media. Because of the expected low biomass deriving from settled aerosols, all of the initial liquid (~0.9 ml) was used for the analyses.

Biomass estimation with ATP

The ATP content of 0.1 ml aliquots was estimated with an AquaSnap™ Total test using a corresponding luminometer (Hygiena, USA). ATP concentration was expressed as RLU – Relative Light Units (where 1 RLU equates to 1 fmol of ATP) and calculated per swabbed surface (RLU /20 cm$^2$).

Biomass of cultivable microbes and identification of coliforms

Samples with corresponding dilutions were plated onto four different media to propagate microbial colonies: nutrient agar (NA, Fluka), malt extract agar (MEA, Fluka), CF-chromID™ Coli agar (CF, Biomérieux) and water agar (WA), which contained 1.5 % agar (Biomérieux) and percolation water that was sampled (26 October 2015) from a permanent active drip (discharge during sampling was 1.1 l/min) in Planina Cave (Planinska jama). Planina Cave, part of which carries the underground Pivka River downstream of Postojna Cave, has a similar geological setting (Zupančič et al., 2011). WA was designed to mimic natural oligotrophic conditions. The sampled water had the following physicochemical characteristics: pH 8.38, SEC 496 μS/cm, temperature 10.6°C, oxygen 10.76 mg/l (101.7%), Cl$: 5.70$ mg/l, NO$_3$: 13.73$ mg/l, SO$_4^{2-}$. 3.45$ mg/l, PO$_4^{3-}$: 0.002$ mg/l$, Ca$^{2+}$+Mg$^{2+}$ vs. Ca$^{2+}$ 1.46, hardness expressed as 267.8 CaCO$_3$ mg/l and alkalinity expressed as 276.3 CaCO$_3$ mg/l. The water was analysed using Standard methods (Clesceri et al., 1998). The same set of media (nutrient-rich NA and MEA, and nutrient-poor WA) was used to estimate microbial biomass for all samples subjected to oligotrophic or eutrophic conditions (Table 2).

Petri plates with NA, MEA and WA were cultivated aerobically in Postojna Cave at 10°C for 28 days, and in a laboratory at 20°C for up to 14 days. Incubation on NA at 20°C served to estimate r- and K-strategists in communities. The general conditions require 3 days to determine r-strategists and an additional 4 to 7 days for K-strategists (Krístufek et al., 2005). A subset of Petri plates with NA and CF was cultivated aerobically at 37°C for 2 days. Visible colonies were quantified in

| Table 2. List of sampling methodologies and biomass estimators for different habitats. |
|----------------|----------------|----------------|----------------|
| **Habitat**    | **Surface**   | **Air**        | **Water**      |
| **Method**     | Swabbing      | Gravity-settling| Direct intake  |
| ATP biomass estimator (units) | RLU/20 cm$^2$ | RLU/20 cm$^2$ | RLU/ml         |
| Cultivable biomass (units)     | CFU/20 cm$^2$ | CFU/20 cm$^2$ | CFU/ml         |
| Medium: temperature: time      |               |               |                |
| NA: 10°C: 28D                 | NA: 10°C: 28D | NA: 10°C: 28D | NA: 10°C: 28D  |
| WA: 10°C: 28D                 | WA: 10°C: 28D | WA: 10°C: 28D | WA: 10°C: 28D  |
| MEA: 10°C: 28D                | MEA: 10°C: 28D| MEA: 10°C: 28D| MEA: 10°C: 28D |
| NA: 20°C: 3°, 7°, 14D         | NA: 20°C: 3, 7, 14D | NA: 20°C: 3, 7, 14D | NA: 20°C: 3, 7, 14D |
| WA: 20°C: 3, 7, 14D           | WA: 20°C: 3, 7, 14D | WA: 20°C: 3, 7, 14D | WA: 20°C: 3, 7, 14D |
| MEA: 20°C: 3, 7, 14D          | MEA: 20°C: 3, 7, 14D | MEA: 20°C: 3, 7, 14D | MEA: 20°C: 3, 7, 14D |
| NA: 37°C: 1, 2D               | NA: 37°C: 1, 2D | NA: 37°C: 1, 2D | NA: 37°C: 1, 2D |
| CF: 37°C: 1, 2D               | CF: 37°C: 1, 2D | CF: 37°C: 1, 2D | CF: 37°C: 1, 2D |

* – r-/K- strategy; D – day(s) of cultivation; † –34 days period of collecting settled aerosols; ‡ –identification of Enterobacteriaceae with Api®20E

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terms of Colony-Forming-Units (CFU) and calculated as CFU/20 cm². Colonies that expressed β-D-galactosidase enzymatic activity typical for coliforms and β-D-glucuronidase typical for Escherichia coli on the CF medium were further confirmed using an Api®20E (Biomérieux) identification scheme. Table 2 summarizes sampling methodologies, biomass estimators, and cultivation media and conditions used in the study.

**Statistical analyses**

Statistical analyses were performed using PAST (Hammer et al., 2001) and Daniel’s XL Toolbox, an open-source add-in for Microsoft Excel (Version 6.60).

**RESULTS**

**Biomass of swabs**

Biomass of swabs in terms of ATP was extremely varying, ranging from 240 to 1,258,800 RLU/20 cm² for cave samples, and from 0 to 114,390 RLU/20 cm² for external non-cave environments (Table 3). The highest in-cave value was for a stalagmite contaminated by touching (1,258,800 RLU/20 cm², No. 6), followed by dead wood (848,880 RLU/20 cm², No. 10), a surface with bat guano droppings (750,600 RLU/20 cm², No. 19) and a tourist footprint (412,200 RLU/20 cm², No. 5). The highest biomass level in the external non-cave environment was attributed to occasionally flooded limestone in a riverbed (114,390 RLU/20 cm², No. 11) and rock colonized by biofilm (106,200 RLU/20 cm², No. 20). The lowest biomass on a natural in-cave surface was on a flowstone in an undisturbed part of the cave (900 RLU/20 cm², No. 4). An analogue on the surface expressed a higher bioburden (17,820 RLU/20 cm², No. 21). A relatively high value was found on a tectonically polished surface (32,040 RLU/20 cm², No. 8). Biomass occurring as cave and aerial biofilms showed a similar order of environmental bioburden expressed in ATP units. Absence of microbial biomass on a tourist footprint just outside the cave entrance was attributed to the application of a cleaning solution on the day of sampling, although 70 tourists walked along the swabbed surface immediately prior to the sampling.

The highest CFU counts were from a swabbed concrete footprint in the cave, 7.45×10⁶ CFU/20 cm² (No. 5) on WA medium (20°C). The same order of microbial concentration on the WA medium was also for a swabbed limestone surface soiled with bat excrement (No. 19), dead wood (No. 10), a stalagmite contaminated by tourist contact (No. 6) and a wooden railroad tie (No. 3). Concentrations of microbial CFU comparable with these samples were also obtained on the NA medium. Using the same method, microbial biomass of the external non-cave surfaces was found to be poorer, with the highest concentration of viable microbes on the WA medium. The highest CFU counts from the external environments were from a swabbed railroad tie, 3.02×10⁴ CFU/20 cm² (No. 15), followed by a limestone subjected to floods (No. 11), a concrete surface with pigeon guano (No. 16) and a limestone with biofilm (No. 20). MEA that supports fungal growth (Campbell et al., 2013) showed concentrations of cultivable fungi up to three orders of magnitude lower compared to bacteria (Table 3).

There was a strong positive correlation between ATP and CFU counts on all media for swabbed surfaces in caves (n = 12), but the statistical significance (p < 0.05) was only for CFU counts on WA medium cultivated at 10°C (r = 0.70, p = 0.012) and on MEA medium cultivated at 10°C (r = 0.71, p = 0.01). When log₁₀ concentrations of ATP was used in the analysis, statistical significant correlations were obtained also for CFU counts on NA (10°C, 20°C) and WA (20°C). A positive correlation was also apparent between ATP and CFU counts for swabbed surfaces (n = 9) from external environments, but the statistical significance was only for CFU counts on NA medium cultivated at 37°C (Table 4).

Many samples incubated at 20°C expressed higher CFU counts compared to those incubated at cave temperature, except for: a limestone with bat guano (No. 19) and limestone colonized with subaerial biofilm (No. 20) on NA medium, a flowstone with dust (No. 2), a metal safety fence in the cave (No. 9), a limestone subjected to floods (No. 11), a limestone monument handled by the public (No. 13) and external dead wood (No. 17) on WA medium, and on MEA medium for a concrete footprint in the cave (No. 5), limestone with cave biofilm (No. 18), a limestone monument (No. 13), limestone with biofilm (No. 20), and the limestone cliff at Predjama (No. 21, Table 3).

Swabbed microbial communities differed in terms of r-strategists. Microbes that initially colonize a habitat are most commonly r-strategists with the highest growth rates that would favour reproductive success at low population densities, which depends directly on the carrying capacity of the environment (Fontaine et al., 2003; Blagodatskaya & Kuzyakov, 2008; Ciccazzo et al., 2015). The highest abundance of r-strategists (≥ 75%) was on dead wood (No. 17), a metal safety fence (No. 9), a limestone with cave biofilm (No. 18), a limestone with fresh bat guano (No. 19), a limestone with biofilm (No. 20) and a stalagmite contaminated by handling (No. 6). The highest microbe abundances in communities growing at 37°C compared to those growing at 20°C were from a swab from a metal fence (No. 9), a piece of dead wood in the cave (No. 10) and limestone with subaerial biofilm (No. 20). The surface of the dead wood in Postojna Cave was largely impacted by organic and faecal pollution related to the Pivka River. Its swabbed surface contained *E.coli* (% ID 98.4, good identification) and *Citrobacter youngae* (% ID 77.0%, good identification to genus).

**Airborne biomass**

Cave air carries a significant quantity of dust and diverse microbes (Mulec et al., 2012b; Martin-Sanchez & Saiz-Jimenez, 2014). An approximately 10-times higher concentration of biomass was retrieved from settled aerosols on a limestone tablet (379 RLU/20 cm²) in the restricted access part of Postojna Cave (No. 24) during a period of 34 days, compared
Table 3. Biomass, percentage of r-strategists and effect of temperature on microbial growth of the swabbed surfaces.

<table>
<thead>
<tr>
<th>Substrat, sample info (No.)</th>
<th>ATP (RLU/20 cm²)</th>
<th>10°C (CFU/20 cm²)</th>
<th>20°C (CFU/20 cm²)</th>
<th>37°C (CFU/20 cm²)</th>
<th>37°C / 20°C (%)</th>
<th>r-strategists (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concrete, footpath† (12)</td>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Concrete, footpath* (5)</td>
<td>412,200</td>
<td>2.00×10⁵</td>
<td>9.64×10⁵</td>
<td>1.20×10⁶</td>
<td>7.45×10⁵</td>
<td>2.00×10⁵</td>
</tr>
<tr>
<td>Concrete, pigeon guano (16)</td>
<td>18,180</td>
<td>1.38×10⁶</td>
<td>1.57×10⁶</td>
<td>1.53×10⁶</td>
<td>1.92×10⁸</td>
<td>1.97×10⁸</td>
</tr>
<tr>
<td>Flowstone, dust* (2)</td>
<td>13,860</td>
<td>1.01×10⁶</td>
<td>2.99×10⁴</td>
<td>2.35×10⁵</td>
<td>1.45×10⁵</td>
<td>4.94×10⁵</td>
</tr>
<tr>
<td>Flowstone, stalactite* (7)</td>
<td>6,930</td>
<td>1.91×10⁵</td>
<td>7.95×10⁵</td>
<td>6.00×10⁵</td>
<td>2.13×10⁸</td>
<td>9.69×10⁵</td>
</tr>
<tr>
<td>Flowstone, stalagmite* (4)</td>
<td>900</td>
<td>2.61×10⁵</td>
<td>3.75×10⁵</td>
<td>0.00</td>
<td>4.50×10⁵</td>
<td>1.95×10⁵</td>
</tr>
<tr>
<td>Flowstone, stalagmite, touched* (6)</td>
<td>1,258,800</td>
<td>1.44×10⁹</td>
<td>1.21×10⁵</td>
<td>5.25×10⁵</td>
<td>1.92×10⁵</td>
<td>8.21×10⁵</td>
</tr>
<tr>
<td>Limestone (21)</td>
<td>17,820</td>
<td>2.22×10⁴</td>
<td>2.67×10⁴</td>
<td>6.60×10⁴</td>
<td>3.96×10⁴</td>
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</tr>
<tr>
<td>Limestone, bat guano* (19)</td>
<td>750,600</td>
<td>2.66×10⁴</td>
<td>4.26×10⁴</td>
<td>6.06×10⁵</td>
<td>3.68×10⁵</td>
<td>3.98×10⁵</td>
</tr>
<tr>
<td>Limestone, biofilm (20)</td>
<td>106,200</td>
<td>8.94×10⁵</td>
<td>1.24×10⁶</td>
<td>9.00×10⁵</td>
<td>1.62×10⁹</td>
<td>7.38×10⁵</td>
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<tr>
<td>Limestone, biofilm* (18)</td>
<td>46,800</td>
<td>9.00×10⁵</td>
<td>9.90×10⁵</td>
<td>5.10×10⁵</td>
<td>1.98×10⁸</td>
<td>1.21×10⁸</td>
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<tr>
<td>Limestone, flood subjected* (1)</td>
<td>1,080</td>
<td>2.08×10⁵</td>
<td>1.20×10⁴</td>
<td>8.00×10⁴</td>
<td>2.44×10⁵</td>
<td>2.34×10⁵</td>
</tr>
<tr>
<td>Limestone, flood subjected (11)</td>
<td>114,390</td>
<td>2.38×10⁴</td>
<td>7.07×10⁶</td>
<td>2.70×10⁷</td>
<td>2.26×10⁴</td>
<td>8.61×10⁷</td>
</tr>
<tr>
<td>Limestone, monument, touched (13)</td>
<td>2,520</td>
<td>7.20×10⁵</td>
<td>2.85×10⁵</td>
<td>2.55×10⁵</td>
<td>2.40×10⁵</td>
<td>3.60×10⁵</td>
</tr>
<tr>
<td>Limestone, tectonic slickenside* (8)</td>
<td>32,040</td>
<td>5.06×10⁶</td>
<td>1.54×10⁴</td>
<td>2.40×10²</td>
<td>7.87×10³</td>
<td>7.18×10³</td>
</tr>
<tr>
<td>Metal, fence* (9)</td>
<td>240</td>
<td>1.00×10⁴</td>
<td>4.00×10⁴</td>
<td>1.60×10⁵</td>
<td>4.00×10⁵</td>
<td>4.00×10⁵</td>
</tr>
<tr>
<td>Metal, handle (14)</td>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Wood, dead (17)</td>
<td>4,320</td>
<td>1.32×10⁴</td>
<td>5.70×10⁴</td>
<td>3.00×10⁴</td>
<td>3.00×10⁵</td>
<td>8.40×10⁵</td>
</tr>
<tr>
<td>Wood, dead* (10)</td>
<td>848,880</td>
<td>1.28×10⁸</td>
<td>3.95×10⁸</td>
<td>9.20×10⁸</td>
<td>3.85×10⁸</td>
<td>9.72×10⁸</td>
</tr>
<tr>
<td>Wood, railroad tie (15)</td>
<td>17,370</td>
<td>2.61×10⁵</td>
<td>1.34×10⁵</td>
<td>5.70×10⁷</td>
<td>3.02×10⁷</td>
<td>1.15×10⁷</td>
</tr>
<tr>
<td>Wood, railroad tie* (3)</td>
<td>59,400</td>
<td>1.45×10⁶</td>
<td>8.35×10⁵</td>
<td>6.92×10⁵</td>
<td>1.61×10⁵</td>
<td>1.00×10⁵</td>
</tr>
</tbody>
</table>

* - cave sample, † - cleaner effect
to the cave section exposed to dust pollutions from the underground train (No. 23) and the external atmosphere (No. 26). The sampling point along the underground railway (No. 23) is close to the Rov pri Mumij passage, where a strong air flow is present in the cold season and is also responsible for local air circulation (Mulec et al., 2012b). Cave air close to alluvial sediments, cave biofilms and aerosols partly originating from the Pivka River (No. 22, Fig. 1) had 3,075 RLU/20 cm² (Table 5). The highest recorded biomass during the same period was in the cave air along the tourist footpath, 7,908 RLU/20 cm² (No. 25).

The highest concentration of biomass expressed as ATP (No. 25) did not correspond to the highest concentration of biomass estimated as CFU (Table 5). The highest CFU count (~ 200,000 CFU/20 cm² on NA at 10°C) during a period of 34 days was for the settled aerosols in the section of the cave with restricted access. The effect of a temperature shift from 10 to 20°C was not clearly expressed in the corresponding increases of CFU counts. The highest CFU count at 37°C was at the site with cave sediment (No. 22); the high level of this count can also be attributed to settled aerosols that also contained high CFU at 37°C originating from the Pivka River (Table 6).

**Biomass in karst waters**

Water samples differed in physicochemical parameters and biomass indicators. Drip water values were below the detection limit or showed very low concentration of biomass, both in terms of ATP and CFU. Ponor rivers bring many viable microbes and abundant organic matter into the underground environment compared to drip water (Table 6). A three-times higher concentration of microbial biomass expressed as ATP for the Pivka River compared to that of the Lokva River was not reflected in all corresponding values of CFU on different media. The Pivka River also deposits faecal microbes on cave surfaces, as was indicated by dead wood surface colonized by *E. coli* (swab No. 10).

**DISCUSSION**

**Surface and subsurface biomass**

Not all microbial colonization attempts are (completely) successful, because of unsuitable surface conditions (Kargar et al., 2014). In a continuous flow of allochthonous organic matter in karst, the new-coming microbes play a significant role in the colonization-succession process (Barton et al., 2013; Brannen-Donnelly & Engel, 2015). A relative high input of biomass in the underground karst occurs in well-fissured areas, as was demonstrated on a tectonically polished surface of an active fault plane inside Postojna Cave in this and a previous
study (Šebela & Mulec, 2011). A high percentage of r-strategists (74.03%) and microbes able to grow at 37°C were detected at this site (Table 3). Rapidly growing r-strategists commonly dominate in uncrowded and unstable habitats where resources are temporarily abundant (Andrews & Harris, 2013). Low abundances of r-strategists were found on external (2.28%, No. 15) and in-cave wooden railroad ties (37.01%, No. 1), an untouched and pristine stalagmite (9.09%, No. 4), and in dust associated with the underground railway (19.82%, No. 2). Dust particles collected along the main passage in Postojna Cave contained increased concentration of heavy metals, Cu, Pb, Zn, Fe and Mn (Muri et al., 2013), which can adversely affect microbes and their metabolism processes (Giller et al., 1998).

Different media and cultivation conditions resulted in different numbers of CFU, which also gave distinct correlations between total biomass estimator – ATP and counted colonies. Particularly for the cave samples, stronger correlations were observed for samples cultivated at the cave temperature rather than at 20°C (Table 4). A stronger correlation between ATP and CFU plate counts at low cultivation temperature (10°C vs. 35°C) has already been established for bacteria from cold environments, for example refrigerators (Chen & Godwin, 2006). Interestingly, a very strong relationship between ATP and CFU on NA (37°C) for above-ground samples was observed, but this cultivation condition enabled growth only of a small proportion of the microbial community (Table 3). In the study, anaerobic plate count was not evaluated because only a smaller portion of the microbial community may be attributed to strict anaerobes, because all the sampling sites were exposed to normal oxygen concentrations.

Results of the study indicate that underground microclimatic conditions might play an important role in the preservation or even the concentration of non-viable microbial biomass and viable microorganisms. This is especially the case for big cave systems where the DAPI total cell count did not change with animal excrement, e.g. from bats (Mulec et al., 2012a) and the presence of visible microbial biofilms (Mulec et al., 2015) are important sources of microbial biomass (Table 3) in the underground. Some caves do not rely only upon the input of organic matter from the surface, but are characterized by in situ microbial biomass production based on chemolithotrophic metabolism (Jones & Macalady, 2016). For example, a metagenomic analysis of surface speleothems from Kartchner Cavern, located in an arid zone (Arizona, USA) revealed the presence of a chemolithotrophic community adapted to low-nutrient conditions (Ortiz et al., 2014). In chemolithotrophy-based cave ecosystems, e.g. Frasassi Cave, Italy, in situ low nitrogen can be surmounted by a diazotrophy (Desai et al., 2013).

Areas where aerosols settle provide high bioburden potential for the cave (Table 5). Locally there are major differences in the sampled presence of organic matter, which can also be explained by the low percentage of r-strategists – for example on a swabbed stalagmite (900 RLU/ 20 cm², No. 4). Interestingly, at the same location (Fig. 1), in only 34 days the settled aerosols showed a rather high biomass (379 RLU/ 20 cm², No. 24, Table 5), which can be attributed to the circulation of biomass-rich air masses in the cave across longer distances. As well as through the movements of air masses, microbes enter caves with both flowing and seeping water, as well as with animals and humans (Mulec, 2015). Not just the major flows of ponor rivers, but also dispersed epikarstic seepage water bring along considerable amounts of organic carbon (Simon et al., 2007).

In comparison to UltraSnap™ (data not shown), which basically consists of cotton swabs, AquaSnap™Total, when used in combination with flocked swabs (FLOQSwabs™) as an ATP biomass estimator tool, showed correlations with CFU counts and thus considerable promise for determination of the microbial biomass in various cave sub-habitats. UltraSnap™ kit has previously been used for surface swabbing in Lechuguilla Cave (New Mexico, USA), where the DAPI total cell count did not change significantly in line with changes in ATP levels (Johnston, 2013). In a previous study in Postojna Cave (Mulec et al., 2012a), similar surfaces were swabbed using RIDA®COUNT test plates directly for swabbing. The swabbing procedure described in this study and the use of comparable nutrient-rich media (NA vs. RIDA®COUNT Total for bacteria and MEA vs. RIDA®COUNT Yeast&Mold Rapid for fungi), with similar cultivation conditions (35°C and 37°C for 48 hours for bacteria, 20°C for 72 hours for fungi) resulted in 3-times up to 80-times higher CFU retrieval. However, more data collection is needed to help develop this procedure as a general estimator for the bioburden of underground habitats. An example of bioburden ranges for Postojna Cave, based upon the ATP levels of swab samples is given in Fig. 1. An ATP biomass estimator (AquaSnap™Total) can be used as the first and easiest step in studying factors that affect microbial transport and colonization underground.

**Human impact in show caves**

Karst caves and karst aquifers are highly susceptible to pollution and biomass input from various sources such as wastewater discharge, agricultural and urban runoff (Mahler et al., 2000; Reed et al., 2011), and tourism (Jurado et al., 2014; Mulec, 2014). Locally high concentrations of biomass in Postojna Cave are related to human activities (Fig. 1). Based on biomass estimates recorded during this study, Postojna Cave seems to be mainly affected by human intervention that is attributed to the surface biomass of wooden railroad ties (~1.30×10¹¹ RLU of ATP, ~3.50×10¹² CFU) and tourist footpaths (~7.80×10¹¹ RLU of ATP, ~1.41×10¹³ CFU). In comparison to the Postojna Cave system as a whole, this surface bioburden can represent up to a 1.6-times greater biomass in
terms of ATP or 6.4-times in terms of CFU, when a standardized bioburden of 900 ATP RLU/ 20 cm² and 4.50×10⁴ CFU/ 20 cm² (Table 3, No. 4 as a reference site) is adopted for the entirety of the surveyed cave surfaces, comprising wall and floor areas but excluding isolated speleothems.

The absence both of UV radiation and desiccating conditions in caves results in the survival of microbes from organic pollution, for example Enterobacteriaceae (Campbell et al., 2011). Enterobacteriaceae, more specifically E. coli and Citrobacter, were retrieved from an occasionally flooded surface in Postojna Cave. These two organisms are particularly relevant in the environment as a source of antibiotic resistance determinants that can spread quickly among different species through horizontal gene transfer (Mulec et al., 2002; Perry et al., 2014). It seems that cave conditions (high humidity, presence of sediments and organic debris) enable longer survival of these bacteria. However, in the long term, survival of enterobacteria in aquatic environments is known to be briefer than their survival in soils (McFeters et al., 1974).

The highest ATP value in the study, recorded from a handled speleothem, can also be attributed to epithelial cells from human skin with an average value inside a human cell of between 3 and 5 mM (Gribble et al., 2000). More so, ATP is also present in extracellular compartments where it operates in cell-to-cell signal transduction (Hayashi et al., 2004). High biomass in terms of ATP for this sample was accompanied by a high percentage of r-strategists (77.45%) and low percentage of microbes able to grow at 37°C compared to at 20°C (Table 3). Nonetheless, human-derived biomass consequently becomes available for exploitation by cave biota.

Application of a cleaning product on walking surfaces, and use of a disinfecting barrier at the cave entrance contribute to the overall reduction of microbial biomass by tourist footprints, though related levels still remain high in the cave (Fig. 1). The low ATP concentration of a swab on a safety fence in the cave (240 RLU/ 20 cm², No. 9) can be attributed to the known toxic effect of copper on biota (Baker et al., 2014), even though the sampling site suffers heavy contamination as a result of tourist contact. Copper surfaces are significantly effective in lowering the bioburden (Schmidt et al., 2015). Heavy metals introduced as a part of the tourist infrastructure, e.g. copper within safety fences, metal particles deriving from the railway, particles from the wear and corrosion of the railway tracks (Muri et al., 2013) very likely have an adverse effect on microbiota.

The direct tourist impact in Postojna Cave is not only evident on contact surfaces, but also in the air quality (Mulec et al., 2012b). Whereas one might expect one of the highest levels of biomass in the air to be along the main passage, with the tourist railway, in the cave, this was not the case. Human-induced and natural air streamlining direct the movement and settling of aerosols. This can explain the relatively low biomass along the route of the tourist railway (dilution) and the relatively high biomass accumulation in the part of the cave with restricted access.

CONCLUSIONS

ATP luminescence assay (Hygiena, USA) was tested successfully on different samples. Using flocked swabs (FLOQSwabs™, Copan) the assay proved its versatility in estimating the bioburden of solid surfaces. Surfaces inside caves displayed similar or even higher levels of bioburden than surfaces exposed to the external atmosphere that can be attributed partly to human impacts. There is a high variability of surface microbial biomass within single cave systems. These cave sub-habitats are colonized by physiologically different microbial communities. The highest in-cave values of ATP and CFU from swabbed surfaces were from a stalagmite contaminated by touching, bat guano and tourists’ footprints. These samples exhibited also high percentages of r-strategists in a community, whereas wood surfaces and untouched pristine stalagmite exhibited low abundances of fast growing bacteria. Not only microbial ATP, but also ATP deriving from human epithelium cells contributed to the highest concentration of ATP from a handled stalagmite. The toxic effect of copper in the metal safety fence can be attributed to low total biomass. A strong positive correlation was recognized between ATP and CFU for swabbed surfaces from caves. Enterobacteriaceae were easy to retrieve from a surface exposed to the underground river, which was contaminated by faecal bacteria. Mass visitation in the tourist part of Postojna Cave resulted in an increased microbial airborne biomass. Microbial biomass on non-cave surface analogues was generally lower.

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Tube coalescence in the Jingfudong lava tube and implications for lava flow hazard of Tengchong volcanism

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Abstract: Tube-fed structure occurs as a general phenomenon in Tengchong basic lavas, such as lava tubes, lava plugs and tube-related collapse depressions. We deduced the development of Laoguipo lava flows, which is the longest lava tube (Jingfudong lava tube) evolved in Tengchong volcanic area. Following the detailed documentation of the tube morphology of the Jingfudong lava tube, we propose that the Jingfudong lava tube was formed through vertical coalescence of at least three tubes. The coalescence and bifurcation process is reconstructed by the interpretation of tube floor continuity, the distribution of remnant tubes and the scales of lava tube branches (shapes of cross sections).

Keywords: Tengchong volcanism, Laoguipo volcano, field investigation, lava tube, tube coalescence


INTRODUCTION

Lava tubes are among the most common features in low viscosity/high effusion rate lava flow, not only on Earth, but also on other planets in the solar system (Greeley, 1992). As a type of cave, lava tubes provide a long-term stability environment, where diverse flora and fauna adapted to the unique ecosystems (e.g., Clarke, 2010; Novak et al., 2012; Pérez-Moreno et al., 2016) and complex aggregation of different minerals could take place (Onac & Forti, 2011). Lava tubes are observed in both Pahoehoe and aa lava flows (Halliday, 2002; Cashman & Mangan, 2014), their formation and development processes have been widely documented by investigating the basaltic lava flow fields (e.g., Wentworth & Macdonald, 1953; Ollier & Brown, 1965; Greeley, 1971; Hatheway, 1972; Peterson & Swanson, 1974).

Walker et al. (1973) suggested that the key parameter controlling the length of lava flow is effusion rate. Harris & Rowland (2009) reasserted this view and emphasized that cooling rates play an important role in lava flow length. Dietterich & Cashman (2014) summarized that the emplacement of lava flow was controlled primarily by effusion rate, underlying slope and rheology of lava (e.g., Dragoni et al. 2002, 2008; Honda, 2004), and proposed that the effusion rate at the vent could not record the lava supply to the flow front in accuracy. Furthermore, the branching, merging and slope also played important roles in lava emplacement and are affected strongly by topography.

In the context of lava rheology, the formation of lava tubes should be taken into account when correlation set up between lava flow length and effusion rate. The role of tube-fed structures in enhancing lava transport in large lava fields has been widely recognized (e.g., Guest et al., 1980; Greeley, 1987; Peterson et al., 1994; Calvari & Pinkerton, 1998; Cashmam & Mangan, 2014). Once the lava tube is successfully formed, it provides an efficient path for the transportation of following lava flows. Lavas has been inferred to flow to long distances in tube-fed transportation, due to efficient thermal insulation in lava tubes (e.g., Helz et al., 2003; Harris & Rowland, 2009; Cashman & Mangan, 2014). Furthermore, drained lava tubes could also act as a transportation path for later lava flows (Schmincke, 2005; Greeley, 1987).

Lava flow could intrude into the older lava tubes by eroding their ceiling or wall, and this is termed as tube coalescence (Calvari & Pinkerton, 1999). We herein report the tube-fed features in the Tengchong volcanoes, Southwest China, and focus on the tube morphology of Jingfudong lava tube, which is the longest lava tube (812 m) known in this volcanic area and developed within the lava flow of Laoguipo volcano. This paper documents the important changes...
of Jingfudong morphology, which is helpful for reconstructing the processed of tube coalescence and bifurcation. This tube is formed by vertically stack of at least three tubes, which means tube coalescence is very important in tube forming processes. The tube morphology changes dramatically with the trend and slope of the lava tube, which has important implication for lava flow behaviors.

**GEOLOGICAL BACKGROUND**

The Tengchong volcanic field is located in Southwestern China (Fig. 1), along the Southeastern margin of the Tibetan plateau. There are several active faults and frequent earthquakes occur in this region as consequence (Lei et al., 2013). Three active magma chambers with size about 19-28 km (horizontal extent) are located at depth 4-12 km under the ground (Zhao et al., 2006).

The origin of Tengchong volcanic field remains controversial and several mechanisms have been proposed. Early researches showed that the eruptions in Tengchong area derived from the subduction-collision (Zhu et al., 1983; Mu et al., 1987), or the post-collision or delayed arc-volcanism in the southeast margin of the Tibetan plateau (Zhao & Chen, 1992), or the mature island-arc area (Cong et al., 1994). Wang et al. (2007) proposed that the source of the Tengchong volcanic rocks ascended from an intracontinental tectonic setting, rather than a subduction or collision zone between Indian and Eurasian plates, on the basis of petrological, geochemical and tectonic investigations. Recent research suggests that the volcanic activity is caused by deep subduction of the Burma microplate (or the Indian plate), revealed by high-resolution tomography (Wei et al., 2012).

Tengchong volcanic field consists of 68 volcanic edifices, and 25 of them are preserved cones with clear crater (Fig. 1). Volcanic activities and the products can be divided into three stages: (1) Miocene-Pliocene basalt (5.5-0.9 Ma); (2) Pleistocene dacitic ignimbrite and intermediate-acid lava flows (0.8-0.01 Ma); and (3) Holocene basaltic andesites (0.01-0.007 Ma) (Jiang, 1998; Wei et al., 2003; Zhao et al., 2012).

The most intensive eruption was the explosive eruption of Daliuchong volcano in the Pleistocene, with major products of strongly welded dacitic ignimbrites (Li et al., 2014). Monogenetic volcanic activities continued from the Pleistocene to Holocene, and erupted basalts to trachyandesite (Fan et al., 1999). The eruptions in Holocene were from the Heikongshan, Dayingshan and Ma’anshan volcanoes (Jiang, 1998; Wei et al, 2003; Zhao et al., 2012), which formed three scoria cones and lava flows covered an area of about 90 km². Many villages are now located in this area. The youngest eruption in this area is supposedly the eruption of Dayingshan volcano in 1609, which was described more like a forest fire in *Travels of Xu Xiake* (Xu, 1642) (whose official name is Xu Hongzu), and recorded in Simkin & Siebert (1994). However, the existence of this event is still under debate.

**TUBE RELATED FEATRURES IN TENCHONG LAVA FLOW**

Many tube-fed structures could be recognized in the Tengchong lava flows. There are Dapengdong (“Shelter Cave”), Bianfudong (“Bat Cave”), Yemaodong (“Wildcat Cave”) lava tubes (Zhao et al., 2009) and other unnamed tubes in the lava flow of Ma’anshan volcano (e.g., Zhao et al., 2012). Some lava tubes are exposed in the Heikongshan lava flows and were used as refuges by Chinese citizens in World War II. Furthermore, many collapse depressions appear on the Heikongshan lava flows (Fig. 2A and B). As far as we know, no drained tubes have been found in the Dayingshan lava flows, but instead of many plugs in the eastern lava fronts (Fig. 2C). The diameter of these structures ranges from 8 m to 12 m, with lengths in excess of 60 m. They are characterized by concentric-circled cleavages formed by shear stress, and the interval between cleavages ranges from 5 cm to 15 cm.

The scoria cone of Laoguipo volcano is located about 5 km west of Tengchong city at an altitude of 1842 m above sea level. The lava flows cover an area of 15
km², with southern parts overlapped by later lava effusion from the Ma’anshan lava flow (Fig. 3A). The K-Ar dating results of lavas from Laoguipo lava flow show that the Laoguipo erupted in the late Pleistocene (0.165 ± 0.08 and 0.204 ± 0.136 Ma, Jiang (1998); 0.239 ± 0.029 Ma, Li et al. (2000)).

Two vents are recognized and shown in Fig. 3. The south vent erupted lavas flowing mainly to south parts, where the Jingfudong lava tube evolved (Fig. 3A). The northwest vent supplied the northern parts of lava flows and the highest ridge in this lava flow field (Fig. 3B, dashed ellipse in Fig. 3A).

Four collapse depressions recognized in the north part of Laoguipo lava flows (Fig. 3A). In the dashed rectangular in Fig. 3, different scale collapse depressions occurred in the lava flows and a remnant lava tube connected with the small pit (Fig. 4A). The lava tube has an entrance with 3 m in width and 2 m in height (Fig. 4B). The walls of the collapses are nearly vertical (Fig. 4C).

**INVESTIGATION OF JINGFUDONG LAVA TUBE**

**Method**

A systematic field speleological survey of Jingfudong lava tube was carried out by using conventional techniques and instruments, including compass and tape measure. The tube has been divided to dozens of segments in observation and documentation. The observation points depended on the morphology changes. In each point, width and height of the tube are measured, and photos of the profile of this segment have been taken while the shape parameters of each profile have been particularly recorded.

The chemical compositions of whole rocks were analyzed by X-ray Fluorescence Spectrometer (AB104-L, PW2404). The compositions of representative minerals were analyzed by JXA-8100 Electron Probe Microanalyzer with the standard of GB/T 15074-2008.

**Results**

Field measurements are plotted in plan view and as profiles (Fig. 5A and B), while the cross sections were obtained from the photos of the same segments, and then scaled by measurement of width and height of the same segment.

The volcanic rocks are black and vesicular. The rocks exhibit porphyritic textures in thin sections. The phenocrysts consist mainly of plagioclase, clinopyroxene and a small amount of olivine. The groundmass consists of volcanic glass and amounts of plagioclase, clinopyroxene and titanomagnetite microlite (Fig. 6A). Some plagioclase phenocrysts are euhedral and have the resorbed sieve core and hyperplasia edges (Fig. 6B).

The An of plagioclase phenocrysts are in the labradorite range. The pyroxene phenocrysts have Mg⁰⁺ values of 71.75 to 81.89 and their end member compositions (Wo₁₀.₀₈-₄₁.₁₁En₁₂.₁₂-₅₃.₈₈Fs₁₀.₉₃-₁₇.₁₁) locate in the augite region according to pyroxene nomenclature scheme (Morimoto et al., 1988). Two olivine phenocrysts have chrysolite values of Fo = 76.85 and Fo = 81.41, respectively. Mineral compositions are shown in Table 1.

Three whole-rock major elements analyses in this work are shown in Table 2, together with five whole-rock analyses from Xiang et al. (2000). The Laoguipo lavas show a range of SiO₂ from
Fig. 3. A) The lava flow direction of Laoguipo volcano, the locations of vents and the Jingfudong lava tube; B) The profile of the highest ridge of Laoguipo lava field, formed by the latest lava flows. Terrain data from SRTM database in Chinese Academy of Sciences.

Fig. 4. A) Collapse depressions and remnant in Laoguipo lava field; B) Entrance of the remnant tube, which is connected with the small pit; C) Vertical wall of the large collapse depression.
51.12 wt % to 54.8 wt %. The lava compositions of Laoguipo volcano fall in a narrow range of basalt-andesite in TAS plot and a region of subalkali series.

**CONFIGURATION AND FEATURES OF JINGFUDON LAVA TUBE**

**Configuration of Jingfudong lava tube**

The Jingfudong lava tube is the longest known in the Tengchong volcanic field. The measured length of the tube is 812 m, and the end of the tube is sealed by road construction. The tube extends forward and is exposed in a private timber mill on the other side of the road. The starting point of the Jingfudong lava tube is characterized by two lava inlets (① in Fig. 5B), which have height less than 50 cm. Lots of raft breakdowns with diameter of ~10 cm were observed on the floor of the lower tube.

The average width of tube floor is 3.6 m, while the first 500 m in upstream has average width of 3.2 m and the downstream (from 500 to 812 m) has a width of 4.5 m. The overall height difference between floors of starting and ending is 119 m, giving out the average slope of tube floor is 8.9° to the horizontal.

The rapid changes of tube width and slope between cross-sections No. 18 and No. 24 are notable. From cross-sections No. 18 to No. 21, the average width is 2.8 m. The average inclination of this segment is 2°. The average width from cross-sections No. 22 to No. 24 is 5.8 m, much larger than the average width of the entire tube. After cross section No 24, the average width of tube is 3.5 m. The average inclination of segment from cross section No. 22 to No. 24 is 18.6°.

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**Fig. 5.** Map of the Jingfudong lava tube with 32 cross sections. A) Plan view of the lava tube; B) Profile of the lava tube. Scales: equal ratio between length and width in plan view; height is shown with 3× exaggeration in profile. The cross sections have appropriately equal ratio exaggeration. Tube coalescence evidence is marked by circled number.

**Fig. 6.** Microphotographs of Jingfudong volcanic rocks (crossed polarized light). A) typical plagioclase, pyroxene and olivine phenocrysts; B) plagioclase phenocryst with resorped sieve core and hyperplasia edge, and matrix plagioclase.
significantly steeper than the average inclination of the tube. Dramatic orientation changes also occur between cross section No. 21 to No. 24.

Features in Jingfudong lava tube

Typical lava tube features have developed in the Jingfudong lava tube, including lava stalactites, lava levees and lava shelf, etc. (Larson, 1992). Some mineral deposits have been observed in the lava tube as well, such as carbonate stalactite, stalagmite and coralloid. We present below some different scale features in Jingfudong lava tube.

Lava stalactites

Some lava stalactites are preserved well on Jingfudong tube ceiling. A few scattered on the tube ceiling, while most of them concentrated between cross section No. 22 and No. 23. The stalactites have V-like shape, with typical length of about 5 cm. A drip-like accumulation of lava occurred on the sharp ends of some lava stalactites (Fig. 7A).

Lining

A lot of linings remain on the tube wall. Thickness of the linings ranges from 1 cm to 6 cm. A small gap less than 0.5 cm separated the linings and the tube walls. The surfaces of these linings are very smooth (Fig. 7B).

Lava levee

A twenty meter long lava levee was identified near the No.18 cross section in Fig. 4B. The height of this levee is about 70 cm, inclining to the tube floor with an angle of about 70°. The width of gap between the lava levee and the tube wall is about 5~8 cm. This lava levee has rough surface, and flow structures could be recognized on the levee (Fig. 7C).

Coralloid

Coralloid is a nodular-like speleothem (Larson, 1992) and forms during sublimation processes (Onac & Forti, 2011). A concentrated cluster of coralloid occurring on the tube wall in Jingfudong lava tube has scale of 20×10 cm, with typical silicate nodule with diameter of 0.5-1 cm (Fig. 7D).

Stalactite and stalagmite

Few carbonate deposits (calcite) and one stalactite with 5 cm length and 3 cm diameter (Fig. 7E) was found on the tube wall. In another part of the tube, a
10 cm long stalagmite (Fig. 7F) is growing on the lower part of tube wall, with 2.5 cm diameter.

Cupola

A few cupolas are found on the ceiling of the Jingfudong lava tube. They have an ellipsoidal shape, and remnant lava shows that they flow down from the upper layer (Fig. 8A). In the central of the recesses of these cupolas, small holes are observed, with diameters of few centimeters. These holes connect with upper layer lava tube. The cupola we show in Fig. 8A is located near cross-section No. 16 in Fig. 5.

Skylight

There are two skylights in the Jingfudong lava tube. One is the tube entrance, with scale of 2×4 m, located near cross-section No. 23 in Fig. 5. Layered lava forms the wall of the entrance. A few breakdowns were found on the floor beneath this entrance. Another skylight of size about 2×3 m is located at cross-section No. 31 in Fig. 5, which is the connecting part of two parallel tubes of approximately similar size. Large breakdowns could be found on the floor, and has the same scale with the skylight.

Lava shelf

A lava shelf overhangs on the tube wall between the cross-sections No. 17 to 18. This lava shelf is about 10 m long, and aligned parallel with the lava tube floor (Fig. 8B). The surface of the shelf inclines toward the tube centerline, with angle of about 10°. There is no evidence of molten lava flow down along the inclination, and the edge of shelf is quite sharp.

A large number of breakdowns are observed on the shelf surface.

**TUBE COALESCENCE OF JINGFUDONG LAVA TUBE**

In the case of Jingfudong lava tube, remnants of former tube vault (e.g. the lava shelf) and tube branch (Fig. 8C and D) are evidence of tube coalescence. The variation of cross section shapes in the Jingfudong lava tube (Fig. 5) and the presence of lava falls also indicate the process of tube coalescence. Jingfudong lava tube consists of several stacked tube branches, which are independent or coalesced at certain locations. We found that the tube heights and shapes of cross-sections varied significantly, for example, the heights near the cross-section No. 8, 16, 17, and 27 were nearly doubled compared to the typical height of the main tube (Fig. 5B). Evidence of the intrusion of lava from upper tube into lower tube, such as lava falls and copulas, are common features at these segments (e.g., Fig. 8D).

There are two lava inlets at the start of the lava tube (① in Fig. 5B). The upper inlet is smaller than the lower one. The tube floor of the lower tube continues for 500 m from the inlets. Remnants of upper tube could be found in segment ①, ②, ③ and ④ (Fig. 5B) and most of the lower tube ceilings were eroded. The diameter of the first 500 m of the upper tube is smaller than the lower one (see cross sections and Fig. 8C). In summary, in the first 500 m segment, the tube consists of two parallel lava tubes, and the upper one is smaller.
The tube floor is not continuous between the cross-section No. 21, 23. Instead, there is a lava sump (⑤ in Fig. 5B) and there is a branch emerging from the lower level. This branch extends in the upstream direction for 15 m, where it is blocked by breakdowns. It could be observed that the tube elongated further for about 10 m upstream through the gaps between blocks. The scale of this branch is similar to the upper one. The tube floor of this branch extends to the end of the entire tube.

After 500 m (downstream), the tube clearly consists of two tubes. The tube floor/vault is partially continuous from cross-section No. 23 to 30 (e.g., remnant of tube vault and large breakdowns could be observed at cross-sections No. 29-30). After cross-section No. 30, the tube consists of two independent tubes, connected by a skylight formed at cross-section No. 31, with breakdown deposits on the lower tube floor. The dimensions of these two tubes are similar (see cross sections).

**DISCUSSION**

**Potential volcanic hazards in Tengchong area**

Tengchong area is a scattered volcanic field, characterized by tens of monogenetic vents and associated features (Fig. 1). The petrology and K-Ar dating results (e.g., Fan et al., 1999; Li et al. 2000) suggest the volcanism resembles the relict subduction zone/post-collisional model (cf., Keskin, 2007; Dabiri et al., 2011), and the initial eruptions occurred in southeastern and northwestern ends of the Tengchong basin, then distributed in the basin. Since the early Pleistocene, volcanism occurred along a NS-trending belt and finally focused on three Holocene volcanoes in the west. This means new volcanism will likely form new vents within the field.

We note that the consideration of lava transport in tubes must be taken into account in assessing the lava flow hazard in the Tengchong volcanic area. The development of new lava tubes and tube coalescence, as well as the existing lava tubes will likely play important roles in future eruptions. We also suggest that unstable lava tube ceiling could suddenly collapse, which could induce accidents in local farm and construction works.

**Lava transportation from Laoguipo volcano**

The development of Laoguipo lava field could be inferred from Fig. 3. Overflows occurred in the initial stages and formed the lava field, followed by the flow of main directions in Fig. 3A (dashed arrows) which delivered new lava to the flow fronts. The topography shows that the initial lavas flowed to the north and then turned to the west, where the two collapse
depressions and remnant tube system evolved. The other early flow direction of lava is represented by Jingfudong lava tube. The latest flow directions are represented by the solid arrow in Fig. 3, and the highest ridge on this lava field (Fig. 3B) was formed during the latest stage.

**Evolution of the Jingfudong Lava Tube**

Lava tube coalescence can be recognized either from the compound shapes of the tube cross sections or features such as lava falls, breached levees and other structures (Calvari & Pinkerton, 1999). In this section, we infer the evolution of tube branches in the Jingfudong lava tube, by considering the tube floor/vault continuity, remnant tube branches, and tube features such as lava falls, lava shelves and copulas. These latter features are shown to provide key evidence for the lava tube being intruded by upper layer lava flows.

The segment between cross-sections No. 20, 23 is complex. There are three distinctive tube branches. The lowest tube has a similar diameter to the main tube, which is blocked by ceiling collapse. We call this branch the “initial tube”. The floor of this tube is continuous to the end of the entire tube. Above the “initial tube” is the “main tube”. This branch has its own inlet (lower inlet) and has a continuous floor from its inlet to cross-section No. 21. The “initial tube” and the “main tube” have similar dimensions. There is another small tube uppermost in this segment (area ④ in Fig. 5B). It has an independent inlet, and intermittent floor (①-④ in Fig. 5B). These evidences suggest that there were at least three tubes comprising the Jingfudong lava tube.

The tube floor from cross-sections No.18 to No. 22 is gently sloped and then there is a significant step from cross-sections No. 22 to 24. We infer that the lava flows in cross-sections No.18 to 22 ponded or merged, caused by underlying topography. After ponding, the lava accumulated until its surface became higher than surroundings, where the lava flows could move rapidly (due to the steep slope from cross-sections No. 22 to 24). According to Cashman & Mangan (2014), formation of a skylight is controlled by underlying slope and stability of lava supply, which suggesting that the steeper slope and unsteady lava supply promote the formation of skylights. So, we could infer that the entrance of Jingfudong lava tube is a skylight caused by slope. In the Jingfudong lava tube, few breakdowns exist on the floor beneath the entrance skylight, which suggests it was formed while the tube was active.

We suggest that the Jingfudong lava tube was formed in three stages. The “initial tube” developed in an early lava flow. This flow ponded near cross-sections No. 22, where there were the gentlest slopes (Fig. 5B). After ponding, it flowed rapidly when advancing, which is implying the steepest tube floor between cross-sections No. 23 and 24. The initial tube floor is continuous from the cross-section No. 21 to the end of the tube.

The next lava flow, which effused from the lower inlet, followed this path and developed another lava tube (the “main tube”). The tube floor was continuous from its start to beyond cross-section No. 22. We suggest that this tube branch remains continuous and was fed only from the lower inlet, because of the distinct two-layer structure and the presence of the remnant floor at ⑥ and ⑦ in Fig. 5B. This tube intruded into the “initial tube” at the lava sump (⑤ in Fig. 5B), and the two layer structure continues after this point (e.g., ⑥ and ⑦ in Fig. 5B).

Finally, a lava tube evolved from a lava flow from the upper inlet. This tube branch is represented by the remnant of tube floor ② and ③ in Fig. 5B) and small tube branch (④ in Fig. 5B). It was small in scale and fully developed, but almost totally intruded into the main tube.

**Implications for flow behavior**

Dietterich & Cashman (2014) proposed that braiding index had a positive correlation with underlying slope and channel width, and flow thickness had a negative correlation with slope. Jingfudong lava tube provides a case for applying their model to the vertical case. In vertically stack tubes, the braiding index is now represented by the stacked tubes, then the more tubes coalesced implying the higher braiding index. Two or three stack tubes were identified at ② and ⑤ in Fig. 5, where the floor slopes are clearly higher than other parts. This suggests that in the vertical direction, vertical braiding has a trend of coalescence. Cross section No. 18-21 has a significant small scale. The gentle slopes and the narrowest tube suggest the horizontal braiding index is high in this segment, which implies that the bifurcation has taken place in these segments.

Another interesting thing is the orientation change near No. 22 and 24 cross section in Fig. 5A, which could be the restraint of pre-existing topography. In Fig. 3A, the topographic contour lines show a depression change. The area of cross section No. 22 is the largest in the tube, and the most complicate coalescence occurs there. Areas of cross sections after No. 24 are obviously less than No. 22 cross section, and tube direction changes again from this point. These indicate that tube bifurcation occurs in these segments. From all above, we suggest that tube coalesce and bifurcation play important roles in Jingfudong evolution.

**CONCLUSION**

On the basis of the well-developed lava tubes in the Laoguipo lava flows, lava tubes in Ma’anshan volcano, lava tubes and collapsing pits in Heikongshan lava flows and tube-like features in Dayingshan volcano, the tube-fed mechanism is important for lava transport in Tengchong eruptions. Lava tubes could significantly enhance lava transportation in active eruptions (see references above).

Detailed morphology of Jingfudong lava tube in Laoguipo volcano lava field was documented, and the tube evolutions were interpreted from the morphology changes. At least three lava tubes evolved well and used the same path. The interpretation of tube coalescence and bifurcation were based on the shape,
slopes and orientation changes within the lava tube, which are helpful for understanding the lava flow behavior.

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High-resolution signatures of oxygenation and microbiological activity in speleothem fluid inclusions

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Abstract: Speleothems frequently host “fossil” fluids that were trapped in small inclusions during growth. Such fluids may provide valuable clues to past microbial, geochemical, and climatic processes during their formation. However, one difficulty is to understand which gases represent background atmosphere and fluids within a given cave system at a particular time, and which may be the product of post-trapping residual microbial activity or abiotic chemical reactions? Do we have any hope of sorting out these differences? The success depends on a quantitative understanding of the gas composition trapped in the inclusions and an understanding of the interactions of cave mineralogy, air and water chemistry, and microbiological processes that may interfere with climatic or geochemical interpretations. Our proof-of-concept project uses time synchronous samples from several sites. We report here on this pilot investigation of speleothem inclusions using a methodology for quantitatively analyzing gases dissolved in inclusion fluids. We use incremental crushing of highly spatially resolved samples by mass spectrometry. Here, we report primarily on CH₄, CO₂, O₂, and N₂, but have included other detectable gases. The detection limit for He within aqueous fluid inclusions is ~0.2 ppm and gas ratios have ~5% precision using natural standards. We used chemically inert argon as a tracer gas to normalize results to air or air-saturated water. This enables interpretation of gas data despite variability in hydrological and geological cave histories. Results are variable. For example, in one case oxygen was depleted while nitrogen was increased, which may be attributable to the breakdown of nitrate or nitrogen-containing biomolecules. In other cases, oxygen is enriched which may be attributed to several factors both geochemical and biological. We suggest potential interpretations between the competing hypotheses with larger future data sets. This first attempt tackles the complex and intertwined speleological questions using the inclusion gas method.

Keywords: fluid inclusions, fluid inclusion gases, microbial activity, speleothems

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INTRODUCTION

Speleothems are secondary mineral deposits that form in subterranean voids (caves, vugs, and mines). They are produced by processes ranging from deposition of minerals from saturated waters (Palmer, 2007) to bedrock breakdown and reprecipitation by microbial processes (Boston et al., 2009). Chemical, physical, and biological conditions can range from highly variable in caves with active streams or high ventilation conditions to very stable over considerably longer periods of time in certain cave systems. The latter highly stable situation would be the case where speleothems have been influenced by hydrologically quiescent systems, especially where water and atmospheric composition are relatively sealed away from surface influences.

In the broad sense, fluid inclusions are microscopic cavities within rocks and minerals that comprise any combination of liquid, solid and/or gas (Roedder, 1984). These fluid inclusions develop during mineral growth or by annealing of secondary fractures and trap the actual fluids, gases, and other compounds that were once present in the greater environment. As
carbonates (and some other minerals) are deposited in caves, they develop small inhomogeneities that result in such inclusions. These can act as micron scale chemical and microbiological time capsules, and can be used to help us examine changes in system condition, by providing portals into former microbial communities and past climate conditions that once occurred in the caves. Besides fluid, both dissolved salts and gases are often typically present. Some inclusions may also contain organic material that has been shown to be useful as a climate signal (e.g., Baudin et al., 2007), and trapped microorganisms that are still alive yielding analyzable DNA (Boston, unpub. results).

Gases trapped in cave speleothems are analogous to several other types of data. For example, ice cores in polar regions trapped fossil atmosphere in snow, and are now used as a standard approach to understanding ancient atmospheres (e.g., EPICA, 2004). Carbon-dioxide and other gases remain trapped in bubbles within the ice and it is assumed that the gas concentrations have not been modified since entrapment, but this may or may not be a legitimate supposition if microbial or geochemical processes alter the abundance of CO₂ within the bubbles. The gas concentrations are analyzed by mass spectrometry and the data is correlated with other data [e.g., ice δD and δ¹⁸O]. These are used to build models of climatic change. This method of evaluating such trapped gases is applicable to polar and boreal conditions, and tropical glacial examples (e.g., from the Andes) have been advanced to explain tropical climate change (e.g., Ginot et al., 2002). However, our understanding of possible atmospheric conditions in the mid-latitude regions where human populations and vegetation are highly concentrated must be based on additional analytical techniques. Caves offer the potential for mid-continental and low latitude paleoclimate proxies (e.g., Martin & White, 2008; Lachniet, 2009). Although the emphasis has been on what these sites can tell us about climate, the potential for microbial processes to interfere with such interpretations by altering either the concentration of gases, transforming gases into other materials as consequences of metabolism, or altering isotopic ratios has been largely ignored. We believe that this must be investigated in order to validate ongoing studies and to uncover any potential pitfalls that might compromise such datasets and climate interpretations.

Fluid inclusions in speleothems were originally studied 30 years ago (Schwarcz et al., 1976) but are recently being regarded anew as valuable paleoclimate proxies, with most work concentrated on the stable isotope composition of water in inclusions (Matthews et al., 2000; Dennis et al., 2001; Fleitmann et al., 2003; Serefiddin et al., 2005; van Breukelen et al., 2008; Verheyden et al., 2008; Dublyansky & Spotl, 2009). Some research has focused on noble gas concentrations (Kluge et al., 2008; Scheidegger et al., 2010). Prior research has shown that δ¹³C and δ¹⁸O stable isotopes reflect changes through time using the U-Th-Pb dating methods to constrain temporal variability (Brook et al., 2006; Dennis et al., 2001; Genty et al., 2002; McDermott, 2004). The potential for detecting radiatively active trace gases (RATG) of biological origin that are dissolved in inclusions has not previously been attempted, but the close coupling of the global biota to the climate system is a topic of intense interest (Armeth et al., 2010). Any approach that might aid interpretation of past instantiations of the Critical Zone would be immensely valuable in interpreting Earth’s past biogeochemical state (NSF, 2011; Nordt et al., 2012).

Analyzing fluid inclusion composition is routinely performed by microthermometry as a first pass method (Shepherd et al., 1991). However, dissolved gases that cannot be detected at low concentration using microthermometry include several of particular interest to understanding microbiological processes (e.g., CH₄, N₂, and other N species, O₂, CO₂, and low-mass organics). The alternative is to conduct quantitative fluid inclusion gas analysis by mass spectrometry, which is the approach used in studies of geothermal systems (Moore et al., 2001; Blamey, 2012; Blamey et al., 2015), hydrothermal ore deposits (Blamey, 2012), metamorphic fluids (Wright et al., 2012), and exotic materials relating to space exploration (Parnell et al., 2011; McMahon et al., 2012; Blamey et al., 2015), and diagenetic carbonates (Azmy & Blamey, 2013).

In general, the solubility of calcium carbonate is controlled by several factors that include concentrations of calcium and bicarbonate ions, CO₂ loss or gain, water loss or gain, pH changes, ionic strength changes of the fluid, and temperature. The solubility of calcite may be summarized by the following simple equation:

$$\text{Ca}^{2+} + 2\text{HCO}_3^- = \text{CaCO}_3 + \text{H}_2\text{O} + \text{CO}_2$$

As simple as this equation may be, in the speleothem environment, several factors may influence and complicate this straightforward reaction. These factors include relative humidity or saturation, CO₂ levels including microbially induced changes, local pH, temperature, fluctuations of calcium and bicarbonate ions, and finally changes in the fluid ionic strength. Isolating the relative contribution of each factor that may control the speleothem growth may be very difficult, however, trapped fluids and gases may help to unravel such influences.

The physical conditions in the cave environment also affect the chemistry of the gas composition and concentration. For example, even in caves with nearly saturated humidity, evaporation still occurs. We see evidence of this in the presence of efflorescent cave minerals like magnesium sulfate (Hill & Forti, 1997) that come and go with season, and bedrock breakdown products that have an evaporative mechanism as well as microbial and chemical degradative processes (e.g., moonmilk, cf. Curry et al., 2009). Thus, evaporatively driven processes contribute to the movement of materials through the
system that further enhances deposition and in some cases also facilitates the breakdown of bedrock (Boston et al., 2001).

We offer the results of this preliminary study, although from a limited dataset, as a promising proof of concept of both the application of a method and as a first attempt to deconvolve the multifarious processes that no doubt occur in fluid inclusions. We believe that the techniques used can be employed to begin to tackle some very knotty issues with the interpretation of ancient and modern subsurface environments and associated climate parameters that are already being deduced from speleothem climate studies (e.g., Wong & Breecker, 2015). Part of the challenge in interpretation comes because there are major heterogeneities that are occurring on very fine spatial scales. Of particular note, the incorporation of gases must reflect the atmospheric composition within subsurface cavities at various spatial resolutions which may not be homogeneous. Additionally, such incorporation could be influenced by microbial transformations at similar spatial scales both prior to capture within the mineral and as a result of residual biological activity after entrapment.

Sample Descriptions

We have opportunistically selected several sample types from significantly different cave environments that we have already been studying in other ways. These sites include deep, ancient carbonate caves in the high Guadalupe Mountains in New Mexico, a sulfuric acid-rich system and a conventional carbonate system in Mexico, and amorphous opal speleothems in granite caves in northern Spain (Fig. 1). We selected these to provide a range of very different environmental conditions during the formation period of the speleothems in order to see if we could understand the results and if the methodology was worth pursuing. We emphasize that this is a pilot effort piggy-backing on other funded studies, thus the selection of materials was constrained by this circumstance, but was optimized within those limits to provide a suite of very different cave environments.

Sampling methods are very simple since the contents of interest are tiny microbubbles within crystalline or amorphous mineral structures, and consist simply of selecting pieces of such material from speleothems of several sorts. Our methodology requires only minute pieces (sub-millimeter in diameter). In this pilot study, we do not have data on the age of the particular speleothems chosen although in several cases there is very minimal dating that partially constrains timescales. Further, we did not attempt to match morphological speleothem type from cave to cave because the caves are so different in fundamental lithologies and in speleogenetic processes that formed them and their speleothems that simple morphological similarity is irrelevant. We chose specimens that were the apparent product of interesting geochemical and environmental juxtapositions.

New Mexico: Endless Cave, McKittrick Hill, New Mexico, USA (Fig. 2), a now-dry fossil cave with extensive speleothem decorations that show evidence of microbiological activity (e.g., microfossils, micritic textures, and light carbon isotopic signals). Poolfingers (i.e. pendant carbonate structures that formed below the water level) show strong influences of microbial processes in geographically related caves (Melim et al., 2001, 2009).

Mexico: Mexican samples include: 1) a conventional appearing stalagmite (PAA-07) from Arroyo Azul Cave, Poana, Mexico; and 2) a carbonate concretion “cave pearl” (labeled Red Stream) from Cueva de Villa Luz, Tabasco, Mexico (Fig. 2). The Poana cave is a small cave system unaffected by any apparent unusual chemistry including no sign of sulfur influence, and the sample was deposited in a subaerial setting but has subsequently suffered some corrosion. In contrast, Cueva de Villa Luz is a sulfuric acid rich cave with a shallow stream flowing through it. A microbial role in many Villa Luz speleothems has been established (Hose et al., 2000; Boston et al., 2006; Rosales-Lagarde, 2012). The cave pearl was sub-sampled to try to establish whether different visually distinct layers within it had different characteristics. Additionally, a control sample from unaltered bedrock in the same cave was analyzed.

Spain: Grey opal-A speleothems on the cm scale were collected from O Folón Cave in northern Spain (Galicia). No further locational information is allowable under the collection agreements that allowed us access
to that cave system. Granite caves are formed in large granitic plutons that intruded into the country rock approximately 350 million years ago. A plate collision during the Tertiary period caused the uplift and subsequent erosion of overlying strata, thus exposing the granite and allowing the subsequent development of the caves. The overlying rock mass eroded away exposing the granite, and elastically decompressing it producing unusual interior spallation of the granite (Vidal-Romani & Vaqueiro-Rodriguez, 2007). The dense microbial mats that cover the insides of the caves greatly alter the pH of the fluids that condense on the cave walls greatly affecting the development of speleothems (Vidal-Romani et al., 2010 a, b) and we hypothesize that it may be a significant geochemical influence on inclusions as well.

**METHODOLOGY**

Fluid inclusion volatile analysis was done in vacuum using the CFS (crush-fast scan) method (Norman & Moore, 1997; Norman & Blamey, 2001; Blamey, 2012; Blamey et al., 2015) at New Mexico Tech’s Fluid Inclusion Gas laboratory. Samples were first cleaned with NaOH to reduce surface organic contamination, and then were rinsed with deionized water, followed by drying at room temperature. Approximately 0.1 gram of material from the New Mexico and Mexico sites, and ~30 mg of sample from the Spanish opal speleothems were incrementally crushed under a vacuum of ~10⁻⁸ Torr yielding two to ten crushes per sample. The analyses were performed by means of two Pfeiffer Prisma quadrupole mass spectrometers operating in fast-scan, peak-hopping mode. The system routinely analyzes for the following gaseous species: H₂, He, CH₄, H₂O, N₂, O₂, Ar, CO₂, SO₂, C₂H₆, C₃H₈, C₄H₁₀, C₅H₁₂, C₆H₁₄, and benzene. The concentration of each gas specie was calculated by matrix inversion and multiplication (described in Blamey et al., 2015) to provide a quantitative analysis. The instrument was calibrated using commercial gas mixtures, synthetic inclusions filled with gas mixtures, and three in-house fluid inclusion gas standards as described by Norman and Blamey (2001) and Blamey et al. (2015).

Precision for this method is better than 5% for major gaseous species and 0.2 % for water/gas ratios using natural aqueous fluid inclusion standards (Norman & Blamey, 2001). These values are considered realistic for natural inclusions owing to an inherent variability between inclusions even in the same sample. In contrast, synthetic capillary tubes filled at ~1 mbar of atmospheric pressure gave a 1-sigma error of 0.5 % for N₂/Ar ratios, which reflects ideal conditions (Blamey, 2012). The detection limit varies for most species and is dependent on burst size (i.e. inclusion gas yield), interference from other species, and instrument alignment (Blamey et al., 2015). However, it is calculated at ~0.2 ppm for inorganic species and 1-5 ppm for most organics, applying the formulae of Blamey et al. (2012, 2015).

**RESULTS**

All fluid inclusion gas analysis numerical data for non-aqueous inorganic species are presented in Table 1. A graphical representation of some of the key data is seen in Fig. 3. In the majority of analyses, water comprises >99 mole%, however, we are focusing on the inorganic species and therefore report the non-aqueous key species. The dominant dissolved gases are N₂, CO₂, and O₂ with minor concentrations of argon as well as CH₄ and other organic compounds. Since argon is essentially inert and its presence is unaffected by biological activity except under extraordinary circumstances of very high pressures (e.g., Abraini et al. 1998), the gas ratios of N₂/Ar and O₂/Ar may be valuable in recognizing variations in gases that are affected by biological activity. Additionally, Ar can also act as a normalizing, unreactive gas specie for geochemical interactions as well.

**New Mexico**

Endless Cave, NM, USA - The poolfinger sample has oxygen levels that are slightly below air-saturated water (ASW) and the N₂/Ar ratios are between ASW and atmosphere (Fig. 3A). However, the trend for these samples seen in the same figure indicates that oxygen depletion correlates with N₂/Ar increase. Additionally, poolfingers have the lowest helium content of all the samples (Fig. 3B) and are in close agreement with atmospheric values. Further, the poolfingers have the lowest CH₄ of all the samples (Fig. 3C). A single stalagmite sample from the same cave measured by V. Polyak (University of New Mexico) yielded a U-series age of ~0.5 Ma (pers. comm., 2013) using the methods reported in Polyak et al. (2004).

**Mexico**

Arroyo Azul Cave, Poana, Tabasco, Mexico - The conventional carbonate stalagmite sample (PAA-07) has higher oxygen levels than either ASW or air (Fig. 3A). In addition, oxygen increase correlates with a decrease in N₂/Ar ratio (Fig. 3A). PAA-07 is the most helium rich of all samples examined.
Fig. 3. Ternary plot comparisons of all studied gases. A. Ternary plot of O₂-N₂-Ar in carbonate samples. The Red Stream Pebbles are oxygen depleted and the N₂/Ar spread may indicate nitrogen release due to protein breakdown. Poolfingers are slightly oxygen depleted whereas the PAA-07 samples are mostly enriched in oxygen that exceeds air-saturated water. Atmospheric gas ratios are labeled as "air" whereas "ASW" represents air-saturated water, the equivalent of water in equilibrium with the atmosphere; B. Ternary plot of He-N₂-Ar in carbonate samples. The poolfinger samples are the most helium depleted with helium concentrations close to atmospheric values. The Red Stream pebbles have higher helium contents whereas the highest helium is measured in PAA-07; C. Ternary plot of O₂-N₂-CH₄ in carbonate samples. The poolfinger samples are the least methane rich whereas the Red Stream samples are oxygen depleted. Each sample plots in separate fields of the diagram indicating that the environments in which they form are distinctly different (Rosales-Lagarde, 2012; Rosales-Lagarde et al., 2014); D. Ternary plot of O₂-N₂-Ar in silica speleothems from Furnas Cave, Galicia, Spain. All samples are oxygen depleted (see text for details). There is an increase in nitrogen in the Distal and Middle samples, possibly attributable to nitrate breakdown (see text for discussion); E. Ternary plot of He-N₂-Ar in silica speleothems from Furnas Cave. Data is quite variable. However, the large sample provides a close match for ASW; F. Ternary plot of O₂-N₂-CH₄ in silica speleothems from Furnas Cave. All samples are more or less depleted in oxygen. There appears to be a trend of increasing methane from proximal to distal samples.
(Fig. 3B). Preliminary dating of the same speleothem from Arroyo Azul, using sample PAA-02, which is more distal (and hence, probably younger) than the analyzed PAA-07 yielded dates of ~20 - 27 ka (J.P. Bernal, unpub. results, pers. comm.). The sample had visible evidence of diagenetic reworking thus we are not presenting this datum with high confidence, however, it may potentially be indicative of approximate age of the outer portion of this speleothem.

Red Stream, Cueva de Villa Luz, Mexico - This area of the cave conjoins water from springs with radically different chemistries into a single stream. The Red Stream pebble samples are clearly depleted in oxygen (Fig. 3A) by approximately five times relative to air-saturated water. The N₂/Ar ratio for ASW is 38 (Norman & Musgrave, 1994). However, only one crush yielded an ASW-equivalent value whereas most N₂/Ar ratios approximate to atmospheric values and higher. Helium levels are moderate relative to the other samples (Fig. 3B) with the highest values from individual crushes associated with the highest N₂/Ar ratios. The Red Stream Pebble sample subset 1-4 exhibits a spatially progressive decrease in water from the core traveling to the rim. In addition, there is a relative and progressive increase in both oxygen and methane from the initial speleothem growth in the center outwards to the rim, whereas the helium decreases over the same traverse. This can be seen directly in Table 1.

Spain

O Folón - The CO₂ for inclusion gases hosted in the opal samples was relatively consistent. Unlike ASW, analyses were oxygen depleted by as much as an order of magnitude and N₂/Ar ratios were twice that of ASW with the spatially distal sample pieces giving N₂/Ar ratios that exceeded 100 (Fig. 3D). Methane is also elevated and is inversely correlated with oxygen (Fig. 3F).

**DISCUSSION**

Although the technique will need to be applied to much larger sample sizes in order to yield statistically robust results, even our initial experiments have shown trends that at least make sense within plausible chemistry and microbiology scenarios for a given setting. We detail the results in this broad interpretive context below.

**Endless Cave, NM, USA** - The variability in N₂/Ar ratios in Endless Cave analyses may be attributable to one or more of several processes: small air bubbles becoming trapped to variable extents within fluid inclusions, or by release of nitrogen during decay of nitrogen-containing biomolecules, or even abiotic breakdown of nitrate. We attribute minor oxygen depletion to the probable respiratory breakdown of organics by trapped heterotrophic organisms thus decreasing the oxygen levels slightly. If a biological interpretation of the oxygen results is correct, then that would perhaps favor a biological process to explain the nitrogen situation, namely the breakdown of nitrogenous biomolecules.

McKittrick Hill, the location of Endless Cave, is now part of the very arid Chihuahuan Desert but was once a much more verdant area with a higher biomass primary productivity than is present today in a desert region (Dick-Peddie, 1999). Thus, at the time of apparent precipitation of the speleothem, a significantly higher dissolved organic carbon load...
was undoubtedly present in the infiltrating surface waters. The single age date of ~0.5 Ma cited in the results (Polyak, pers. comm., 2013) constrains the time period of interest to at least a limited extent placing it significantly before the end Pleistocene climatic events.

**Arroyo Azul Cave, Poana, Tabasco, Mexico** - PAA-07 data shown in Fig. 3A exhibit an oxygen enrichment to the left bottom corner of the O$_2$-N$_2$-Ar ternary diagram. Such a result is obviously only achieved if oxygen is increasing. The most plausible explanation is the generation of oxygen within the inclusion. However, in a dark cave we cannot expect photosynthesis to occur directly within the cave itself. It is possible that the fluid entering the cave at time of inclusion entrapment was enriched with oxygen, possibly from a stream or other source at the surface where photosynthetic organisms might have been producing high levels of oxygen. This area is currently heavily vegetated tropical forest and has been proposed to form part of a tropical high rainfall regime from Pleistocene time (2.5 Ma) (Wendt, 1987; Salazar-Conde et al., 2004; INEGI, 2005). Alternatively, we consider the possibility that chlorine gas associated with the nearby volcano might oxidize water to produce oxygen by the following reaction:

\[
2\text{H}_2\text{O} + 2\text{Cl}_2 = 4\text{HCl} + \text{O}_2
\]

However, any decay process of organic matter trapped in inclusions would actually depress oxygen as it was used to mineralize the organic carbon back to CO$_2$ rather than enrich it. Another alternative explanation for oxygen elevation could be the breakdown of oxyhydroxides during the formation of red hematite staining, as has been seen in other instances where oxygen exceeds ASW values (McMahon et al., 2012). There is definitely red hematite present in this cave, coating a number of speleothems and being incorporated as visually detectable banding in some specimens (Spilde & Boston, 2010, 2014 unpub. results).

**Red Stream, Cueva de Villa Luz, Mexico** - Red Stream Pebbles exhibit strong oxygen depletion. The Red Stream Pebbles occur at the confluence of slow moving shallow streams of low oxygen content, and the cave’s atmosphere which also has measurably low oxygen at that locality (Hose et al., 2000; Rosales-Lagarde, 2012) thus matching the fluid inclusion data but not precisely proportionately. We know on the basis of prior work, that sulfide-rich, anaerobic waters mix with oxygen-rich, non-sulfide waters in the entire regional and cave hydrological systems (Rosales-Lagarde et al., 2007, 2008, 2012). The oxygen levels in the Red Stream part of the cave can range as low as 14% in the air, while elsewhere in the cave O$_2$ levels as low as 9.6% have been recorded (Boston, unpub. res.), and 0.1 mg/l in the water (Hose et al., 2000) although it can be as high as 5.7 mg/l (saturation at typical surface conditions is ~9.1 mg/l; Rosales-Lagarde et al., 2007, 2008). Unlike the previously described samples, the Red Stream Pebble sample has highly variable N$_2$/Ar ratios with some analyses exhibiting N$_2$/Ar ratios that are greater than atmosphere. One might expect a limit of 83.6 for N$_2$/Ar ratios thus matching the atmosphere but we consider two alternatives as potential explanations for these data.

Firstly, nitrate reduction is a potential process that would generate diatomic nitrogen. Nitrate reduction can be expressed by a chemical reaction in which nitrate consumes acid in an oxygen-poor environment to produce nitrogen:

\[
2\text{NO}_3^- + 12\text{H}^+ + 10\text{e}^- = \text{N}_2 + 6\text{H}_2\text{O} \quad (1)
\]

An acid and a reducing environment will drive this reaction to the right by Le Chatelier’s principle (Krauskopf & Bird, 1994). The Red Stream environment is known to have high concentrations of sulfuric acid the magnitude of which varies on a continuous basis, and it is very oxygen poor (Malaska & Kelly, 2014 unpub. results). Thus, we consider nitrate reduction as a plausible mechanism whereby, within an oxygen-poor environment, fluids may become enriched in nitrogen.

The second alternative hypothesis is that influx of condensing magmatic volatiles from El Chichón Volcano (~30 km distant) are responsible for the gases associated with the Red Stream. Volcanic gases are expected to be rich in sulfur species and the Red Stream contains significant dissolved sulfuric acid from both aqueous reactions in the stream itself and from wall rock interactions of thin fluid films with H$_2$S emitted from numerous springs (Hose et al., 2000; Spilde et al., 2004; Rosales-Lagarde, 2012). In addition, the stream is strongly oxygen depleted which matches volcanic gas composition. A definite correlation of gases within the cave with gas data from the El Chichón Volcano (Spilde et al., 2004) has shown that there must be some physical connection between the two geological features (Rosales-Lagarde et al. 2007, 2008) and thus we suggest that volcanic gases are plausibly involved in the fluid inclusion results shown here. Finally, magmatic gases typically have high N$_2$/Ar ratios (Norman & Musgrave, 1994; Blamey, 2012) and the analyses of the Red Stream samples have N$_2$/Ar ratios that exceed 100. Fluctuating contributions of atmosphere and volcanic volatiles can explain the spread within the data.

Curiously, helium levels are variable (Fig. 3B). We know that a balance exists in our atmosphere between helium being lost to space versus helium that is produced within the crust. Such variability is uncommon in geological systems where helium levels are generally in the ppm range. Whereas most speleothem samples are within one order of magnitude higher in helium than atmosphere, both the Red Stream pebbles and PAA-07 have abnormally elevated helium. Close proximity to a volcano might explain the elevated helium in the Red Stream pebbles because of the demonstrated relationship to El Chichón Volcano (Spilde et al., 2004), but PAA-07 has no obvious volcanic connection. Although difficult to prove, a much longer flow path could conceivably explain the
elevated helium from deeper sources (Norman and Musgrave, 1994). However, determining the length of such a flow path in a hydrologically intermittent environment where dye tracing is not possible is a tricky calculation and poorly constrained.

Interestingly, the six sample time-series subset collected from the Red Stream pebble confirms a progressive change in fluid inclusion gas chemistry through time. The increase in oxygen and decreasing helium may be explained by changing contributions at the confluence of the anaerobic and the aerobic streams such that flow from the anaerobic stream has relatively decreased. The progressive change in methane may well reflect biological activity (either methane production by methanogens, or consumption by methanotrophs) but this avenue of research needs significant refinement before any interpretation can be made. However, the change in gas chemistry with distance from inside to outside layers of the specimen does confirm that in principle, we can distinguish such changes in speleothem inclusions through time. It also confirms that analyzing dissolved fluid inclusion gases offers a means to record changing environmental conditions within a cave system and potentially compare cave systems to one another. The potential for microbial processes to interfere with such environmental and climatic interpretations has been largely ignored so far.

Granite Caves

Figure 3E shows moderately elevated helium in several of the granite cave samples. We believe it unlikely that residence time is relevant here, however, as early as 1950, emissions of alpha particles from granites were shown to be higher than expected based solely on uranium and thorium content because of the emitting effects of high surface area (Hurley, 1950). The medium to coarse-grained granites comprising the Spanish cave provide a great deal of surface area which would be consistent with some elevated helium results. In Fig. 3F, the variation in CH₄ proportion relative to N₂ and O₂ may be related to different amounts of organic matter when particular fluid inclusions where trapped. The heavy lichen coating on the granite surface outcrops above the cave, and the forested surface setting produce significant surface biomass and this is a plausible external source of organic carbon. This is significant in this environment because the granite itself does not possess any intrinsic organic content to help support methanogenesis as might be the case in some other lithologies, namely carbonates, which often do contain organics that were incorporated at the time of deposition (Wald et al., 2012; Thauer, 1998). As everyone appreciates, the common conception of methanogenesis involves strictly anaerobic conditions (e.g., Houghton et al., 2001), however, the controversial notion of aerobic methanogenesis has been reported at least for plants (Keppeler et al., 2006). Perhaps more importantly, the presence of anaerobic microsites in soils with overall bulk conditions, or in proximity to aerobic microsites has been documented in a variety of settings (e.g., Verchot et al., 2000; Li et al., 2000). Either of those circumstances, could result in elevated methane in some inclusions dependent upon highly local and possibly very transient circumstances. When oxygen was also present in the system, then the methane would subsequently react and draw down the oxygen. The scale at which such competing redox processes could operate vis a vis the scale of the inclusions themselves is unknown at this point, although once again we emphasize that it is well known that in soils there is the simultaneous presence of aerobic and anaerobic microsites (e.g., Tiedje et al., 1982). Although methanogens are notoriously oxygen sensitive in the common wisdom, it has been shown that this is not always the case (e.g., Kiener & Leisinger, 1983; Mayer & Conrad, 1990; Fetzer & Conrad, 1993; Fetzer et al., 1993; Ueki et al., 1997).

Relative Merits of Normalizers

The presence of dissolved argon in all specimens is an excellent way to normalize the data to gas values dissolved in air-saturated water (ASW), as a basis for comparison of potential biologically produced gases. Many of the dissolved gaseous species vary in composition and tracking changes is complicated. The abundance of oxygen and nitrogen may be controlled by biological processes, and tracking these increases or decreases requires a gas species that remains unaffected. For this reason we utilize argon, as its abundance is well within the detection of the mass spectrometer system and it is neither added to nor removed by biological activity. The only way to increase the argon concentration is by proximity to K-bearing minerals (McDougall & Harrison, 1988) that are subjected to thermal conditions above the closure temperature of the mineral that is highly unlikely in speleothems. This type of process is restricted to high temperature metamorphic environments and is negligible compared to the relatively large concentration of argon in ASW. We therefore consider that tracking gases relative to argon is a viable means to monitor changes of nitrogen, oxygen and methane.

Helium is also a noble gas with trace quantities in the atmosphere and very low abundances in ASW, thus it could also be considered to play a similar role to argon. However, helium may increase due to several radioactive sources within the crust, thus it is less desirable as a normalization species because of this non-exclusivity of origin.

CONCLUSIONS

We have confirmed that speleothem fluid inclusion gas compositions are not homogeneous. Changes in fluid chemistry trapped within speleothem materials are measurable as is demonstrated by our analyses of a variety of speleothem types occurring in a variety of different geochemical and lithological cave settings. In some cases, oxygen levels within inclusions were enriched above air-saturated water levels and in contrast, other samples showed oxygen depletion. High nitrogen in the Red Stream samples indicates either breakdown of nitrogenous organic matter or nitrate to produce excess nitrogen, or it may confirm an influx
of volcanic volatiles from a nearby volcanic source. We have demonstrated the plausibility of using dissolved fluid inclusion gas analyses as a possible avenue to help understand paleoenvironmental conditions in cave systems at the time of fluid inclusion formation in speleothems. We have pointed out that there are several alternative explanations for each of our preliminary results involving a mixture of chemical and biological processes and conclude that our methodology will need to be employed on larger sample suites, perhaps with the addition of other techniques to sort out the many potential influences on inclusion contents. Besides the intrinsic value of understanding paleoenvironmental cave conditions, and residual microbial activity once trapped in inclusions, the emphasis on speleothems as paleoclimate proxies make the identification of any confounding microbial or geochemical fluid inclusion processes important to understand to help with interpretation of that type of climate data.

ACKNOWLEDGMENTS

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The morphology and development of Kalahroud Cave, Iran

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Abstract: Kalahroud Cave is located in central Iran, ~50 km north of Isfahan. The landscape is a typical mountain desert morphology of cuestas dissected by ravines and gorges created during rare surface run-off events; crest lines are ~2800 m asl and lowlands at ~2100 m asl. Kalahroud Cave (4500 m of mapped passages, ~60 m deep) is entered through breakdown in the eastern wall of a gorge. The host rock is a Cretaceous limestone and mudstone formation 60 m in thickness, underlain by sandstones and conglomerates and overlain by weakly permeable calcareous marl strata, all dipping 15-20°. Below the entrance breakdown, two corrosion notch chambers give access to a rectilinear, quasi-horizontal maze of joint-guided passages extending ~500 m eastwards. Rock solution morphology created by slowly flowing phreatic waters predominates (solution pockets, partitions, paragenetic forms, etc). Seven shafts are known that discharged water into the maze and chambers from inaccessible passages below. From XRD analysis, the paragenetic sediments derive from the mudstone interbeds. There are small displays of frostwork, helictites and thin flowstones typical of vadose speleothem deposition in arid caves. Below the level of the corrosion notch, more complex sub-aqueous and shelfstone calcites are overlain by accumulations of calcite rafts up to 70 cm in depth. Raft deposition continues today. It is proposed that the cave is of hypogene origin, serving to discharge interformational groundwaters into the gorge, and becoming de-watered as the latter was deepened. The corrosion notches are due to cessation of deepening. From U series dating, the modern phase of raft deposition began about 10,000 years ago. The sequence and ages of older events will be investigated in future work.

Keywords: rectilinear maze, corrosion notch rooms, hypogene cave, calcite rafts, arid climate, Iran

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INTRODUCTION

Iran is a large and mountainous country, tectonically a part of the active Alpine-Himalayan orogenic belt. The full range of the standard soluble rocks (the carbonates and evaporates) is to found in the area. Outcrops of salt in the Hormoz region (the southern Zagros Mountains) are especially well-known because of the occurrence of namakiers (glaciers of salt being extruded from diapirs) there (Jennings, 1985). However, carbonate rocks are predominant in outcrop, covering about 185,000 km² or approximately 11% of Iran's land area (Raesi & Laumanns, 2003). Most of their occurrences display some karst landforms and they are of significant importance for water supply in many regions because of the aridity of much of the country. In spite of this large extent, the number and lengths of known caves are less than might be expected. There are many highland karst aquifers without any discovered cave systems at present, although big springs emerge at their base levels of erosion. Multiple levels of cave development are to be expected as results of the rapid rates of tectonic uplift and the high local relief. The intensely deformed (folded and faulted) geologic structures and the limits placed by aridity on development of mature karst aquifers are probably the major reasons for the small number of known caves and their minor lengths.

Organized amateur caving began in 1945 (Javanshad, 1995). British cavers discovered and explored a major vadose shaft and drain cave, Ghar Parau, in the central mountains in the 1960s (Judson, 1973); it was the deepest known Iranian cave for many years but recently has been surpassed by Jojar...
Cave at -806 m. Czech cavers and the Geological Survey of Iran organized more systematic surveying campaigns in the 1990s and early years of the present century, focusing on the Hormoz region where they discovered remarkably long caves in salt; one of them, Tri Nahacu, is currently the longest salt cave known anywhere at 6,580 m (Bosák et al. 1999; Bruthans et al., 2006a,b). Subsequently, there have been speleological training expeditions organized by some of the leading Iranian mountaineering clubs, and the Union Internationale de Spéléologie (UIS) has helped to overcome the lack of international contacts for the Iranian caving community (ISEI, 2008). The first “Iran Cave Directory” in English, published by Raeisi & Laumanns (2003), provided details on some 550 caves. A 3rd edition increased this to more than 2000 caves (Raeisi et al., 2012). In 2011 an Iranian Cavers and Speleologists Association (ICSA) was founded and currently has more than one hundred members from all over the nation.

In addition to cave exploration, speleological studies are also being undertaken in a few Iranian caves. Hydrogeological investigations in Shapour Cave showed that the Shapour River was the principal source of water creating the cave but that, in addition, meteoric seepage waters from a much broader catchment area made important contributions to the development of a large regional system. What we see as Shapour Cave today is the relic of a once much greater cave that has been destroyed by surficial erosion over time (Raeisi & Kowsar, 1997). Rezaei and Nakhaei (2008) studied the genesis of Kattale Khour Cave, showing that this large cave with its anastomosing passages was formed by flood waters and allogenic recharge; the water table shifted position, inducing cave formation at three different levels. There is no direct evidence of any hypogene activity, but further studies are warranted at this cave. Research at Khasse Tarash Cave (Sabokkhiz et al., 2012) focussed on the geotourism analysis and mineralogy of the speleothems. They did not make detailed cave genesis and morphology in this case. Hejazi et al. (2015) studied Yekke Chah Cave, a small cave located in Golpayehan, Isfahan province, and proposed a hypogene origin. “An Introduction to Kalahroud Cave, north of Isfahan, Iran” (Bahadorinia, 2013) was a study undertaken as an MSc degree project by the first author of this paper that reported on initial sedimentary, speleological and morphological investigations carried out during several visits to the cave; the paper presented here includes its findings and expands upon them.

THE SETTING OF KALAHROUD CAVE

Kalahroud Cave is located at Lat. 33°22'21" N, Long. 51°34'35" W, in the Karkas and Kohroud River Basin in the mountains of the Central Iran tectonic zone, approximately 300 km south of Tehran and 50 km north of Isfahan (Fig. 1a). The regional landscape is typical of warm to hot desert mountain morphology, being dominated by steep ravines and gorges created by stream dissection of the massifs, with detrital fans and alluvial flats around their margins, all created during the rare (a-periodic) surface flood events. The Basin crest lines are between 2800 and 2900 m asl and the outlets into surrounding lowlands are at ~2100 m asl.

Fig. 1. a) Geological map of the locality around Kalahroud Cave, with the cave indicated (yellow dot); b) Location of Kalahroud Cave in Iran; c) Google Earth oblique image showing the bedrock formations. The cave entrance is marked with a circle.
The region is semi-arid to arid, with 163.7 mm average annual precipitation and a mean temperature of 22.1°C being measured at the village of Kalahroud, one km downstream of the cave (Fig. 2). The ‘wet season’ there extends from November to April, but only one substantial flood event has been reported during the past twenty years. It destroyed a shrine building located in the river bed just below the cave entrance. The floods come from the local ranges and the greater Karkas highlands to the north. There is little vegetation except around artificial oases maintained by groundwater. Farms and small villages obtain their water from historic systems of ‘qanats’, drainage capture tunnels dug long and deep into the alluvial fans and other deposits.

The local geology (Zahedi, 2005) is shown in Figs. 1b-c, 2, and 3. The bedrocks are a regularly bedded, mixed clastic-carbonate sedimentary sequence aggregating 180 m in thickness. At the base are sandstone and shale with Ammonitic limestone intercalations (unnamed formation, Late Jurassic), overlain by 80 m of conglomerate and red sandstone (Sangestan Formation, Early Cretaceous). Above this, the rock that hosts the cave is a thick to massively bedded Orbitolina limestone with lesser, thinner beds of arenaceous limestone and silty shale (Taft Formation, Aptian) aggregating ~40 m in thickness. This is overlain by 50 m of thinner-bedded, marly limestones with Orbitolina and Ammonitica, that function as a weakly permeable caprock. On top are a final ~10 m of stronger limestones with minor shale and marl (Late Cretaceous; see Fig. 1b).

Structurally this area is located on the southwestern margin of the Urumieh - Dokhtar Magmatic Belt of the Central Iran tectonic zone (Berberian et al., 1982). Early movements on Kalahroud Fault, a major fault to the north of the cave, flexed and folded the local strata to create an anticlinal structure. As Figs. 2 and 3 show, fluvial dissection has entrenched deeply into its core and the cave is developed in a shallow cuesta that survives on the southern flank. The dip of the beds is generally 15-25° to the southeast in the vicinity of the cave entrance but becomes locally shallower in angle to the east. There are two principal joint sets, centered on bearings of 355 and 88°, which control the orientation of the northward- and eastward-trending cave passages respectively. Some joints are vertical, while others are normal to the dipping beds. A local, low-angle thrust fault from the southeast is important in the entrance section of the cave (Fig. 3a).

The crest of the Kalahroud cuesta is ~2400 m above sea level. A torrential, a-periodic stream, ‘Kalahroud River’, has entrenched a shallow gorge across it (Fig. 2). It is now aggraded, with a floor of sand and gravels at ~2295 m asl that is known to be about 10 m deep and burying a bedrock channel floor beneath it. There is one alluvial terrace five meters above the modern dry channel. The cave entrance is in cliffs at 2323 m asl, ~28 m above the dry valley floor. Local people report that there is one small perennial seep or spring at ~2310 m asl in the foot of cliffs north of the cave (Fig. 3a; Point a) and one occasional spring at ~2303 m further down the valley (Fig. 3a, Point b).

**THE MORPHOLOGY AND DEPOSITS OF THE CAVE**

**Morphologic overview**

Kalahroud Cave has a surveyed length of ~4503 m of explored passages (Figs. 3 and 4). There are three morphologically distinct parts:- (i) from the entrance (Fig. 5a) a steep breakdown passage or ramp extends broadly down the thrust fault to a depth of ~45 m. There the form changes to (ii) two large horizontal solution notch chambers beveling the dipping strata, with multiple notches, solution pockets and pendants, tapering ramiform extensions upwards and laterally, and blind pits (shafts) in the floors. Two constricted tributaries enter at the notching level to give explorers access to (iii) The Maze, which is a joint-guided rectilinear maze with irregular, predominantly phreatic, form. There have been periodic pools many meters in diameter in the two large chambers, indicated by stranded calcite raft debris and many tower cones built up to precise paleo-water surfaces.

[Fig. 2. Google Earth oblique image of the local area, looking south. The cave entrance is located in the eastern wall of a gorge cut through a limestone cuesta by an a-periodic desert stream, ‘Kalahroud River’. The village of Kalahroud, in a qanat-supported oasis is seen downstream.]
Fig. 3. a) Google Earth oblique image looking east along the cuesta, with an oriented image of the Kalahroud Cave map superimposed on it. The dry channel of Kalahroud River is seen clearly. Points a and b indicate a perennial and an occasional spring. The trend of the thrust fault at the cave entrance is marked by the dashed yellow line; b) The long section of the cave. Black dots show the bottoms of some of the shafts.

Fig. 4. Plan of Kalahroud Cave with spot elevations in meters. Locations of phreatic lifting shafts and waterline corrosion notches in The Maze are also shown. A-A' and B-B' are projected sections across the east-west trend of the cave.
The Entrance Ramp

No evidence of a solutional origin is preserved in the entrance breakdown passage, as Fig. 5 shows clearly. The breakdown blocks are large, the biggest being >100 m$^3$ in volume and incorporating multiple beds of the limestone. Separation occurred along joints, thrust faults (Fig. 5c, d) and a few of the bedding planes. This collapse can be attributed to solutional undermining of the rock below the thrust fault. The consequent fracturing extends above it, including some further local falls. Significant amounts of finer-grained detritus with angular shards can be seen in places: studies show that they are colluvium intruding from the overlying hill slopes via the fracturing.

The Maze

The joint-directed maze of solutional passages beyond the notch chambers is the predominant component of the cave (Figs. 3, 4). The topographic long section in Fig. 3b shows that the accessible passages in this maze are tightly confined to a zone little more than 30 m in height. The highest ceilings rise only to ~2295 m asl, although the host Orbitolina limestone (Taft Fm) extends up to 50 m higher than this along the northwest side of the cuesta. The lowest known point is at 2263 m asl. There is accessible multi-storey development (upper and lower levels as much as 18 m apart) in several parts of the maze. Although appearing to be nearly horizontal when projected in long section (Fig. 3b), The Maze is tilted very gently southwards down the stratal dip. It is important to note that all of the passages are located within roughly the mid-section of the 40+ m of the Taft Fm. The explored maze appears not to be confined in any manner by the overlying and underlying, less permeable and soluble strata, which is in strong contrast to the situation in many other joint maze caves described in the literature.

The bedrock morphology of the maze is dominated by solution forms (speleogens) that are particularly associated with dissolution in phreatic waters that were flowing slowly (e.g., Klimchouk, 2000; Ford & Williams, 2007; Bella & Bosak, 2012; Audra & Palmer, 2015). Figure 6 shows examples of the most common cross-sectional form, a passage initiated along the junction of a joint with a bedding plane that was then enlarged primarily upwards and downwards in the joint. The amount of enlargement up or down (e.g., the height of the ceiling) can vary irregularly along a particular joint, as seen in Fig. 6a and b, or it may maintain a more steady height until the water flow switched to a different bed (Fig. 6c, d).

Figure 7 shows examples of the ‘partitions’ and ‘windows’ that have developed in some of the larger joint passages, or in small rooms formed at their junctions. “Partitions are thin separations between adjacent passages that may be in the local bedrock or formed by various kinds of planar resistant structures exhumed by dissolution, such as the lithified fill of fractures or faults and paleokarstic bodies. Sometimes they are only a few centimeters thick so that a “window” can be created by a punch with the hand. They attest to origin by slow, selective solution of the rock” (Klimchouk, 2007).
There are many joint-controlled solution pockets extending upwards above general ceiling levels in the passages. The majority are sharply tapered (Fig. 8a), rather than having a broadly rounded ‘cupola’ form like many of the pockets described in recent literature on caves considered to be of hypogene origin (e.g., Calaforra & de Waele, 2011; Temovski et al., 2013). Other pockets are shafts of more uniform diameter; the example shown in Fig. 8b is discharging seepage that has passed through the marly cover strata, and is now depositing calcite and aragonite speleothems.

Seven well-developed sub-circular downwards shafts are known in the explored cave (see Figs. 4 and 3b). Others may be buried by the abundant deposits of silt and clay found everywhere in The Maze, while some more elongated forms may not have been recognized as such. The known shafts are 5-8 m deep, mostly too narrow to explore but apparently floored in mud or calcite raft fillings. One larger shaft in the Second Chamber (Fig. 8d) has always had standing water at the bottom when observed. In all cases it appears that the shafts served to channel flow upwards from below (‘phreatic lifts’) rather than being shafts of vadose origin draining downwards. The two examples shown in Figure 8c, d are typical of the circular form.

‘Paragenesis’ occurs when a filling of insoluble silt or clay, settled from flowing water, builds upwards in volume to protect the cave floor and so direct any further solution of the bedrock upwards into the ceiling, or laterally into the walls if the stream is at the water table (Pasini, 1973, 2012; Renault, 1968; Ford & Williams, 2007). Such fillings are abundant in The Maze, where they have helped to create rounded arch forms or lateral widening and undercuts. Four examples are shown in Fig. 9; in viewing them it should be understood that the fillings seen today have shrunk considerably from their volume when actively accumulating, due to de-watering.

Kalahroud Cave is dry today except in some of its lowest places. Figure 10 shows examples of the few evidences of the more rapid flow of phreatic waters or of local vadose entrenchment of highs in bedrock floors in The Maze that occurred during flood recession stages.

**The Corrosion Notch Chambers.**

The First and Second chambers are the largest voids of solutional origin in the cave (Fig. 4). Their enlargement is due primarily to the lateral extension of a principal corrosion notch at 2285 m asl, approximately the same elevation as the buried bedrock floor of the Kalahroud Gorge ~80 m further west. The morphology of the chambers is illustrated in Fig. 11. We consider these scenes to be “textbook examples” of the late stages of enlargement in a cavern that is being flooded sporadically at low filling rates. At highest water levels the chambers were filled, and solutional pockets were then etched into the ceilings by gravity-driven convection currents under near-static flow conditions. The relationships to the dipping beds are seen clearly in Fig. 11a and c. The flood waters drained down slowly to the externally-controlled 2285 m asl level, where they were retained for longer periods and the principal notch developed. Later lowering with lesser notching may be attributed to regional water table fall below the bedrock floor in the gorge, or to evaporation in the cave, or to a combination of these two processes.

**Clastic deposits in the cave.**

Kalahroud Cave contains quite a wide range of clastic and chemical sediments deposited by different mechanisms, making it a good site for sedimentary studies. The classification of White (2007) was used to categorize the clastic sediments, and standard particle size, texture and fabric methods applied to study them. Several different categories of chemical precipitates were noted. Most of them remain to be investigated in the future but an initial set of calcite raft samples was collected for U series dating.

The clastic sediments may be categorized as follows: *Allochthonous* deposits, including entrance talus, infiltrates (in-washed soil), debris flows and mud masses (Fig. 12a-c). These are comparatively young sediments, derived from floods in the gorge and other
events of the recent past. The finest-grained infiltrates form some mud mounds around the cave and small patches filling parts of solution pockets in the ceiling. An important feature of the sediments in the First and Second chambers with large notches is that they are not laminated and commonly have patchy calcite layers within them. Some contain white, hardened particles of small pebble size: microscope study determined that these were thin calcite rafts which probably were in the first stages of growth. These mixed clastic and precipitate sediments appear to have originated from river inflows in recent wet periods. On the walls they are covered by thin layers of dusty gray calcite precipitated from seepage from leaking joints, or from condensation waters in or close to the breakdown entrance ramp (Fig. 13d).
Autochthonous sediments include the breakdown in the entrance area and paragenetic deposits of water-borne fines observed throughout The Maze. Samples were collected from the paragenetic clays and compared with others from intact mudstone beds within the host limestones. From the XRD analyses it was clear that the large volumes of mud in The Maze originate from weathering and erosion of mudstone layers in the host Taft Formation: there was no evidence of contributions from the underlying conglomerate and sandstone of the Sangestan Fm. (Fig. 12b-d).

The speleothem precipitates
The speleothems are either sub-aerial or pool deposits. A representative selection of the types is illustrated in Figs. 13-15. The sub-aerial deposits are quite widespread (e.g., as also seen in Figs. 6 and 10 in The Maze). They include small calcite stalactites, helictites, flowstones and popcorn, aragonite needles and frostwork. Thin veneers on the walls (e.g., Fig. 13d) possibly include some hydromagnesite and moonmilk but are dominated by precipitated skins of dust-laden calcite. Considered together, these are a suite of deposits typical of caves that are periodically wetted by seepage down joints but also experience dry, strongly evaporitic conditions, i.e., desert caves like many in Arizona, Nevada, New Mexico, etc.

The pool deposits are also varied. Figure 14a and b show examples of substantial pisolite and flowstone accumulations that imply deposition in long-lasting pondings. Figure 14c and d are nodular protrusions that possibly originated as shelfstones at the waterline, and later accreted sequences of silt, manganese and calcite layers as water levels fluctuated around them over comparatively long spans of time. They appear...
quite similar to sub-aqueous wall deposits in the extensive Jewel and Wind maze caves in South Dakota (Ford et al., 1993; Palmer & Palmer, 2015) and will be investigated further in the future.

A final category of pool deposits are the large accumulations of calcite rafts and raft cones (cones of raft debris sunk beneath fixed drip points above a pool - Hill & Forti, 1997) that are illustrated in Fig. 15. The principal deposits are in the First and Second chambers but there are lesser ones at and around the small pools in The Maze (see Fig. 4). Figure 15a shows the largest accumulation, which is in the First Chamber. Its waterline is at 2283 m asl, two meters below the principal corrosion notch. In the modern era it is known to fill to this level following floods.

In May 2012, a pit was dug where the piles of raft debris appeared to be deepest. It reached a silt and clay bottom at -70 cm (Fig. 15d). Samples were taken from depths of 10, 35, and 70 cm and sent to the U-series dating laboratory at the Department of Earth Sciences, Oxford University (UK). The results are summarized in Table 1. The samples have a low content of radiogenic $^{234}$U and $^{238}$U derived from solution of the bedrocks, and a comparatively high content of detrital (non-radiogenic) $^{232}$Th that is probably from the dust particles about which initial calcite raft crystals precipitate. This is a common finding when calcites are dated in desert caves, as Kaufmann (2002) found in a study of shelfstones in the Alisadr notch cave. $^{232}$Th (and likely companion detrital $^{230}$Th) are contaminants that reduce the precision of the radio decay measure, $^{234}$U→$^{230}$Th, requiring that an empirical correction be applied. In Table 1 it is seen that the uncorrected and corrected ages overlap at two standard deviations. The results may be considered decisive - the sampled raft deposit is of Holocene age, about 10,000 years old at the base and modern at the top, accumulating at an average rate of about seven cm per one thousand years.

## DISCUSSION

### General morphology

To summarize, Kalahroud Cave has three morphologically distinct parts (i) an entrance ramp of breakdown in the eastern wall of the Kalahroud River gorge, extending from 2323 m asl down to (ii) a pair of large corrosion notch chambers with a principal notch at 2285 m asl; (iii) behind the chambers and extending ~500 m eastwards, a rectilinear, quasi-horizontal maze of generally narrower, joint-guided passages: The highest points in The Maze are northern, up-dip, ceilings at ~2295 m and the lowest are shaft bottoms at 2263 m asl, so that the cave may be said to pass broadly through the middle of the 40+ m thickness of the southeasterly-dipping limestones of the Taft Formation.

<table>
<thead>
<tr>
<th>sample</th>
<th>$^{234}$U activity ratio</th>
<th>$^{238}$U activity ratio</th>
<th>$^{230}$Th activity ratio</th>
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<tr>
<td>Sr cone</td>
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<td>3.25257</td>
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<tr>
<td>FR pit base</td>
<td>0.00862</td>
<td>0.19401</td>
<td>0.00364</td>
<td>5.97008</td>
</tr>
</tbody>
</table>

Table 1. U series ages of calcite rafts and cones in First Chamber, Kalahroud Cave.

Fig. 11. Features of the main corrosion notch in the First and Second chambers. a-b) First Chamber, showing some past pool levels; c) Second Chamber, with the water level indicated; d) Partition morphology and a window formed at the water line, First Chamber.
Fig. 12. Representative samples of the clastic sediments shown with their XRD spectra. a, c) Allochthonous silt and clay wall and floor veneers in the First and Second chambers, from recent inflows from the Kalahroud River gorge; b, d) Autochthonous sediments on the floors and ledges in The Maze, derived from weathering and erosion of the local mudstone interbeds.
Origin of the Maze – by floods from the Kalahroud River gorge or by hypogene groundwater outflow into the gorge?

The wide variety of characteristic solution features associated with slowly flowing phreatic waters that have been described above (ceiling height irregularities, deep solution pockets, bedrock pendants and partitions, etc), plus the paragenetic development involving fine-grained sediments derived entirely from mudstone beds within the local Taft Fm. limestone, are considered to be strong evidences that The Maze is of hypogene origin, rather than being a river bank floodwater maze formed in mixed vadose-phreatic conditions where much more rapid rates of flow would be expected. As far as the cave is known today, the sources of the water appear to be the basal shafts shown in Figures 3b and 4 that were created by rising groundwaters (phreatic lifts), plus others possibly concealed by the clastic sediments, and perhaps other inputs further to the east or down the dip that may be discovered in future exploration. Basal lifting shafts are prominent features in perhaps the majority of the hypogene maze caves being described in the literature today. It is quite possible that much of basal recharge into Kalahroud Cave was from water rising through the underlying, insoluble but well-fractured, sandstones and conglomerates of the Sangestan Fm, rather than laterally through the basal few meters of the Taft limestone that lie beneath the bottoms of the shafts.

However, The Maze is constrained within a narrow vertical range (2295 to 2263 m asl) that is strikingly discordant to the stratal dip when viewed at the scale of a long section (Fig. 3b). This must be due to the entrenchment of the Kalahroud Gorge through the cover strata at ~2350-2450 m asl. This permitted groundwater flow through the Taft Fm aquifer, with discharge in the earliest springs at around 2300 m.

With continued entrenchment of the gorge, the upper parts of The Maze drained first, then the system stabilized at the main notch level (2285 m asl) when the gorge became shallowly aggraded. The weaker notches seen in The Maze are also at or within one meter of 2285 m asl.

Why is the main corrosion notch limited to the First and Second chambers?

As noted, the main corrosion notch is found at approximately 2285 m asl, which is also the elevation of the bedrock channel floor in the Kalahroud River gorge, a mere 80-100 m west of the First Chamber. The notch is continuous and deep in the two chambers (Fig. 11). Further back in The Maze there are four examples of merely local corrosion notching, also at ~2285 m asl (Fig. 4).

The limitation of deep main notching to the downstream end of the cave differs sharply from the pattern seen in Alisadr Cave (Hamedan), which Ford and Williams (2007) have cited as an ideal example of corrosion notch cave development in dry regions. At Ghar Alisadr, a high but narrow ridge of tightly folded and steeply dipping Jurassic limestones and schists is surrounded by modern desert fans and alluvium. A perennial spring with very high $P_{CO_2}$ rises through the alluvium a short distance away. Kaufman (2002) proposed that the cave is of hypogene, enriched aqueous $CO_2$ origin, with a deep bedding plane- and joint-guided solutional maze of passages that was discharged over a natural bedrock dam at the outlet. Deep lateral notching at the level of the dam extends throughout the full (~400 m) extent of the tourist cave. The hypogene waters may also have been refreshed by recharge entering overhead and at many different points around the perimeter of the ridge. The notching induced local collapse that left some large breakdown chambers above the waterline.

Fig. 13. Examples of the speleothems in Kalahroud Cave. a) Aragonite needles and frostwork; b) Calcite helictites; c) Calcite flowstone; d) Dust-laden calcite veneer on a wall.
These contain conventional dripstone speleothems. Many sections of the Ghar Alisadr notch are now filled with multiple generations of calcite shelfstones that extend over a height of ~1.5 m, suggesting that the waterline fluctuated within this narrow range for a significant period of time. Erosion of the natural dam then lowered the waterline one meter or more below the notch, where it is now stabilized artificially. The shelfstones are Holocene in age (Kaufman, 2002).

By analogy, in Kalahroud Cave deep corrosion notching was limited to the First and Second chambers (and probably to points downstream of them now inaccessible because of the collapse) because only there could the chemically saturated hypogene waters flowing from the inner cave be refreshed by lateral and overhead recharge from the adjoining gorge. These latter intruding waters were chemically aggressive in their own right and could also give a mixing corrosion boost to the hypogene flow. The creation of the breakdown entrance ramp in the cave is best attributed to this combination of aggressive intruding water and deep corrosion notching that undermined the local bedrock.

Interpreting the precipitates below the main notch

In distinct contrast to the situation at Alisadr Cave, however, no shellstone has been deposited in the main notch in the First and Second chambers of Kalahroud Cave. Instead, below the notch there are (i) widespread deposits of older subaqueous layered or nodular calcites (some with probable Mn-rich bands), plus a few small subaerial stalagmites and flowstones (Figs. 13c, 14a,b): where natural breakage has exposed examples of the subaqueous forms in cross-section they do not appear to contain any calcite raft material. Overlying many of these deposits and burying them in places are (ii) a final stage of calcite raft formation and deposition, including construction of the tower cones (Fig. 15). Many cones terminate at one of two pond levels, the higher of which is two meters below the main notch and ~0.50 meters below what is believed to be the modern maximum flood level.

Our provisional interpretation is that the older subaqueous calcites were precipitated either together with the development of the corrosion notch or immediately after it. During a flood in which autogenic waters from The Maze mixed with allogetic waters from the gorge, the two chambers were first filled to ~2290 m asl, allowing some convectional solution in the ceilings. They drained slowly until arrested by an external dam at 2285 m asl: the waters were still acidic, the main notch was created at this waterline, suspended sediments settled out beneath it and the first calcites may have begun to precipitate from locally denser, saturated solutions descending from it. Following this, a combination of very slow seepage loss below the level of the dam and evaporative loss into the two chambers would have lowered the waterline further and accelerated the rate of calcite deposition – until the next flood came along to repeat the cycle. Our future research will include U series dating of representative samples of the Stage (i) subaqueous calcites.

The dated calcite rafts of Stage (ii) are from two tower cones and the bottom of the sample pit dug in the floor of the First chamber, where it was believed that their accumulation was deepest. It is possible that there are deeper accumulations that might yield slightly greater ages elsewhere in the two chambers. In the sample pit it is seen that the rafts rest on a base of Stage (i) clays and that there are no further clay deposits higher up within them (Fig. 15e). The dates indicate firmly that the raft accumulation began there between 9,000 and 10,000 years ago and that it continues episodically today.

The switch from Stage (i) clay and subaqueous calcite deposition to Stage (ii) no clay and the...
predominance of waterline raft formation, was abrupt. We believe that this may have been caused primarily by the bedrock collapse event beneath the entrance. This greatly reduced or entirely eliminated intrusion by clay-laden waters from the Kalahroud River gorge. Now there was only the a-periodic inflow of autogenic (hypogene) water from the local lifting shafts, plus minor contributions from overhead seepage through the marl cover beds. An alternative explanation is that because Holocene warming increased the air temperature in the cave (our estimate is by five degrees C or more) the evaporation rate increased sufficiently for raft formation to overwhelm the other processes. This possibility does not account for the apparently complete cessation of clay deposition in the pools in the two chambers, however, and thus is considered to be a secondary factor at most.

As noted, in the 70 cm sampling pit the rafts accumulated at a mean rate of about 7.8 cm/1000 years. However, the individual floods and the formation of rafts on their receding waters are a-periodic events, so that mean rates are much generalized representations of what has taken place recently in the cave. An alternative approach is to speculate on the likely number of raft-forming floods required to build the 70 cm accumulation over the last 9000 years or so. The range of individual calcite raft thicknesses is ~1-4 mm in our experience. Allowing for 30% porosity in the 70 cm accumulation this yields between ~500 (maximum) and ~120 (minimum) events, or one every 18 to 72 years on average. This is broadly in accordance with the local report that there has been only one flood through the Kalahroud Gorge during the past 25 years or more. This is crude estimation but, it is hoped, will give speleologists some appreciation of the frequency of formative events in arid regions.

CONCLUSIONS

Kalahroud Cave is a relatively extensive, phreatic/paraphreatic, maze cave in a semi-arid to arid mountainous region near the city of Isfahan. It is formed in Cretaceous limestones and calcareous mudstones that overly siliceous conglomerates and sandstone and are capped by weaker limestone marls. Surface a-periodic stream entrenchment along the strike of the SE-dipping rocks created an east-west cuesta. A larger torrential stream from the north, ‘Kalahroud River’, entrenched a shallow gorge across the cuesta, permitting development of strike-oriented groundwater flow in the limestones, with discharge into the gorge. A joint-guided rectilinear maze of solutional passages developed, fed by hypogene (phreatic lifting) shafts from below. The maze is quasi-horizontal, with a vertical extent of ~30 m that is slightly discordant to the stratal dip. Continued entrenchment of the gorge began to drain the highest parts of the maze but was halted by a stillstand in the channel erosion. In the downstream sector of the cave, mixture of phreatic waters from the maze with occasional floodwaters from the river created deep lateral corrosion notching at the stillstand level. This was succeeded by channel aggradation at the surface and, probably, the beginning of shelfstone and sub-aqueous calcite deposition below the notch. Undermined by the notching, part of the gorge wall collapsed into the downstream end of the cave. Since that event, formation and deposition of calcite rafts has predominated in the a-periodic pondings, the earliest being about 10,000 years BP in age. Raft waterlines have been one-two meters or more below the level of the main corrosion notch, indicating that the karst ground waters are now leaking away downstream of the gorge, presumably into the alluvium that helps sustain the qanats of the village of Kalahroud.
The relationships of gorge and cave enlargement, stillstand and aggradation in the river channel, thick shelfstone and thin raft formation in the cave, to the tectonic and climatic histories of the region will be investigated further in future work.

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Two years of cave monitoring investigate ventilation processes in Cueva Larga, a tropical cave in Puerto Rico. The cave is 1,440 m long with a large main passage (about 120,000 m$^3$). Cave air $pCO_2$ in the main passage varies seasonally, between 600 ppm in winter and 1,800 ppm in summer. The seasonal variability in cave $pCO_2$ permits the estimation of a cave air exchange time of $36 \pm 5$ days and a winter ventilation rate of $3,300 \pm 1,000$ m$^3$/day for the main cave passage. Calculations of virtual temperature and differences between cave and surface temperature indicate that the seasonal temperature cycle is the main driver of the alternation between a well-ventilated winter mode and a near-stagnant summer mode. The winter mode is characterized by a positive buoyancy contrast at night leading to maximal cave ventilation, while cave ventilation is at a minimum during summer. Between winter and summer, a transitional mode of partial cave ventilation is observed. On shorter time scales (diurnal to weekly), cave $pCO_2$ is also influenced by atmospheric pressure but this variation is one order of magnitude lower than the seasonal $pCO_2$ change. The cave morphology of Cueva Larga including its large volume, tubular shape and the obstructed cave entrance geometry are important boundary conditions for the observed ventilation patterns. Our findings emphasize that cave systems with varying morphology have to be studied individually in order to correctly describe ventilation processes.

Keywords: environmental monitoring, cave ventilation, carbon dioxide, virtual temperature, air exchange time

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INTRODUCTION

Monitoring of cave environments is necessary to improve understanding of speleothem growth processes (Fairchild et al., 2007; Fairchild & Baker, 2012), improve paleo-climatic reconstructions (James et al., 2015), and enhanced archeological site conservation (Fernández et al., 1986). Deep cave environments exhibit near-constant temperatures over annual and diurnal cycles and relative humidity values close to 100% (Lachniet, 2009; Fairchild & Baker, 2012). Speleothems growing in these conditions make ideal climate archives because kinetic isotope effects and annual variations in the temperature dependent fractionation factor are considered to be extremely low (Kim & O’Neil, 1997; Mickler et al., 2006; Mattey et al., 2010; Deininger et al., 2012).

Variations in CO$_2$ partial pressure ($pCO_2$) of the cave atmosphere (Boch & Spötl, 2008; Baker et al., 2014) can affect commonly used paleo-climatic proxies of speleothems e.g., growth rates, stable isotope ratios and trace element ratios (Dreybrodt, 1999; Wong et al., 2011; Deininger et al., 2012; Breitenbach et al., 2015). Thus, it is valuable to investigate cave ventilation patterns prior to analytical work and speleothem paleoclimate interpretation (James et al., 2015). This study helps to improve the paleoclimatic interpretation of tropical speleothems by investigating the connection between atmospheric changes outside the cave and the cave interior.

Cave air ventilation processes are affected by regional differences, such as latitude (James et al., 2015) and altitude (Mattey et al., 2016). In addition, cave morphologies interact in a complex manner with...
physical drivers such as variations in atmospheric temperature, air pressure and wind flow (Breitenbach et al., 2015; Mattey et al., 2016). On short time scales from hours to a few days local changes in atmospheric air pressure (e.g., during tropical low pressure systems, or via changes of wind speed and/or direction at the cave entrances) are known to have an effect on cave ventilation (Baldini et al., 2006; Cowan et al., 2013; Breitenbach et al., 2015; Ridley et al., 2015; Mattey et al., 2016). During the passing of low pressure systems cave air expands and leaves the cave, when normal pressure conditions are reached again atmospheric air is forced into the cave. Wind field changes are linked to cave air movements via the Venturi effect causing underground pressure changes (Kowalczk & Froelich, 2010; Breecker et al., 2012). We discuss changes in the temperature difference ($dT$) between the temperature inside and outside the cave and compare $dT$ to virtual temperature ($T_v$) which combines the effects of elevated relative humidity and $pCO_2$ levels to air buoyancy (Sánchez-Cañete et al., 2013). Cave monitoring took place over two years at Cueva Larga, including atmospheric $pCO_2$, temperature ($T$) and relative humidity ($RH$) both inside and outside the cave. The monthly observations detect a seasonal cycle in $pCO_2$ which allows us to estimate cave ventilation rates and to calculate $dT$ and $T_v$.

**SITE DESCRIPTION**

Cueva Larga (CL; Fig. 1), is located in an area characterized by sinkholes and mogotes 350 m above mean sea level (amsl) in the north central karst region of Puerto Rico in the northeastern Caribbean (N 18°19' W 66°48'). The area above the cave is covered by thick tropical forest with thin soil cover that is nearly absent on the higher elevated and exposed locations. The cave is dominantly a vadose cave with some phreatic features. It is located in the massive dense Oligocene Lares Limestone (Giusti, 1978; Monroe, 1980). The entrance of CL is located along an inclined surface belonging to a sinkhole at the lower edge of a small hill. CL has a horizontal extension of about 1,440 m from west to east (Miller, 2010) and a single entrance which is formed like a narrow vertical pit whereas the main passage has a tubular morphology with ceiling heights of up to 30 m. The entrance area and the main chamber are separated by two U-shaped obstacles along the cave ceiling and one depression on the cave floor (Fig. 1B). The cave ends in the Collapse Room, a chamber where the roof has collapsed. It is separated from the main chamber by a rise in the cave passage floor. The Collapse Room is subdivided in a small lower level passage and a large upper level passage which reconnect at the end of the cave (Fig. 1 insert 3). Due to the remote location recreational cave visits are limited and therefore CL is well suited for studying natural cave ventilation processes.

**METHODS**

**Weather observation**

A Vaisala WXT 520 weather sensor connected to a Campbell Scientific 200 data logger was placed in November 2012 on top of a mogote, directly over CL, at a height of 405 m amsl. Vegetation surrounding the
Cave monitoring

Five cave monitoring sites inside CL were visited at near monthly intervals (Fig. 1). Site A is located in the entrance of CL (0 m to cave entrance) below a cave-ceiling-overhang and a height of 60 cm to the floor, sites B, C, D and E are inside CL with a distances to the cave entrance of about 50 m, 160 m, 250 m, and 425 m, respectively. The height above the cave floor varied among sites between 0.6 to 1.5 m (site B-1.5 m, C-1.3 m, D-0.8 m and E-0.6 m). The sites are located near the lowest part of the main cave passage except site D which is located at a climb along the cave wall with a distance of about 10 m to the floor of the main cave passage. Air pressure measurements during 5 individual field trips show that the absolute vertical height of each station does not exceed a difference of more than 12 m to the cave entrance. The individual measurements inside CL were taken at the same location during each visit.

Cave parameters (pCO₂, T and RH) were measured at each site during each monthly cave visit. An Amprobe CO₂-100 handheld carbon dioxide meter (accuracy of ± (30 ppm + 5% of reading) for pCO₂ between 0 and 5,000 ppm; ± 0.6°C for T and ± 5% for RH above 90%) was employed from January 2013 to July 2013, while from July 2013 to January 2015 we used a handheld Vaisala GM 70 with a 2,000 ppm CO₂ probe (accuracy of ± (30 ppm + 2% of reading) for pCO₂ between 0 and 2,000 ppm) and a HM70 humidity and temperature probe (accuracy of ± 0.2°C for T and ± 1.7% for RH above 90%). During two cave visits both devices collected data at the same time. The measurement...
results of both devices were in agreement with each other considering their accuracy ranges.

To detect daily pCO$_2$ variability in the cave a CM-0018 data logger from CO$_2$ Meter Inc. (accuracy of ± (30 ppm + 2% of reading) for pCO$_2$ and 0.4°C for T) was placed at site E from 4 November 2013 to 29 November 2013 and recorded the pCO$_2$ and T value in hourly intervals. The data logger was kept in a breathable nylon bag together with a moisture absorber to prevent condensation inside the data logger.

Virtual temperature calculation

Virtual temperature ($T_v$) is the temperature that dry air must reach to have the same density as moist air (Sánchez-Cañete et al., 2013). Thus, $T_v$ is used to determine buoyancy contrasts between two air masses with different humidity contents. The air mass with the higher $T_v$ has higher buoyancy. Kowalski and Sánchez-Cañete (2010; Equation 1) expanded the definition of $T_v$ by including the effect of high CO$_2$ values. This is important in cave environments, where CO$_2$ values are up to several orders of magnitudes higher than in the atmosphere (Kowalski & Sánchez-Cañete, 2010; Sánchez-Cañete et al., 2013).

$$T_v = T \times (1 + 0.6079r_v - 0.3419r_v)$$  (1)

where: $T$ = temperature in degrees Celsius, $r_v$ = water vapor mixing ratio, and $r_v$ = carbon dioxide mixing ratio.

We calculated $T_v$ outside and inside the cave to investigate buoyancy differences between cave and outside atmosphere using the excel template provided by Sánchez-Cañete et al. (2013). Required input parameters were $RH$, pCO$_2$, and $T$. For the cave air $T_v$ calculation $RH$ was set to 100% as suggested by Sánchez-Cañete et al. (2013) and indicated by the field observations (Fig. 2C). Choosing any $RH$ value above 90% changes $T_v$ by only up to 0.7% resulting in a negligible change on the calculated outcome of $T_v$ inside the cave. The range of RH at the weather station has a small influence on the $T_v$ calculation as $T_v$ changes by less than 3% when the maximum and minimum $RH$ values are used.

The annual cycle in outside air $T_v$ was calculated using the CL weather station daily average $T$ and $RH$. No $RH$ data was available from the AO weather station to fill the gaps of the CL station. Instead we used the average daily $RH$ value of 82 ± 5% (SD) from the CL weather station data. This is reasonable because the data do not show a seasonal cycle (Fig. 2C). We used a value of 400 ppm pCO$_2$ to calculate outside $T_v$ because our measurements at the weather station above the forest agree with 400 ppm which is within the accuracy range of the device and this is also the global atmospheric value (Tans & Keeling, 2014). More precise pCO$_2$ measurements could allow for the detection of a seasonal pCO$_2$ cycle, for example a seasonal variation of 8 ppm is observed at the Mauna Loa observatory (2013/14). This seasonal pCO$_2$ variation is negligible in the outside $T_v$ calculation because a pCO$_2$ change of 8 ppm causes a variation of 0.005% in outside $T_v$. To calculate diurnal outside $T_v$ variations, we used the 10 minute data recorded at the CL weather station for each day that the cave was visited. At cave site E, $T_v$ was calculated using $T$ and pCO$_2$ that were measured manually at this site on the days the site was visited and during November 2013 the data logger observations were used.

RESULTS

Atmospheric changes at the weather station and inside Cueva Larga

The mean annual temperature (MAT) measured over two years at the CL weather station was 22.5 ± 0.1°C (22.5°C between November 2012 and November 2013 and 22.4°C between November 2013 and November 2014; including adjusted AO data to fill the gaps of the CL weather station). The daily average temperatures at the CL weather station ranged from a minimum of 17.5°C (25 December 2013) to a maximum of 27.2°C (1 October 2013). The annual range of daily average temperatures at the CL weather station was 9.7°C. Differences between daily minimum and maximum temperatures reach up to 15°C.

Monitoring sites near the cave entrance (A, B and C) exhibit variations in temperature exceeding 2°C. At site D (240 m from the cave entrance) and site E (425 m from the cave entrance) the temperature variations were smaller than 1°C. The mean temperature at site E (22.5 ± 0.2°C) is indistinguishable from the MAT (22.5°C) measured outside the cave (Fig. 3). Outside temperature variations and possibly changes in the extension of thermal different air masses inside the entrance area appear to influence the first 150 m of the cave. No pronounced temperature variability is detected at distances greater than 240 m from the cave entrance.

Fig. 4A shows the seasonal variation in daily average temperatures at the CL weather station compared to the temperature recorded inside the cave at site E. The point measurements of temperature at site E are always close to the outside MAT (22.5°C) with a deviation not greater than 0.4°C (22.2 to 22.9°C).

![Fig. 3. Temperature observations in Cueva Larga. Points mark the average, minimum and maximum T measurements at the monitored sites. Error bars of the average T represent the standard deviation. The black line represents the MAT outside the cave.](image-url)
Relative humidity inside CL ranges between 82.2% and 100% (Fig. 2C). Values below 90% are encountered up to 150 m inside the cave (Site C). At greater distances to the cave entrance the measurements are indistinguishable from 100% considering the measurement error. At the distant site E the minimum value is 92.7%, the maximum is 100% and the average value is 99.0%.

Monthly \( pCO_2 \) measurements recorded in CL (Sites B, C, D, and E) throughout the year (Fig. 4B) are in phase with the annual temperature cycle outside the cave (Fig. 4A). In CL’s main passage the highest \( pCO_2 \) values (1,600 to 1,850 ppm) occur during the warmer summer months between July and the beginning of September. Unlike temperature, the annual \( pCO_2 \) pattern is not symmetrical. The \( pCO_2 \) values show a gradual rise from May to August and a rapid decrease in September. The lowest \( pCO_2 \) values are observed from December to March. At station A the \( pCO_2 \) pattern is similar with a less pronounced summer maxima and mostly lower \( pCO_2 \) values (1,000 ppm in summer and 400 ppm in winter). At the cave entrance (station A) outside atmosphere dominates during the winter when it seeps into the cave. During the summer higher values inside the cave are accompanied by high values at the entrance linked to the slow dispersion of cave air during the summer when ventilation is low or absent. Since November 2013 the upper passage of the Collapse Room at the end of the cave has been visited monthly as well. Here the \( pCO_2 \) values show the same seasonality as in the main passage and are elevated reaching about 900 ppm in winter and up to 2,200 ppm in summer (Fig. 4B). The highest \( pCO_2 \) values have been measured in the smaller and lower passage below the main passage in the Collapse Room. Infrequent visits indicate that the \( pCO_2 \) values seem to range between 2,300 and 3,600 ppm.

Reconstructing ventilation patterns via temperature difference \( dT \) and calculation of \( T_v \)

The temperature difference between cave and outside temperature \( dT \) is a commonly used indicator for buoyancy differences between cave and outside air (Fairchild & Baker, 2012; Breitenbach et al., 2015; Ridley et al., 2015). A more precise measure of buoyancy is virtual temperature \( T_v \) because \( T_v \) includes the effect of variations in the primary molecular composition of each air mass to their buoyancy. Figure 5 shows the monitoring results from 2013 and 2014, including the seasonal \( pCO_2 \) variation at site E, daily average barometric pressure, \( dT \), \( T_v \) for cave site E and \( T_v \) for the external atmosphere.

The temperature difference \( dT \) was calculated between the cave temperature at site E (~22.5°C) and
the daily average outside temperature. Negative values occurred between October/November to May/June reaching as low as −3.6°C. Positive values dominated during the rest of the year reaching values as high as 5.3°C.

Virtual temperature $T_v$ was calculated for inside and outside the cave (Equation 1). Outside the cave $T_v$ range was calculated at 10°C for the daily average data collected at the weather station. Minimum $T_v$ and maximum $T_v$ were calculated at 19.0°C on 21 December 2013 (17.2°C, 82% RH and 400 ppm $pCO_2$) and 29.0°C on 20 September 2013 (25.8°C, 82% RH and 400 ppm $pCO_2$), respectively. Cave $T_v$ was calculated for site E because it is the most representative site for undisturbed cave conditions inside the main passage due to the distance to the cave entrance and nearly invariant temperature. At site E, $T_v$ ranges only between 25.2 to 25.8°C (Fig. 5) despite the seasonal $pCO_2$ cycle. Using constant values of $T = 22.5°C$ and $RH = 100%$ the influence of the $pCO_2$ variations on $T_v$ inside the cave was calculated to account for only 0.2°C $T_v$ for the monitored period. The annual outside temperature cycle highly influenced the outside $T_v$ results (Fig. 5).

Outside’s air buoyancy (represented by $T_v$) is greater than the buoyancy inside the cave when temperatures outside the cave are larger than inside the cave (represented by a negative $dT$).

To estimate the daily and monthly variability in $T_v$ we used the hourly $T$ and $pCO_2$ measurements of the $CO_2$ data logger at site E, which are available from 4 November 2013 to 29 November 2013. Fig. 6 shows the calculated $T_v$ for measurements at Site E and at the weather station during 17 November 2013 and 18 November 2013. $T_v$ outside the cave is strongly dependent on the diurnal $T$ cycle while the $T_v$ inside the cave is invariant. During night-time atmospheric $T_v$ is below cave $T_v$ creating favorable conditions for more buoyant cave air to exit the cave. Despite possible night time ventilation, no significant variation in $pCO_2$ has been recorded at the distant cave site E. This suggests daily atmospheric temperature variations do not lead to noticeable ventilation in the main cave passage distant from the cave entrance because the cave air exchange time needed to effectively ventilate the cave is likely longer. During the 25 days of hourly observation the $pCO_2$ inside the cave varies over a range of 200 ppm on a multi-diurnal to weekly time frame. This short-term variability is not co-occurring with changes in $dT$, but it occurs together with changes in atmospheric pressure. At low (high) pressure high (low) $pCO_2$ values occurred at cave site E. The long term observations (Fig. 5) show no seasonal cycle in atmospheric pressure and no co-variation between atmospheric pressure and long term $pCO_2$.

Seasonal changes in diurnal buoyancy contrast were estimated by calculating the $T_v$ contrast between the cave and outside ($T_v$ Cave - $T_v$ Outside) for twelve individual days from which actual field observations were available (Fig. 7). Positive buoyancy contrast (blue shading) indicates that there should be a high tendency for more buoyant cave air to leave the cave and to be replaced by less buoyant outside air. Negative buoyancy contrast (red shading), on the other hand, means that there is a high tendency of cave air to remain inside the cave. Our results can be grouped into three buoyancy contrast modes: winter (column 1), transition (column 2) and summer (column 3).

The winter mode is characterized by a positive buoyancy contrast exceeding +5°C in $T_v$ during the night. A positive gradient of several degrees lasting up to 18 hours per day seems favorable for convective air exchange between the cave interior and exterior (winter mode). This likely generates the low $pCO_2$ values measured in winter (Fig. 4). During the summer no marked positive buoyancy contrasts exists. Even during night hours the air outside the cave is occasionally more buoyant than inside and free convective air exchange is limited. The main differences between winter and summer mode are represented in insert 2 in Fig. 1 where arrows represent ventilation generated by more buoyant cave air leaving the cave during winter and the more stagnant...
Fig. 6. Results of successful CO$_2$ data logger deployment at site E. Top shows the observations during two days (17 to 18 November 2013). Bottom shows the complete deployment period (5 to 29 November 2013). The hourly log of pCO$_2$ (red) is compared to the average atmospheric pressure per hour (green), the temperature difference between cave and outside $dT$ (black, the dashed line marks $dT = 0$) and the calculated $T_v$ inside the cave (brown) and outside the cave (blue).
summer conditions when highest $pCO_2$ values are represented by no air motion. The transition mode is characterized by a switch between diurnal dominant negative buoyancy contrast to positive buoyancy contrasts (favorable for ventilation) at night and negative (stagnant ventilation) during the day. Here the positive buoyancy contrasts does not exceed $+5^\circ$C. Intermediate $pCO_2$ values are measured during the transition mode inside CL.

Cave air exchange time and ventilation rates

Monthly monitoring data allows estimating cave air exchange times and ventilation rates for CL. To do so, the dynamic CL ventilation pattern was simplified to a linear time invariant system. In this system the cave atmosphere responds to step changes in cave air ventilation rate from no ventilation in summer (ventilation rate = 0) to maximum ventilation in winter. The resulting ventilation rate is a minimum estimate value because it may be possible that some ventilation may occur during the summer. In this case, the ventilation rate would be greater than our estimate. Two assumptions have been made: 1) The CO$_2$-input is constant throughout the year; and 2) cave ventilation with the outside air is the only way to remove CO$_2$ from the cave. The change in seasonal ventilation patterns can be described by an exponential decrease during fall/winter in cave atmosphere $pCO_2$:

$$pCO_2(t) = (pCO_2^i - pCO_2^f) \times e^{-t/\tau} + pCO_2^f$$

Winter $pCO_2$ Fall:

where: $pCO_2(t)$ = partial pressure of CO$_2$ at time $t$, $pCO_2^i$ = initial partial pressure of CO$_2$ in ppm, $pCO_2^f$ = final partial pressure of CO$_2$ in ppm, $\tau$ = system time constant in days, and $t$ = time in days.

The system time constant $\tau$ (Equation 2) corresponds to the cave air exchange time (Frisia et al., 2011). It was calculated via a non-linear least-square fit model by minimizing the sum of squared residuals for all $pCO_2$ measurements from 2013 and 2014, taking the individual measurement errors into account (Fig. 8). To compare the result, the fit was also performed for each single season and year, respectively (Table 1). The start of the winter fall in the CO$_2$ curve was set to the last maximum measurement before the exponential decline = 1,880 ppm (04-Sept-2014) and the end of the exponential decline was set to the lowest measured value of = 620 ppm (06-Jan-2014). For seasons of the single years, the initial and final values where chosen in an analogous manner. For a better overview the results of
all fits are shown in Table 1. The resulting values for cave air exchange time during the winter ventilation season is \( t_{\text{winter}} \approx 36 \pm 5 \) days. Fig. 8 shows the resulting fit curves for all cases. Our cave air exchange time agrees with the cave air exchange time estimates for each single year within the range of the standard error, respectively (Table 1), ensuring that our estimation does not vary among years. The performed least square estimation of the cave air exchange time is well constrained because all measurements with one exception fall within the 1 MSE confidence level (Fig. 8).

Table 1. Results of the nonlinear least square fit of \( pCO_2 \) measurements for different time periods in Cueva Larga to estimate the cave air exchange time \( \tau \). Errors are calculated as mean squared error of the residuals (MSE) for the fit curve \( pCO_2(t) \) and as standard error (SE) for the estimated parameter \( \tau \).

<table>
<thead>
<tr>
<th>Year</th>
<th>( pCO_2 ) (ppm)</th>
<th>( pCO_2 ) (ppm)</th>
<th>( \tau ) (days)</th>
<th>SE (days)</th>
<th>MSE (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2013/14</td>
<td>1880 (18-Aug-2013)</td>
<td>620 (06-Jan-2014)</td>
<td>36.87</td>
<td>6.03</td>
<td>116.7</td>
</tr>
<tr>
<td>2014/15</td>
<td>1880 (04-Sep-2014)</td>
<td>620 (19-Apr-2015)</td>
<td>35.18</td>
<td>8.25</td>
<td>122.6</td>
</tr>
<tr>
<td>Both years</td>
<td>1880 (18-Aug-2013)</td>
<td>620 (6-Jan-2014)</td>
<td>35.56</td>
<td>4.41</td>
<td>103.0</td>
</tr>
</tbody>
</table>

Using an estimation of the cave volume based on survey data from Miller (2010) allows a rough calculation of the ventilation rate during winter. The volume is approximately 120,000 m³ with a conservative error estimation of 20%. Dividing the cave volume by the cave air exchange time \( (36 \pm 5 \) days) results in a cave ventilation rate of 3,300 ± 1,000 m³/day.

**DISCUSSION**

Two years of monitoring provide evidence for cave ventilation conditions during winter and near-stagnant ventilation conditions during summer in CL, Puerto Rico. The seasonal ventilation changes are accompanied by high maximum \( pCO_2 \) values in late summer and minimum \( pCO_2 \) values in the winter. When air buoyancy is higher in the cave than outside the cave (positive \( dT \); greater cave \( T_a \)) air convection (cave ventilation) is induced and cave air moves from the cave towards the outside and is replaced by less buoyant outside air with “low” atmospheric \( pCO_2 \) concentration. Conversely, when buoyancy is lower in the cave than outside the cave (negative \( dT \); lower cave \( T_a \)), the air column is stable and cave ventilation is at a minimum due to stagnation of temperature driven ventilation. Without temperature driven ventilation surface pressure variations can cause minimal ventilation (Baldini et al., 2006) which in the case of CL does not match the \( CO_2 \) input and \( CO_2 \) accumulates inside the cave (Fig. 5).

The seasonal temperature change outside CL appears to be the principal controller on the observed annual \( pCO_2 \) cycle. Outside the cave \( T_a \) follows the seasonal temperature cycle and its range is an order of magnitude greater than in the cave. In CL, \( dT \) and \( T_a \) calculations show the same seasonal and diurnal buoyancy changes between cave and outside. At this site \( dT \) calculations are valid estimation of buoyancy differences between cave and outside air despite the fact that \( dT \) does not take variations of \( pCO_2 \) and RH into account. The buoyancy effect of seasonal \( pCO_2 \) variations in CL does not oppose the seasonal \( dT \) cycle, because the summer \( pCO_2 \) maxima inside CL, similar as the higher summer temperature outside CL, decreases the cave air’s buoyancy compared to the buoyancy of outside air. Significant deviations between \( T_a \) and \( dT \) buoyancy examinations are expected at locations with temporal different cave \( pCO_2 \) variations and where cave \( pCO_2 \) reaches higher values than in CL (Sánchez-Cañete et al., 2013).

Our method to collect point measurements inside CL once every month to investigate seasonal changes inside the cave appears appropriate because the \( pCO_2 \) logging data does not show any diurnal \( pCO_2 \) cycle (Fig. 6). The hourly logging observations were taken in November of 2013 during the transition from summer to winter ventilation mode. If a diurnal \( pCO_2 \) cycle did occur in the main chamber in CL,
it would be expected to be especially pronounced during the transition ventilation mode because the shift towards the well ventilated winter mode would occur predominantly during the night when unstable density conditions between cave and outside air favor air exchange. During this time no diurnal variation in \( p\text{CO}_2 \) is detected, indicating that the main chamber is ventilated via a slower air exchange process filtering out the diurnal change from stable (day) to unstable (night) air density gradient. Thus, our quasi-monthly point measurements represent a time period of at least one day.

A model study by James et al. (2015) predicts little or no seasonal changes in ventilation and cave atmosphere \( p\text{CO}_2 \) for tropical sites. In contrast, CL exhibits a seasonal cave atmosphere \( \text{CO}_2 \) cycle similar in magnitude to observations from temperate regions (Frisia et al., 2011; Spötl et al., 2005). Other caves from higher latitudes with similar ventilation systematics but different \( \text{CO}_2 \) amplitudes have been observed such as in Austria (Boch & Spötl, 2008), Ireland (Baldini et al., 2008), France (Bourges et al., 2006), Arizona, USA (Buecher, 1999), Texas, USA (Cowan et al., 2013) and Germany (Meisner et al., 2010). Yok Balum Cave in tropical Belize also shows seasonal ventilation differences (Ridley et al., 2015). Similar as in CL, low \( p\text{CO}_2 \) values occur in Yok Balum during the winter where ventilation is more continuous than during the summer where diurnal variations cause \( p\text{CO}_2 \) values of about 700 ppm. Unlike CL, Yok Balum Cave has two entrances at different altitudes preventing ventilation stagnation despite the on average higher outside temperatures during the summer due to density driven flow through the cave (Ridley et al., 2015). These two tropical sites show that seasonal or higher resolution field observations are inevitable for a correct site description and prediction of cave ventilation processes.

Other types of \( p\text{CO}_2 \) systematics have also been documented. A very dynamic ventilated cave with diurnal and seasonal ventilation patterns is Mawmluh Cave in northeast India (Breitenbach et al., 2015). Stable \( p\text{CO}_2 \) conditions exist in Castañar de Ibor Cave, Spain (Fernandez-Cortes et al., 2009) and in Bunker Cave, Germany (Riechelmann et al., 2011) which has constant ventilation during the whole year likely caused by two cave entrances at different altitudes. Caves in Gibraltar, near the sea level such as Ragged Staff Cave show a similar seasonal \( p\text{CO}_2 \) cycle as in CL and elevated caves above sea level such as St. Michaels Cave show an opposite \( p\text{CO}_2 \) seasonality where maxima occur during the winter (Mattey et al., 2010, 2016). Similar observations come from Candamo Cave, Spain (Hoyos et al., 1998). The host rock of both locations has high porosity allowing air from the surface to reach the cave during the summer; this is not the case in the dense Lares Limestone hosting CL. Here, the air exchange with external air is restricted to the single cave entrance.

Numerous examples indicate that cave geometry is an important controlling factor on cave ventilation patterns (Geiger, 1961; Pflitsch & Piasecki, 2003; Fairchild & Baker, 2012; Gregorić et al., 2013). Diurnal variations in temperature and \( p\text{CO}_2 \) have been observed in other caves such as Ballynamintra Cave, Ireland (Baldini et al., 2008) and caves in central Texas (Cowan et al., 2013). In Ballynamintra Cave, diurnal \( p\text{CO}_2 \) changes of up to 1,200 ppm have been observed. It has two entrances, located at the upper and lower cave level, and is significantly smaller than CL. This geometry seems to be responsible for the strong diurnal ventilation changes and near atmospheric \( p\text{CO}_2 \) concentrations during the night. Buoyant cave air is likely to escape the cave through the upper entrance during the night while less buoyant air flows into the lower entrance inducing a rapid air stream through the cave. This is not the case in CL. In tropical settings, as CL, the seasonal variation in daily atmospheric average temperatures at the surface is smaller than the daily temperature cycle between day and night (Tahirule-Lips & Ford, 1998). Although we only have hourly \( p\text{CO}_2 \) measurements for 25 days, there is no indication that the \( p\text{CO}_2 \) in CL’s main passage responds to the daily temperature cycle because additionally to the hourly measurements in November 2013 there is no spatial variation on \( p\text{CO}_2 \) throughout the main chamber at any point in time. Thus, we deduce that ventilation processes are driven primarily by seasonal differences between buoyancy inside and outside the cave as we have shown for the monthly observations. It is likely that the particular cave geometry of CL prevents pronounced diurnal cave ventilation. Fig. 1 (insert 3) shows CL’s vertical cross-section. Two ”U” shaped structures are visible at the cave roof about 150 m into the cave between the entrance and site C (Fig. 1). Probably fast air movement is restricted due to the obstacles in the cave’s entrance similar to observations by Baldini et al. (2006). In other caves, evidence exists for daytime ventilation even during the summer (e.g., Cowan et al., 2013; Breitenbach et al., 2015), while in CL the obstructed entrance and the lower summer cave air’s buoyancy create a calm and stable air-mass in the entrance. We confirmed this on a summer day in 2016 via vertical temperature measurements (constant temperature) and a free falling plastic foil, which showed no horizontal drift while falling through the air column. Effective exchange between cave and outside air in CL would require overcoming the ”U” shaped obstacles. This seems to occur predominantly during winter when the air buoyancy in the cave is greater than outside. Especially during winter nights the buoyancy contrast between cave and outside air is positive and cave air moves more easily through the ”U” shaped flow path out of the cave. The lowest \( p\text{CO}_2 \) value measured in CL was 580 ppm. Thus under current climate conditions the cave never becomes completely flushed with atmospheric air (~400 ppm). This might have been different during the geological past. If the temperature contrast between cave and outside had been greater than today, especially during the well ventilated winter season, more intense ventilation than at present could occur. Such a scenario may have been possible during cold events where temperatures have been shown to decrease by about 4°C in the tropics (Arienzo et
Higher than atmospheric winter $pCO_2$ values seem to be linked to the cave morphology of CL. The single entrance and the “U” shaped roof structures trap air inside the cave and hinder rapid air exchange under present climate conditions. Note that the buoyancy contrast curve for 30 September 2013 (Fig. 7) shows only negative values, but the $pCO_2$ value inside the cave has already reached an intermediate value indicative of some cave ventilation (Fig. 5). This observation is most likely linked to colder weather periods before the field trip causing a short term increase in cave ventilation. The monitoring in 2014 shows similar observations. The $pCO_2$ rise ceases in July 2014 where mean outside $T$ were similar to cave $T$, possibly allowing night time ventilation. The onset of the 2014 decrease in $pCO_2$ also takes place in September similar to 2013. Several consecutive days in September have lower mean outside $T$ values than cave $T$, values, likely promoting cave ventilation and the onset of the transition mode. Thus our estimation of cave air exchange time is a maximum estimate since weather phenomena lasting several days seem to affect the ventilation system. More frequent observations are needed to get a more robust cave air exchange estimate.

In the main passage no spatial variation in cave atmosphere CO$_2$ concentration was observed as documented for other caves (Baldini et al., 2006; Cowan et al., 2013). The lack of diurnal and spatial $pCO_2$ variations are linked to the long cave air exchange time of about 36 ± 5 days because diurnal ventilation processes are dampened inside the large main passage of CL leading to a well-mixed and homogeneous cave atmosphere. A simple measurement of vertical temperature profiles during a summer day in 2016 near the entrance and at the end of the main passage revealed no significant temperature variations. This strengthens the interpretation of a homogeneous cave atmosphere. Cave air exchange time of similar magnitude (54 days) and air exchange rate (2,400 m$^3$/day compared to 3,300 m$^3$/day in CL) have been estimated for the Throne Room in the Karchner Caverns (Buecher, 1999). In contrast, smaller caves seem to have faster exchange times. Cave chambers of flank margin caves on nearby Mona Island are estimated to have air exchange times of less than one hour (Vieten et al., 2016). These caves have several openings towards the island’s cliff and sky lights which promote fast air exchange. Another smaller cave than CL is Grotto di Ernesto (about 13,000 m$^3$) which has an estimated exchange time of 1.4 days (Frisia et al., 2011). The large volume of CL, its tubular shape and the obstructed cave entrance geometry seem to be the key parameters for the observed seasonal ventilation pattern with well-mixed cave atmosphere. To understand the effect of the obstructed cave geometry in more detail higher spatial monitoring in the entrance zone should be conducted to investigate also the possible formation of distinct thermal zones, obstructing the ventilation process.

This study did not investigate soil CO$_2$ production. Baldini et al. (2008) found evidence for soil-temperature induced changes in soil $pCO_2$ production driving seasonal cave air $pCO_2$ variations at a temperate location in Ireland. Changes in $pCO_2$ production in the soil could be an addition to the observed seasonal $pCO_2$ pattern, but seem to be of minor importance compared to changes in cave ventilation at this tropical site. Similar conclusions have been made at numerous other sites (De Freitas et al., 1982; Pfiftsch & Piasecki, 2003; Spötl et al., 2005; Kowalczk & Froelich, 2010; Cowan et al., 2013; Gregorčič et al., 2013). Mattey et al. (2016) found that decay of organic matter washed down into the unsaturated zone appears to be a $CO_2$ source of similar importance, which is likely to be annually constant. Drip site studies in CL (Vieten et al., accepted) show that variations in element concentration in the drip water appear constant, implying that the $CO_2$ input remains constant throughout the year. Additional support for our assertion of seasonal temperature variations controlling cave ventilation is the strong drop in cave air $pCO_2$ from about 1,900 ppm to about 600 ppm during the summer-winter transition because increased ventilation transports atmospheric, low $pCO_2$ air into the cave. During the winter-summer transition the gradient between cave and atmospheric air is small compared to the summer-winter transition, thus the decreasing ventilation is accompanied by a less rapid increase in $pCO_2$.

Mammals and aerobic bacteria are another source of CO$_2$ inside CL, besides the CO$_2$ input from drip water degassing into the cave atmosphere. Their CO$_2$-producing activities include respiration and the decay of fecal and biological matter introduced into the cave. Bats and rats live in CL and their population size is approximated to range between 200 and 500 individuals. We consider their influence to the seasonal $pCO_2$ cycle as an insignificant contribution because their population size does not vary seasonal at this site and estimating the input of CO$_2$ by respiration of bats via the field metabolic rate (FMR) and the energy balance shows that a few hundred thousand bats should live during the summer in the cave to explain an annual CO$_2$ increase of more than 1,200 ppm during transition from winter to summer (Neuweiler, 2000).

The highest $pCO_2$ values are measured in the Collapse Room at the end of the cave. This is not surprising because usually CO$_2$ accumulates with distance from the cave entrance (Baldini et al., 2006; Cowan et al., 2013). In CL the ventilation of the Collapse Room seems to be less effective compared to the main passage because an elevation on the cave floor (Fig. 1) divides the Collapse Room from the main
passage. Maximal $pCO_2$ values are reached in the lower passage of the Collapse Room which suggests that it’s small geometry at the cave end forms a calmer air mass with less effective ventilation as in the upper Collapse Room and the main passages of CL, similar to other caves (Baldini et al., 2006; Badino, 2009).

Atmospheric pressure variations have an influence on the $pCO_2$ concentration at site E on timescales of days to weeks. Over the logging period we observed a $CO_2$ variability of up to 200 ppm related to atmospheric pressure changes. The hourly $pCO_2$ log from November 2013 (Fig. 6) shows that low atmospheric pressure systems are accompanied by high $pCO_2$ values at site E and vice versa. Similar as in Ballynamintra Cave, Ireland (Baldini et al., 2006), this observation appears to be relate to the air mass with high $pCO_2$ values at the end of the cave in the Collapse Room. When the atmospheric pressure decreases, the air in the back of the cave expands into the main cave passage where it increases the $CO_2$ concentration. In addition, the low pressure might pull out air from fractures and fissures which has high $pCO_2$ values. While a high pressure system provides additional cave ventilation by pushing atmospheric, low $pCO_2$, air into the cave which lowers the cave atmosphere $pCO_2$. In Castañar de Ibor Cave a major atmospheric low pressure event lowered cave $pCO_2$ via increasing cave ventilation (Fernandez-Cortes et al., 2009), such a major event did not occur over the monitored time frame in CL. Similar observations come from another site in Spain, Candamo Cave, where low atmospheric pressure systems during the winter are related to lower cave $pCO_2$ (Hoyos et al., 1998). The long term observations (Fig. 5) do not show a relationship between the seasonal $pCO_2$ cycle and atmospheric pressure, thus the primary driver of long term cave ventilation is the seasonal temperature change outside the cave. On a daily time scale (Fig. 6) a semidiurnal oscillation appears in the hourly $CO_2$ log, but its amplitude is smaller than the logger’s accuracy. This signal appears to be authentic, because it shows a similar anti-correlation to the atmospheric tides (Lindzen, 1979) as the monthly $CO_2$-log does to the atmospheric pressure systems, discussed above. Evidence for atmospheric tides influencing cave temperatures has been found in caves in France (Bourges et al., 2006). In the Roseland Tunnel in the French Alps (Richon et al., 2009), atmospheric tidal signal is evident in the underground radon-concentration and in drip water flow rates, similar to Père Noël cave in Belgium (Genty & Deflandre, 1998), but at these locations no related variation in the underground $CO_2$ concentrations were documented.

Speleothems are frequently used as climate archives (Wang et al., 2001; Lachniet et al., 2004, Winter et al., 2011). Cave monitoring has important implications for the robust interpretation of speleothem climate records. In general, cave temperatures are assumed to be constant over the year and equal to the MAT outside the cave (Fairchild et al., 2006). It is well known that seasonal and diurnal temperature variations are encountered near the cave entrances. Temperature measurements in CL show considerably temperatures variation (> 2°C) near the cave entrance which can cause noise in the speleothem proxy record of up to ~0.23%/°C (Lachniet, 2009). Annual constant temperatures exist at a distance greater than 200 m from the entrance (Fig. 2). In CL and in similar settings speleothems should be taken at larger distances than 200 m of the cave entrances, if they cannot be analyzed at seasonal or higher resolution.

Seasonal differences in cave atmosphere parameters can change carbonate precipitation rates (e.g., Kaufmann & Dreybrodt, 2004; Fairchild et al., 2006; Baldini et al., 2008). The $CO_2$ concentration of cave atmosphere is directly linked to the growth rate of stalagmites (Dreybrodt, 2012; Baker et al., 2014). Growth rate variations can bias the speleothem climate record and lead to changes in the incorporation of trace elements into the crystal lattice (Fairchild et al., 2006; Gabitov & Watson, 2006). Increased cave ventilation leads to lower $pCO_2$ values in the cave atmosphere and accordingly lower calcium equilibrium concentration which increases carbonate precipitation rates (Baker et al., 2014). Even though it rains more in the summer, speleothems in CL are expected to grow faster during the low $pCO_2$ winter season, all other things being equal. This is important if the drip site has a fast response time to rainfall events above the cave. In the extreme case, speleothems in CL could only grow during the winter and fast responding drip sites will only record a winter environmental signal in the speleothem’s carbonate. To quantify the seasonal growth bias in CL further studies are needed which investigate the Ca$^{2+}$-ion concentrations in the drip water throughout the year, the water film thicknesses and drip rates (Baldini et al., 2008).

**CONCLUSION**

Monthly monitoring of the cave atmosphere and high-resolution logger data enabled us to quantify changes in cave air $pCO_2$ in CL, Puerto Rico. The largest variation occurs on the seasonal scale with $pCO_2$ maxima in summer and minima in winter. This seasonal pattern is mainly driven by the seasonal cycle in surface temperature, as is evident from calculation of temperature differences and virtual temperature contrasts between the cave and the surface. During summer, when surface temperature is warmer than cave temperature, cave ventilation is suppressed, leading to accumulation of $CO_2$. During the transition from summer to winter, and especially during winter, the positive buoyancy contrast between the cave and the surface promotes cave ventilation leading to lower $pCO_2$. On shorter time scales (diurnal to weekly), cave air $pCO_2$ is also influenced by atmospheric pressure changes.

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Characterization of antimicrobial activity of culturable bacteria isolated from Krubera-Voronja Cave

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Abstract: In the present study we aimed to perform the first analysis of antimicrobial activity of bacteria isolated from Krubera-Voronja Cave, with the main focus on their activity against Gram-positive bacteria, including Gram-positive pathogens. Using five different media, in total 874 heterotrophic cultures were isolated from water and sediment samples collected in Krubera-Voronja Cave at a depth from 220 m to 1640 m. 14.0% of all isolates demonstrated antibacterial activity against Gram-positive and Gram-negative test microorganisms. Our results show that this percentage was not uniform: it increased with the sampling depth and was the highest in the lower part of the cave. 24 isolates were active exclusively against Gram-positive test strains Micrococcus luteus and Bacillus thuringiensis. Two isolates, namely strains 1350R2-TSA30-6 and 1410WF1-TSA30-2, were chosen for the further work because of the high and comparable activity against both Gram-positive test microorganisms.

Keywords: antimicrobial, Bacillus, cave microorganisms, Krubera-Voronja Cave, volatile compound

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INTRODUCTION

Antibiotic resistance of pathogenic bacteria became one of the greatest threats to human health over the last few decades (Blair et al., 2015). A few different approaches are used to resolve this problem: chemical synthesis of novel antibiotics (Wright et al., 2014), discovery and development of novel agents capable of circumventing or neutralizing the existing resistance mechanisms (Blair et al., 2015), as well as search for new natural antimicrobials (Lamprinou et al., 2015). Considering the last-mentioned approach, caves represent one of the most attractive environments with a strong potential for the discovery of novel antimicrobials. Antimicrobial activity of different cave microorganisms against pathogenic bacteria was reported previously (Herold et al., 2005; Nakaew et al., 2009; Rajput et al., 2012; Cheeptham et al., 2013; Rule & Cheeptham, 2013; Tomova et al., 2013; Lamprinou et al., 2015); a new genus of bacteria, producing antimicrobial substances, was identified in a cave in Thailand (Nakaew et al., 2012).

Ecological conditions enabling cave microorganisms to produce antimicrobial compounds are unclear. Montano & Henderson (2013) showed that the frequency of human visitation does not affect the antimicrobial activity of cave microorganisms, while the increasing depth, and consequently increasing oligotrophy do. Energy-starved conditions in the caves prompt the complex interactions (competitive or cooperative) between different microorganisms resulting in the production of secondary metabolites.
such as pigments, siderophores, and antibiotics, which are used as informational cues (Rajput et al., 2012; Gabriel & Northup, 2013). Krubera-Voronja Cave is the Earth’s deepest cave (Sendra & Reboleira, 2012). Because of its depth, high antimicrobial activity of microorganisms living in it could be expected, but it has not been investigated so far.

Antimicrobial potential of cave actinobacteria and especially *Streptomyces* was investigated (Herold et al., 2005; Nakaew et al., 2009; Hodges et al., 2012; Rajput et al., 2012; Cheeptham et al., 2013; Rule & Cheeptham, 2013; Nimaichand et al., 2015), but the antimicrobial compounds of bacteria from other phyla – *Cyanobacteria* (Lamprinou et al., 2015), *Proteobacteria* and *Bacteroidetes* (Tomova et al., 2013) – were also of scientific interest. Our previous cultivation-independent analysis of bacterial diversity in Krubera-Voronja Cave showed that Gram-positive phyla *Actinobacteria* and *Firmicutes* were among the most numerous phyla in this cave (Kieraite-Aleksandrova et al., 2015). This indicates during the evolution the indigenous Gram-positive bacteria of Krubera-Voronja Cave could have evolved the mechanisms to compete and/or collaborate specifically with other Gram-positive bacteria. The production of antimicrobial compounds could be regarded as one of those mechanisms. Antimicrobial activity of the indigenous Gram-positive bacteria of Krubera-Voronja Cave against other Gram-positive bacteria is still unknown and should be evaluated, the rather that the search for novel antimicrobials targeting Gram-positive pathogens is of major importance. These bacteria most commonly cause severe nosocomial infections, and the resistance of these pathogens to conventionally used antimicrobials continuously increases and strengthens (Herold et al., 2005). Therefore, screening for novel narrow-spectrum antimicrobials, that would be active against Gram-positive pathogens, is vital.

In the present study we aimed to perform the first analysis of antibacterial activity of microorganisms isolated from Krubera-Voronja Cave, mainly focusing on their activity against Gram-positive bacteria including Gram-positive pathogens.

**MATERIAL AND METHODS**

**Site description and sampling**

Krubera-Voronja Cave is located in the Arabica Massif (43.4184 N 40.3083 E, Western Caucasus). Sampling in Krubera-Voronja Cave was described explicitly in our previous work (Kieraite-Aleksandrova et al., 2015). It was performed on 30th July to 7th August 2012, at depths of 220 m to 1640 m. At the time of sampling, humidity ranged from 69-76% in the upper part (220-230 m in depth) of the cave to >90% in the middle (500-700 m in depth) and the lower (1215-1640 m in depth) parts of the cave. The temperature in the cave ranged from 3-4°C in the upper and middle parts to 6-8°C in the lower part of the cave.

In total, 26 samples were collected from Krubera-Voronja Cave. The collected sample materials included soil and clay from cave walls, sediment, speleothems, drinkable water from the underground camps as well as coloured spots from cave walls. All samples were collected using aseptic techniques and placed into sterile microcentrifuge tubes. The samples were transported in a cooler and stored at -20°C until microbial analysis was performed.

**Isolation of bacterial cultures**

For isolation of culturable microorganisms from water samples, 100 μL of each sample was spread over different solid microbiological media dispensed in Petri dishes. For isolation of culturable microorganisms from sediment samples, 300 mg of each sample was suspended in 700 μL of saline, and 100 μL of each suspension was spread over different solid microbiological media. Tryptic Soy agar (TSA) (Merck Millipore), Hickey-Tresner (HT) agar, Actinomyces Isolation (AI) agar, Starch Casein Nitrate agar (SCNA), and Diço™ ISP medium 4 (ISP4) were used. The cultivation media were selected according to Cheeptham et al. (2013) and Hodges et al. (2012) as well as the previous data on the bacterial diversity in Krubera-Voronja Cave (Kieraite-Aleksandrova et al., 2015). It was previously shown that *Actinobacteria*, *Firmicutes*, and *Proteobacteria* were the dominant phyla in this cave. HT agar, AI agar, SCNA and ISP4 are usually used for the isolation and cultivation of *Actinobacteria* and TSA is routinely used for the isolation and cultivation of *Firmicutes* and *Proteobacteria*. Two different temperatures were chosen for the isolation of microorganisms from the cave samples: 4°C and 30°C. The first one was selected according to the temperature profile inside the cave (3-8°C, see above); it is suitable for the growth of psychrophiles that were believed to thrive in the cave. The second temperature is suitable for the growth of psychrotolerant microorganisms that have an optimum temperature between 20°C and 40°C, but could be metabolically active at the cave’s temperature. Microorganisms were cultivated at 4°C for 7 days and at 30°C for 3 days. Only those microorganisms that stood apart in colony morphology and in ability to excrete soluble pigments were chosen for further work.

**Primary screening of microbial isolates for antibacterial activity**

In order to identify microbial isolates with antibacterial activity, a modified version of agar diffusion test (cross-streak assay) was used (Montano & Henderson, 2013). Cave microorganisms were streaked and cultivated under the same conditions and on the same solid medium which they were isolated. For example, bacterial cultures 1350R2-TSA30-6 and 1410WF1-TSA30-2 were initially isolated on TSA at 30°C. Therefore, for the primary screening for antibacterial activity, they were restreaked on TSA and cultivated at 30°C. For screening, the plates with the streaks of the cave microorganisms were covered with a layer of sterile TSA. Test microorganisms were streaked on sterile TSA perpendicularly to the already grown streaks of the cave microorganisms. Test
microorganisms were cultivated at 30°C for 24 h. The zone of growth inhibition of the test microorganisms near the cave isolate streak indicated antimicrobial activity of the cave microorganisms. No inhibition was defined as absent interruption of the target test microorganism streak where it crossed the cave isolate streak. Gram-positive (*Micrococcus luteus* and *Bacillus thuringiensis* TL8) and Gram-negative (*Escherichia coli* BL21(DE3) and *Pseudomonas* sp. VR1) bacteria served as test microorganisms.

**Genotyping of the active isolates**

The bacterial genomic DNA was extracted from fresh cell culture using the GeneJET™ Genomic DNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. BOX-PCR was performed in 50 μL of reaction mixture containing DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific), 0.5 μM BOXA1R primer (5'-CTA CGG CAA GGC GAC GCT GAC G-3') and 10 ng of bacterial genomic DNA. BOX-PCR was conducted under the following conditions: initial denaturation at 95°C for 2 min followed by 29 cycles, each consisting of 95°C for 1 min, 53°C for 2 min, and 72°C for 3 min with a final extension step at 72°C for 7 min. Products of amplification were analysed by electrophoresis through 1% agarose gel.

**Phylogenetic analysis of active isolates**

Amplification of 16S rRNA genes was done according to Kuisiene et al. (2002). PCR products were cloned into *E. coli* DH5α using the CloneJET™ PCR Cloning Kit (Thermo Fisher Scientific). Recombinant plasmids were isolated using the GeneJET™ Plasmid Miniprep Kit (Thermo Fisher Scientific). 16S rRNA genes of strains 1350R2-TSA30-6 and 1410WF1-TSA30-2 were sequenced at the DNA Sequencing Centre (Vilnius University, Institute of Biotechnology, Lithuania); the obtained sequences were edited and sequence similarity was determined using the SEQBUILDER and MEGALIGN components of LASERGENE 6 (DNASTAR). 16S rRNA gene sequences were subjected to phylogenetic analysis using ARB (Ludwig et al., 2004). The size of the 16S rRNA gene used for the analysis was 1372 nt. The tree was rooted using the 16S rRNA gene sequence of *Paenibacillus polymyxa* DSM 367 as an outgroup.

**Nucleotide sequence accession numbers**

16S rRNA gene sequences of strains 1350R2-TSA30-6 and 1410WF1-TSA30-2 were deposited in the GenBank under the No. KU513788 and KU513789 respectively.

**Evaluation of antimicrobial activity of active isolates by agar-well diffusion method**

Test microorganisms were cultivated on TSA medium at 30°C for 24 h and then resuspended in saline. The correlation between the optical density of bacterial suspensions at 600 nm and the number of CFU/mL after the TSA plate counts was established for test microorganisms. For the evaluation of antimicrobial activity, the suspension of the test microorganism was mixed with molten TSA to achieve final 10⁶ CFU/mL. Inoculated TSA was poured into the Petri dishes (20 mL per dish). 5 mm diameter wells were cut in the inoculated agar surface and filled with 100 μL of the sample. The zone of the growth inhibition of test microorganisms indicated antimicrobial activity of the sample and was recorded as a zone diameter (in millimeters) around the wells, minus 5-mm diameter of the well (Choma & Grzelak, 2011; Tomova et al., 2013).

**Optimization of culture growth parameters for production of antibacterial compounds**

Effects of the concentration of nutrients, growth medium pH, temperature and aeration on the culture growth were examined in order to optimize the production of antibacterial compounds. Effects of the concentration of nutrients were examined using 1xTryptic Soy broth (TSB), 0.5xTSB and highly nutritious growth medium Brain Heart Infusion broth (BHIB). To test the effect of pH, growth medium was prepared in 50 mM Tris-HCl buffer (pH 7, 8, and 9/20°C). For determination of the effect of temperature, 30°C (the isolation temperature), 16°C, 8°C (the upper temperature limit of the cave), and 4°C (the lower temperature limit of the cave) were chosen. The effect of aerations was determined at 180 rpm and 250 rpm in an orbital shaker Multitron Standard (INFORIS HT).

Inoculation (5% vol/vol) was performed using bacterial cultures grown for 12 h on TSA plates at 30°C. Inocula were prepared in the respective sterile growth medium. Bacteria were cultivated in 250 mL Erlenmeyer flasks, and the samples were aseptically removed every 2 h to determine culture growth and production of antimicrobial compounds. Growth of the culture was monitored by measuring optical density at 600 nm, and antimicrobial activity of the culture was assayed in the culture supernatant by agar-well diffusion method (see above). Cells from the liquid culture were removed by centrifugation (7000×g, 20 min, 4°C).

**Extraction of antibacterial compounds by salting-out with ammonium sulphate**

Active bacterial strains were cultivated in buffered 0.5xTSB for 12 h at 30°C at 180 rpm in the orbital shaker Multitron Standard (INFORIS HT). Cells were removed by centrifugation (7000×g, 20 min, 4°C), and solid ammonium sulphate was added to the supernatant to achieve 80% saturation. The precipitate was recovered by centrifugation (12000×g, 20 min, 4°C), then dissolved in 20 mM Tris-HCl buffer (pH 7). The dissolved proteins were evaluated for the antimicrobial activity using agar-well diffusion method. The dissolved proteins were also treated with recombinant proteinase K (Thermo Fisher Scientific) according to the manufacturer's instructions. Antimicrobial activity of hydrolysed proteins was also evaluated using agar-well diffusion method. Protein concentration was determined using the Pierce™ Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.
Extraction of antibacterial compounds by organic solvents

Active strains were cultivated in 100 mL of buffered 0.5xTSB for 12 h at 30°C at 180 rpm in the orbital shaker Multitron Standard (INFORS HT). Cells were removed by centrifugation (7000xg, 20 min, 4°C). Antibacterial compounds in the supernatant were extracted with equal volume of acetone, chloroform, ethyl acetate, methanol, and hexane in a separating funnel. Different supernatant samples of the same culture were separately extracted with each solvent. The organic solvent extracts were evaporated to dryness and dissolved in chloroform (Sanghvi et al., 2014).

Thin layer chromatography and bioautography

Thin layer chromatography (TLC) of chloroform extract was done on Silica gel 60 F254 plates (Merck Millipore). The antibacterial compounds were separated with ethyl acetate-methanol (3:1, vol/vol) solvent system. Chromatograms were observed under UV light and exposed to iodine vapours. Bioautography was used for the localization of antibacterial compounds in chloroform extract (Attimarad et al., 2012). TLC plate with the separated antibacterial compounds was dried, put in sterile Petri dish, and overlaid with the inoculated TSA. Inoculated TSA was prepared as described above for the agar well diffusion method. M. luteus was used for bioautography as the test microorganism. After incubation at 30°C for 24 h, the zone of inhibition around the spot of antibacterial activity was determined. The fractions with antibacterial activity were recovered from TLC plates, disssolved in ethyl acetate and subjected to further analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis of antibacterial compounds

Gas chromatographic (GC) analysis of the fractions with antibacterial activity recovered from TLC plates was performed on a PerkinElmer Clarus 580 series gas chromatograph coupled to a PerkinElmer Clarus 560 S mass spectrometer (PerkinElmer, Shelton, USA). The GC system was equipped with Elite-5MS capillary column (30 m x 0.25 mm id, 0.25 µm film thickness) coated with methylpolysiloxane (5% phenyl). Helium was employed as a carrier gas with a constant flow of 1 mL/min. GC conditions were as follows: the oven temperature was programmed: 40°C for 1 min, from 40 to 250°C at 3°C/min and held at 250°C for 8 min; the injector temperature was held at 250°C. Injection was performed in pulsed splitless mode (pulsed to 4 mL/min until 1.5 min, split 10:1 open at 1.55 min). The capillary column was connected to the ion source of the mass spectrometer by means of the transfer line maintained at 280°C. The electron ionization ion source conditions were: electron energy 70 eV and temperature 180°C. GC-MS in full scan mode was used. The analyses were carried out with a filament multiplier delay of 3 min, and the acquisition was performed in the range of m/z 33 - 600. The qualitative identification of different compounds was performed by comparing their mass spectra with those stored in the NIST (The National Institute of Standards and Technology) library. The relative amounts of individual compounds were expressed as percentages of the peak area relative to the total peak area. GC-MS identified compounds were compared with the bacterial organic volatile compounds included in the Microbial Volatile Organic Compounds (mVOC) database (Lemfack et al., 2014) as well as with those in the SuperScent database (Dunkel et al., 2009).

Evaluation of antibacterial activity against pathogens and microorganisms of the cave

For evaluation of antibacterial activity against pathogens and microorganisms of the cave, the active fractions, recovered from TLC plates and dissolved in ethyl acetate, were used.

Antibacterial activity was determined against Gram-positive pathogenic bacteria: Bacillus cereus ATCC 10876 (Firmicutes), Enterococcus faecalis ATCC 29212 (Firmicutes), Listeria monocytogenes ATCC 7644 (Firmicutes), Staphylococcus aureus subsp. aureus ATCC 25923 (Firmicutes), and Rhodococcus equi ATCC 6939 (Actinobacteria). Agar-well diffusion method was used for determination of the minimal inhibitory concentration (MIC). The inocula of the pathogenic bacteria were prepared in saline and matched 0.5 McFarland standard. 19 mL of melted and cooled down TSA medium was inoculated with 1 mL of such suspension and poured into Petri dish. The final concentration of cells in TSA was 1 x 10^8 CFU/mL. 5 mm diameter wells were cut in the inoculated agar surface and filled with 100 µL of the sample. Six different concentrations of antimicrobial compounds were used for determination of MIC: 4.66 mg/mL (the undiluted sample), 2.33 mg/mL (1:3 dilution), 1.165 mg/mL (1:7 dilution), 0.5825 µg/mL (1:15 dilution), and 0.29125 µg/mL (1:30 dilution).

In the current work antibacterial activity of the active fractions was also examined against bacteria isolated from Krubera-Voronja Cave: Gram-positive strain 28TSA30-8, Brevibacillus sp. 1410WF1-HT30-5, Paenibacillus sp. 23TSA30-6, Paenibacillus sp. 28ISP30-5, and Streptomyces sp. 1350R2-SCNA30. Modified agar-well diffusion method was used for this test. Sterile TSA (20 mL) was poured into the Petri dish, and the well was cut in agar in the centre of the Petri dish. Bacterial cultures were streaked from the edge of the well to the border of the Petri dish. Then the well was filled with 100 µL of the sample containing antimicrobial compounds at a concentration of 1.165 mg/mL. Growth inhibition indicated the antibacterial activity of the tested compounds.

RESULTS

Isolation and antibacterial activity of microorganisms from samples of Krubera-Voronja Cave

In total, 874 heterotrophic cultures were isolated using five different media: TSA, HT agar, AI agar, SCNA, and ISP4. 108 isolates were from water samples, and 766 – from sediment samples collected in Krubera-Voronja Cave at the depth from 220 m
to 1640 m (Kieraite-Aleksandrova et al., 2015). The largest number of cultures was isolated on TSA (93 isolates at 4°C and 141 - at 30°C) and HT agar (207 isolates at 30°C). The smallest number of isolates was obtained using Al agar – 138 at 30°C. In general, temperature of 30°C was more suitable for the growth of heterotrophs – 714 cultures were obtained at this temperature, while only 160 cultures – at 4°C. Only TSA and ISP4 were suitable for growth at the latter temperature.

In order to examine antibacterial activity of the isolated cultures, primary screening using agar diffusion test was performed. Out of 874 cultures, 122 isolates (14.0%) demonstrated antibacterial activity. 108 active isolates were obtained from sediment samples, and 14 – from water samples. Most (63.9%) of the active isolates inhibited growth of a single test microorganism, while four isolates possessed a broad activity spectrum, being antagonistic against all test microorganisms. Only 9.3% of isolates from the upper part of the cave (sampling at 220-230 m in depth) showed antimicrobial activity, while 11.1% and 14.1% of all isolates from the middle (sampling at 500-700 m in depth) and lower (sampling at 1215-1640 m in depth) parts of Krubera-Voronja Cave, respectively, inhibited growth of at least one test strain.

Our previous cultivation-independent analysis showed that Gram-positive phyla *Actinobacteria* and *Firmicutes* were among the dominant phyla in Krubera-Voronja Cave (Kieraite-Aleksandrova et al., 2015). Therefore, we particularly focused on the isolates that exhibited antagonism only against Gram-positive but not against Gram-negative test microorganisms. In total, 31 isolates inhibited growth of two different test strains, and 24 of them were active only against Gram-positive ones. Activity of most of these 24 isolates against both Gram-positive test strains was very weak; somewhat stronger activity was observed only for seven cave isolates. Two cave isolates, 1350R2-TSA30-6 and 1410WF1-TSA30-2, were chosen for further experiments because of their high and comparable activity against both Gram-positive test strains – *M. luteus* from phylum *Actinobacteria* and *B. thuringiensis* TL8 from phylum *Firmicutes*. Both strains were isolated on TSA at 30°C from samples 1350R2 and 1410WF1 that have been collected in the lower part (1350 m and 1410 m depth) of Krubera-Voronja Cave. Sample 1350R2 was the sample of white clay from the rarely visited branch of the cave, and sample 1410WF1 was the sample of water from the underground camp (Kieraite-Aleksandrova et al., 2015).

**Genotyping and phylogenetic analysis of active isolates**

In order to determine whether isolates 1350R2-TSA30-6 and 1410WF1-TSA30-2 represent two different strains, BOX-PCR genotyping was performed. Our analysis clearly showed that these two isolates belong to two different strains (Fig. 1).

16S rRNA genes of these two strains were cloned, sequenced and analysed. The BLAST search showed that both strains belong to the family *Bacillaceae* in phylum *Firmicutes*. Both strains were assigned to the genus *Bacillus* (Fig. 2). 16S rRNA gene of the strain 1410WF1-TSA30-2 was most similar to those of *Bacillus aerophilus*, *Bacillus altitudinis* and *Bacillus stratosphericus* with 99.9% sequence similarity. Strain 1350R2-TSA30-6 clustered with *Bacillus simplex*, *Bacillus frigoritolerans* and *Bacillus muralis* with 99.8%, 99.6% and 99.5% sequence similarity respectively.

**The effect of culture growth parameters for the production of antibacterial compounds**

Optimization experiments showed that growth of a culture as well as production of antibacterial compounds depended on the concentration of nutrients in a culture medium. For the strain 1350R2-TSA30-6, the highest optical density (OD600) as well as the largest antimicrobial activity were observed in 0.5xTSB (data for BHIB not shown) (Fig. 3). For the strain 1410WF1-TSA30-2, similar maximum OD600 and antibacterial activity were obtained in TSB and 0.5xTSB, but the growth rate was higher in 0.5xTSB (Fig. 3). BHIB was the worst choice for the strain 1410WF1-TSA30-2 in terms of both culture growth and antibacterial activity (data not shown). Therefore, for both strains further optimization experiments were carried out in 0.5xTSB.

![Fig. 1. BOX-PCR electrophoretic profiles. M - GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific); 1 - 1350R2-TSA30-6; 2 - 1410WF1-TSA30-2. The results of this test indicate that isolates 1350R2-TSA30-6 and 1410WF1-TSA30-2 are distinct.](image-url)
Both the growth of a culture and the production of antibacterial compounds were influenced by pH of a culture medium. The best pH for the strain 1350R2-TSA30-6 was pH 7, and for the strain 1410WF1-TSA30-2 it was pH 9. Aeration did not influence the results of our experiments; highly similar results were obtained for both 180 rpm and 250 rpm. Low (16°C) temperature was not suitable for the growth of both strains – the strain 1410WF1-TSA30-2 did not grow, and OD600 for the strain 1350R2-TSA30-6 at this temperature was ~3.3 times lower than at 30°C. Consequently, the growth in the liquid culture at 8°C and 4°C was not tested.

Based on these results, both strains were further cultivated in buffered 0.5xTSB at 30°C at 180 rpm. pH of the medium was adjusted to 7 for cultivation of the strain 1350R2-TSA30-6 and to 9 for cultivation of the strain 1410WF1-TSA30-2. Growth curves and antimicrobial activity, obtained after the optimization experiments, are shown in Fig. 3. It is noteworthy that for both strains the production of antimicrobial compounds started in the exponential growth phase and continued during the growth of the culture.

**Extraction of antimicrobial compounds**

Two different approaches were used for the extraction of antimicrobial compounds from the culture supernatant – salting-out with ammonium sulphate and extraction by organic solvents.
Characterization of antimicrobial compounds

Antimicrobial compounds of strains 1350R2-TSA30-6 and 1410WF1-TSA30-2 were analysed using TLC and bioautography. Both strains showed two different fractions on TLC plates, but only a single fraction of each strain showed antimicrobial activity against Gram-positive bacteria *M. luteus* (Fig. 4). Retardation factor (*R*) of the compounds with antimicrobial activity differed. *R* for the compound of the strain 1350R2-TSA30-6 was 0.85, and for the strain 1410WF1-TSA30-2 it was 0.93. The active compounds were recovered from the TLC plates and analysed by GC-MS.

GC-MS was used to determine a chemical composition of the two active TLC fractions. Main components of the active fraction of the strain 1350R2-TSA30-6 were pyrrolizidines (Table 1, compounds No. 8-12). Their total amount was 60.3%. For four peaks (RT 46.23, 49.58, 50.19, and 58.70 min, compounds No. 8-11 in Table 1), the highest match with the library data was for pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-. Clearly, those peaks should represent four different compounds. We could suppose that pyrrolo[1,2-alpyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- corresponds to the compound with RT 50.19 min (Table 2, compound No. 10), as its mass spectrum shows the highest match with the library data. Other three peaks should correspond to the compounds with very similar structure, as their mass spectra are quite similar with a very intense fragment peak at *m/z* 154 that could be attributed to hexahydro pyrrolo[1,2-alpyrazine-1,4-dione (molecular mass 154). When harsh electron ionization is applied, the compounds undergo fragmentation and their molecular ions are not intense. Two of the compounds (with RT 49.58 and 50.19 min – Table 2, compounds No. 9 and 10 respectively) have the same molecular ion with *m/z* 210 indicating that they could be isomers of pyrrolo[1,2-alpyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-. A compound eluting at 46.23 min (Table 2, compound No. 8) has a molecular ion of *m/z* 196 and probably could be identified as pyrrolo[1,2-alpyrazine-1,4-dione, hexahydro-3-(2-isopropyl) -. A compound eluting at 58.70 min (Table 2, compound No. 11) has a low intensity molecular ion peak at *m/z* 343. Unfortunately, very few mass spectra of related compounds can be found in the library, thus more precise identification was not possible. It should be noted that pyrrolopyrazines pyrrolo[1,2-alpyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- and pyrrolo[1,2-al pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- are semivolatile cyclic dipeptides also called cyclolipeptides.

Salted-out extracellular proteins of strains 1350R2-TSA30-6 and 1410WF1-TSA30-2 showed antibacterial activity against *M. luteus* (data not shown). The treatment with proteinase K did not diminish this activity. These experiments allowed us to suggest that antimicrobial compounds produced by both strains are not peptides or proteins.

Acetone, chloroform, ethyl acetate, methanol, and hexane were used separately for extraction of the different aqueous samples of the same culture by organic solvents. All extractions were successful; different extracts of the strain 1350R2-TSA30-6 were the most active in the case of strain 1410WF1-TSA30-2 exhibited comparable activity for all solvents used, while hexane and chloroform extracts were the most active in the case of strain 1350R2-TSA30-6 (Table 1). So, chloroform extracts were used for characterization of antimicrobial compounds produced by both strains.

Table 1. Antimicrobial activity of organic solvents’ extracts. *Micrococcus luteus* was used as the test microorganism in this agar-well diffusion assay, the width of the inhibition zone is shown in millimeters.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ethyl acetate</th>
<th>Acetone</th>
<th>Hexane</th>
<th>Chloroform</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1350R2-TSA30-6</td>
<td>1.5</td>
<td>3.0</td>
<td>4.0</td>
<td>3.5</td>
<td>2.0</td>
</tr>
<tr>
<td>1410WF1-TSA30-2</td>
<td>1.0</td>
<td>1.5</td>
<td>1.5</td>
<td>1.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>
The concentration of the antimicrobial compounds (Table 4) was based on the well-known antibacterial activity of the members of phylum Actinobacteria (Rule & Cheeptham, 2013). Nevertheless, the percentage (14.0%) of Krubera-Voronja Cave isolates, that produce antimicrobial compounds, was quite low and comparable to some shallow caves: Backcountry Cave (6 m in depth), Spider Cave (44 m in depth), and Fort Stanton Cave (134 m in depth) – 21.1%, 19.8% and 8.6% respectively (Montano & Henderson, 2013). On the other hand, data for some other shallow caves show higher percentage of active isolates than of Krubera-Voronja Cave: 66% for the Left Hand Tunnel passage in Carlsbad Cavern (316 m in depth), 40% for Lechuguilla Cave (489 m in depth) (Montano & Henderson, 2013), 57% for Helmckens Falls Cave (Rule & Cheeptham, 2013), 73.9% for Magura Cave (56 m in depth) (Tomova et al., 2013). Our results also showed that the percentage of bacteria capable of antagonistic activity increased from the upper to the lower part of the cave. These results were in accordance with the findings of Montano & Henderson (2013) who revealed that the increasing depth influenced the antibacterial activity of the cave isolates. To the best of our knowledge, our present study is the first report on the antibacterial activity of bacterial isolates in Krubera-Voronja Cave.

### DISCUSSION

For isolation of microorganisms from Krubera-Voronja Cave, general purpose media (TSA) as well as the growth media designed for the isolation and cultivation of actinobacteria (HT agar, AI agar, SCNA, ISP4) were used. The choice of the latter four media was based on the well-known antibacterial activity of the members of phylum Actinobacteria (Rule & Cheeptham, 2013). The active TLC fractions of strains 1350R2-TSA30-6 and 1410WF1-TSA30-2 showed two different fractions on TLC plates, but only a single fraction of each strain showed antimicrobial activity. The active TLC fraction of strain 1350R2-TSA30-6 was resistant to antibacterial compounds produced by the strain 1410WF1-TSA30-2.

### Spectrum of inhibitory activity of antimicrobial compounds

The active TLC fractions of strains 1350R2-TSA30-6 and 1410WF1-TSA30-2 were evaluated for their antibacterial activity against Gram-positive and Gram-negative bacteria as well as against bacteria isolated from Krubera-Voronja Cave. The highest activity of active TLC fraction of the strain 1350R2-TSA30-6 was observed against R. equi ATCC 6939, while the lowest activity was recorded against S. aureus subsp. aureus ATCC 25923 and B. cereus ATCC 10876 (Table 4). The concentration 0.291 mg/mL was still inhibitory for all tested pathogenic strains, except for S. aureus subsp. aureus ATCC 25923. The MIC for the strain of S. aureus subsp. aureus was 0.583 mg/mL, while for other four pathogenic strains it was 0.291 mg/mL (Table 4). It should be noted, that the active TLC fraction was the only partially purified mixture of compounds, therefore, the inhibitory concentrations could not be evaluated for the discrete active compounds.

Antibacterial compounds of the strain 1350R2-TSA30-6 also inhibited growth of other bacteria of Krubera-Voronja Cave: Streptomyces sp. 1350R2-SCNA30, Brevibacillus sp. 1410WF1-HT30-5, and Paenibacillus sp. 23TSA30-6 (Fig. 5). The active fraction of strain 1410WF1-TSA30-2 showed no antimicrobial activity against the tested pathogenic bacteria. But antimicrobial compounds of this strain had inhibitory effect on the growth of other bacteria isolated from Krubera-Voronja Cave (Fig. 5). Only Streptomyces sp. 1350R2-SCNA30 was resistant to antibacterial compounds produced by the strain 1410WF1-TSA30-2.
The other focus of our research was the antibacterial activity targeting Gram-positive bacteria. It should be noted that Gram-positive pathogens are a major cause of nosocomial bacterial infections. Staphylococci, enterococci, streptococci, and Clostridium difficile are the most important species of clinical interest (Rossolini et al., 2014). Antibiotic resistance issues are common among Gram-positive pathogens, and the percentage of nosocomial infections caused by antibiotic-resistant Gram-positive bacteria is increasing. For example, in the United States, approximately 60% of staphylococcal infections are now caused by multidrug-resistant S. aureus, and these percentages continue to rise (Rice, 2006). Therefore, the development of novel antibiotics that would be active against Gram-positive pathogens is imperative. Only a small portion (24 out of 874) of bioactive isolates were antagonistic exclusively against Gram-positive bacteria, moreover – the bioactivity of the most of these 24 isolates was very weak. Two most active strains 1350R2-TSA30-6 and 1410WF1-TSA30-2, isolated from the lower part of the cave, were chosen for detailed characterization of the antimicrobials. It is of interest that these strains, although antagonistic against Gram-positive test strains M. luteus and B. thuringiensis TL8, were also Gram-positive - both strains were assigned to the genus Bacillus.

Bacteria of the family Bacillaceae are mainly known for their antimicrobial peptides – both ribosomally and nonribosomally synthesized peptide antibiotics. Nonribosomally synthesized antibiotic bacitracin A is produced by Bacillus licheniformis, lipopeptides surfactin, iturin, bacillomycin, and fengycin are produced by several strains of Bacillus subtilis. Well-known ribosomally synthesized peptides (bacteriocins) of Bacillaceae include subtilin and subtilosin A from B. subtilis; coagulin from Bacillus coagulans; bacthuracin F4, thuricin 17, entomocin 9, and tochinin from B. thuringiensis; cerecin 7 from B. cereus; bacillacin 490 from B. licheniformis (Sumi et al., 2015). On the other hand, bacilli also produce non-peptide antimicrobial compounds, such as bis (2-ethylhexyl) phthalate, synthesized by Bacillus pumilus (Moushumi Priya & Jayachandran, 2012); macrolide antibiotics macrolactins 1-3 produced by Bacillus sp. (Mondol et al., 2011); macrolides gageomacrolactins 1-3 produced by B. subtilis (Tareq et al., 2013). Therefore, production of both antimicrobial peptides and non-peptide antibiotics was expected for bioactive strains from Krubera-Voronja Cave.

The antimicrobials of strains 1350R2-TSA30-6 and 1410WF1-TSA30-2 differed significantly. The active compounds behaved differently in some organic solvents during extraction as well as had different Rf values in TLC. It was also noticed that antimicrobial substances of the strain 1350R2-TSA30-6 were stable, and the inhibition zone was visible constantly during the growth of the test microorganism in bioautography experiments, while antimicrobial substances of the strain 1410WF1-TSA30-2 inhibited the test microorganism only at the first stages of its growth, but disappeared through further cultivation due to evaporation of active compounds or their inactivation by the growing culture of M. luteus (data not shown). Activity of antimicrobials of the strain 1350R2-TSA30-6 was higher than of strain 1410WF1-TSA30-2. The common characteristics for both strains included poor production of antimicrobials in a highly rich growth medium BHIB as well as continued production of active compounds during the growth of the culture starting in the exponential growth phase. The first characteristic can be associated with the need to produce antimicrobials under starvation, but not under the nutrient-rich growth conditions (Montano & Henderson, 2013). The second feature (the time of production) indicated that antimicrobial compounds (or at least some of them) were primary metabolites (Sanchez & Demain, 2008).

Chemical compounds in the active TLC fractions of both strains differed markedly. The main antibacterial agents of the strain 1350R2-TSA30-6 were 2,5-diketopiperazine antibiotics. Most of 2,5-diketopiperazines are stable to proteolysis (Pérez-Picasso et al., 2009), and we have also observed this effect after treatment of the salted-out extracellular proteins of the strain 1350R2-TSA30-6 with proteinaise K. Antitumor, antifungal, antiviral, and antibacterial activities of cyclic dipeptides are well-known (Yan et al., 2004; Pandey et al., 2010; de Carvalho & Abraham, 2012; Kumar et al., 2014). Cyclic dipeptides are usually produced by Gram-negative bacteria, but have been also isolated from Gram-positive. Among the genera of the family Bacillaceae, they were so far identified only in the genus Bacillus (de Carvalho & Abraham, 2012; Leyton et al., 2012). Because of their activity spectrum, cyclic dipeptides attract much attention as potential pharmaceuticals. Our results clearly showed that antibacterial fraction of the strain 1350R2-TSA30-6 was active against Gram-positive pathogenic bacteria B. cereus, E. faecalis, L. monocytogenes, S. aureus subsp. aureus, and R. equi. Cyclic dipeptides are secondary metabolites (Leyton et al., 2012), therefore, if they would be the only compounds with bioactivity, then the antimicrobial

Fig. 5. Antibacterial activity of active TLC fractions against bacteria isolated from Krubera-Voronja Cave. The concentration of antimicrobial compounds in ethyl acetate was 1.165 mg/mL: 1 – Streptomyces sp. 1350R2-SCNA30; 2 – Brevibacillus sp. 1410WF1-HT30-5; 3 – Gram-positive strain 28TSA30-8; 4 – Paenibacillus sp. 28ISP30-5; 5 – Paenibacillus sp. 3TSA30-6.
effect would appear only in the stationary phase of the culture growth. But antimicrobial activity was also detected in the exponential growth phase of the strain 1350R2-TSA30-6. We have concluded that other volatile compounds, identified in an active fraction of this strain, were responsible for this early effect. For example, antibacterial, antiviral, or antifungal activities were previously reported for 1,2-benzenedicarboxylic acid, diisooctyl ester (Rameshthangam & Ramasamy, 2007; Maruthupandian & Mohan, 2011) and 1,3-dimethyl-benzoate (El-Shoumy et al., 2014). Some compounds (ethylbenzene, p-xylene) were previously identified in mixtures of bacterial volatiles (Tenorio-Salgado et al., 2013), while others (cyclohexanone) - in plant extracts (Kim et al., 2011) possessing antimicrobial activity.

GC-MS results for the strain 1410WF1-TSA30-2 markedly differed from the results for strain 1350R2-TSA30-6. The only common antimicrobial compound was 1,2-benzenedicarboxylic acid, diisooctyl ester. Cyclic dipeptides were not found, but a range of other compounds with different bioactivities were identified. Antibacterial, antiviral, antifungal, or antioxidant activities were previously reported for dibutyl phthalate (Maruthupandian & Mohan, 2011), nonadecane (Fernando et al., 2005), phenol, 2,4-bis(1,1-dimethylethyl)- (Varsha et al., 2015). Some compounds (hexadecane, octadecane) were previously identified in antimicrobial or nematocidal mixtures of bacterial volatiles (Gu et al., 2007; Karanja et al., 2010), while others (1-nonadecene, 2-methyl-ecosane) - in plant extracts with antibacterial activity (Boussaada et al., 2008). The main compounds in antibacterial mixture of volatiles of the strain 1410WF1-TSA30-2 were benzoic acid, octadecyl ester and 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester. We could not find any information about putative antimicrobial activity of octadecyl benzoate; it was not listed among bacterial volatiles in the mVOC and SuperScent databases either. But the esters of 1,2-benzenedicarboxylic acid (bis(2-ethylhexyl) ester, bis(2-methylpropyl) ester, butyl-2-methylpropyl ester, butyl-2-ethylhexyl ester, diisooctyl ester, etc.) are frequently the main components of antibacterial and antifungal mixtures of volatiles from different sources: algae (Sivakumar, 2014), plants (Joshi et al., 2011), fungi (Nakalembe & Kabasa, 2012), bacteria (El-Mehalawy et al., 2008). Actually these esters are believed to be responsible for antimicrobial activity of these mixtures of volatiles. Antiviral, antifungal, and antibacterial activities were demonstrated for 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester; while antimicrobial, antioxidant, and antitumor activities were shown for 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester (Kavitha et al., 2009; Karthikeyan et al., 2014). Antibacterial and antifungal activities were confirmed for 1,2-benzenedicarboxylic acid, bis(5-ethylheptyl) ester (Kavitha et al., 2009). 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester, found in active antibacterial fraction of the strain 1410WF1-TSA30-2, had been also identified in a few other representatives of the genus Bacillus (Hao & Lu, 2007; Malliaiah & Muthamilan, 2015).

It is known that bacteria use volatile metabolites for inter- and intraspecific communications as well as in order to respond and adapt to environmental changes (Audrain et al., 2015). Microorganisms in caves form multispecies communities with complex interaction networks among their members, and bacterial volatile mixtures can participate in these interactions. For example, it had been previously shown that Bacillus sp. can start producing cyclic dipeptides when challenged by a co-culture of other Bacillus species (de Carvalho & Abraham, 2012). Our results showed that antimicrobial volatile mixtures of strains 1350R2-TSA30-6 and 1410WF1-TSA30-2 could be also involved in the competition and engagement with other bacterial species in Krubera-Voronja Cave. The strains of Gram-positive genera Paenibacillus and Brevibacillus (phylum Firmicutes, family Paenibacillaceae) were sensitive to active antimicrobial fractions of both strains isolated from the same cave. The main difference between volatiles of both investigated strains was in their antagonism against actinobacterial strain of the genus Streptomyces. It is interesting that antimicrobial compounds of the strain 1410WF1-TSA30-2 were antagonistic against bigger number of cave strains than antimicrobial compounds of the strain 1350R2-TSA30-6. This is contrary to the zero antimicrobial activity of volatiles of the strain 1410WF1-TSA30-2 against pathogenic bacteria. It was supposed that the primary role of volatiles of the strain 1410WF1-TSA30-2 is the competition with the indigenous Gram-positive microorganisms of the cave.

In conclusion, our results clearly demonstrated that while screening for novel bioactive compounds, examining the lesser-studied phyla, such as Firmicutes, rather than the commonly examined Actinobacteria is an important and promising approach. Cave actinobacteria are usually the main targets of bioactivity assays, and therefore the antimicrobial potential of other bacteria is underestimated. Detailed analysis of two Firmicutes strains, isolated from Krubera-Voronja Cave, revealed two different mixtures of compounds with antibacterial activity. The main antibacterial compounds of the strain 1350R2-TSA30-6 were pyrolopyrazines, and the main antibacterial compound of the strain 1410WF1-TSA30-2 was bis(2-methylpropyl) ester of 1,2-benzenedicarboxylic acid. To the best of our knowledge, our study is the first report on chemical characterization of antimicrobial mixtures of Firmicutes isolated from caves.

ACKNOWLEDGEMENT

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Hybrid Taguchi-Objective Function optimization approach for automatic cave bird detection from terrestrial laser scanning intensity image

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Abstract: This paper proposes an optimized Taguchi-objective function segmentation-based image analysis to detect bird nests in a cave from high resolution terrestrial laser scanning intensity images. First, the Taguchi orthogonal array was used to design 25 experiments with three segmentation parameters: scale, shape, and compactness, each having five variable factor levels. Then, a plateau objective function was computed for each experiment using their respective level combinations. A merger of the factor level combination in the orthogonal array and the computed plateau objective function values was used to generate main effects and interaction plots for signal-to-noise ratios, which provided a measure of robustness for scale, shape, and compactness factors. The optimized parameters were used in the segmentation process in eCognition. The image object was then classified into nest and cave-wall on the basis of laser return intensity and area index using knowledge-based rule sets, and the detection accuracy was evaluated. The result produced area under ROC curve of 0.93 with P<0.0001 at 95% confidence level. This indicates that the proposed method is effective for distinguishing birds from cave-wall with high precision. The classification result was transferred to ArcGIS where the detected nests were counted after post-classification editing. A total number of 25,959 nests were counted from the seven scan scenes used. This shows that the fusion of Taguchi and objective function is indeed an effective method to determine optimal segmentation parameters to group image objects as small as birds within a segment. Moreover, the use of segments’ spectral intensity value and area index increased classification accuracy significantly. Further, the method was tested for reliability using six additional images. The test of heterogeneity using Cochran’s Q and Inconsistency tests produced a P value of 0.384 and I² value of 5.10% at 95% confidence interval, respectively. This shows that the method is consistent with non-significant difference among the trials.

Keywords: Biospeleology, remote sensing, optimization, Gomantong, feature extraction, ecology, terrestrial laser scanning

INTRODUCTION

Until lately, ecological research, especially species population census of the inhabitants of caves, has been largely stalled due to perpetual darkness, inaccessibility, and harsh environmental conditions of this unconventional ecosphere (Idrees & Pradhan, 2016). Gomantong is one of the caves in north Borneo Island with large population of Black and White Nest swiftlet birds (Aerodramus maximus and Aerodramus fuciphagus) and wrinkle-lipped bats (Chaerephon plicata) sharing their dark home (Chasen, 1931). Although the bats and birds rarely intermingle freely, they may roost a meter or few away from each other. The cave is constantly alive with swiftlet birds, bats, and other smaller creatures such as rats, insects, and other invertebrates. Bat and, to some extent, swiftlet droppings (guano) support the cave ecology and sustain the predator-prey relationship within the micro eco-climate (Abdullah et al., 2007; Price, 2014). Economically, the high commercial value of edible bird nests which are made entirely or partly of glutinous saliva containing soluble glyco-proteines and cellular elements has attracted pivotal socio-economic attention of both the indigenous people and the nation for centuries. Furthermore, the cave...
is a popular destination that receives a large number of both local and foreign tourists that stream into experience the thrilling swiftlet birds’ nest harvesting, astounding bat swarm, and the cave powerhouse, guano (Price, 2007; Kingston, 2010). In spite of the centennial interaction of people with the cave and the apparent threat posed by incessant harvesting, it has not been possible to keep track of the actual number of bats/swiftlets birds in the cave until now due to absence of a practical counting method.

Interestingly, however, the growing use of high resolution terrestrial laser scanning (TLS) within caves has recently triggered a new perspective in biospeleology, particularly roosting bats and birds. This field of enquiry pioneered by Azmy et al. (2012) and McFarlane et al. (2015) demonstrates the potentiality of TLS datasets for population surveys as well as understanding the relationship between roosting and the cave morphology. The methods adopted by the researchers are inventive; nonetheless, they have their own limitations. For example, the strength of intensity TLS images lies in the reflectance of the laser pulse which is a function of the reflective ability and properties of the target surface (Remondino, 2003; Haddad, 2011). It is, therefore, unlikely that rocks with different compositional elements will always exhibit uniform reflectance that will constantly produce a distinctively bright cave background. In that case, using intensity value alone as the basis to detect objects as performed by Azmy et al. (2012) will certainly lead to conflicting representations. Also, the method employed by the latter researchers require full resolution scans (McFarlane et al., 2015). Data collection at full resolution is practically time consuming and costly. Furthermore, processing the resulting volume of point cloud is computationally intensive, thus, limiting the application of the method to relatively small areas. In the phase of rising concern for ecologically endangered species, this paper demonstrates that remote sensing approaches will facilitate quantitative spatial ecometrics of valuable tropical caves using laser return intensity images.

Advances in computer vision and artificial intelligence have made object-based image analysis (OBIA) a widely accepted technique in the field of remote sensing for object recognition, scene classification, and information retrieval (Martha et al., 2011; Cheng et al., 2013). A number of applications have benefitted from this method (Blaschke, 2010; Anders et al., 2011; Lahousse et al., 2011; Blaschke et al., 2014). In OBIA, unlike pixel-based image analysis, feature classification employs a knowledge-driven approach by exploring spectral information (e.g., color, intensity value), spatial properties (e.g., shape, size), together with associated textural and contextual primitives to represent objects in the scene in a way that best depicts the imaged reality.

Segmentation is the foundation and first major step in image object classification in object-oriented image analysis. The widely used region-growing segmentation technique partitions the image into homogeneous contiguous regions that enclose identical pixels as objects within each segment based on the assumption that an image pixel most likely belongs to the same object as its neighboring pixels (Moosavi et al., 2014). The region-growing process identifies candidate pixels as building blocks for the initial segments and, iteratively, the pixels are amplified with neighboring pixels that satisfy user-supplied homogeneity criteria. In fact, the image classification accuracy is directly controlled by the quality of the segments. And the quality of segmentation itself is controlled by the fitness of the defined parameters (Martha et al., 2011).

In eCognition (eCognition9.0, 2014), segmentation quality depends on selection of suitable values for scale, shape, and compactness parameter settings (Tumuhairwe, 2011; Kurtz et al., 2014). The scale factor determines the size of the thematic layer of segments that is composed of a group of homogeneous pixels. On the other hand, shape and compactness factors, which are associated with color density and smoothness, specify how much spectral information should be aggregated to build segments. These parameters synchronously determine the sensitivity of feature detection and classification accuracy.

Until Espindola et al. (2006) proposed the objective function to select appropriate parameters that can produce the best quality segmentation based on intra-segment homogeneity and inter-segment reparability, selection of segmentation parameters have conventionally been achieved by trial-and-error basis. Even so, most applications that employed this method (e.g., Gao et al., 2011; Martha et al., 2011; Moosavi et al., 2014, Pradhan et al., 2015) have consistently emphasized on the scale parameter alone while other factors are kept constant, despite the fact that they collectively contribute to the overall segment quality.

In this paper, the statistical Taguchi (Chou et al., 2009) and objective function (Espindola et al., 2006) robust methods were combined in a single processing flow to optimize segmentation parameters for the detection of edible white-nests in Simud Putih (the Gomantong’s upper level cave). The situation is even much more complex involving objects such as birds of sub-centimeter area scale, located closely to one another in a dark cave environment. Therefore, merging of the statistical and spatial optimization process creates intrinsic sensitivity to the image pixels and their spatial relationship and the strength of these properties is utilized to obtain the desired quality. The principal motivation for this hybrid approach is the awareness that segmentation is not affected only by controllable factors but also by factors that are difficult to control (i.e. clustering of pixels belonging to the same object).

STUDY AREA AND DATA

Gomantong Cave is situated in one of the tower-like limestone outcrops in Sabah, North Boneo. The limestone hill sits within the 3000 hectares Gomantong Forest Reserve (118° 04'E, 5° 32'N), some 34 kilometers south of Sandakan and about 34.1 kilometers east of the state capital, Kota Kinabatangan. To the south of the hill is the Lower Kinabatangan River that...
flows in the northeast direction into the Sulu Sea (Fig. 1). The profile of the hill from the peak is a tower-like outcrop where the limestone folds to form a syncline of near-vertical joints. Around the cave, the limestone dips between 20-30° to the north-west which provides entrance along one of the major faults (Lundberg & McFarlane, 2012). The hill is penetrated by complex cave systems that consist of two major halls, one above the other. The more accessible lower cave, Simud Hitam (or Black Cave), opens to the base of the hill close to and level with the bank of a small stream (Fig. 1) while the entrance to upper cave, Simud Putih (or White Cave), is located about 85 m above the floor of the entrance to the lower cave (Abdullah et al., 2005; McFarlane et al., 2013). Gomantong Cave, like every other cave, is dark and humid, providing an excellent microclimate habitat that is suitable for swiftlet birds and bats to live as far back as 600 years ago (Abdullah et al., 2005; Kingston, 2010).

Data used
The entire Gomantong cave system was scanned in July, 2014, using FARO Focus3Dx130 instrument at ¼ resolution mode (= 244,000 points/second) to generate a point cloud spacing of 12.5 ± 2 mm ranging error and intensity image. ¼ scan resolution mode increases the efficiency of the scan because it balances the speed of data acquisition and quality of the scan. Furthermore, the unique small weight (~5 kg), high distance accuracy and ease of use (Idrees and Pradhan, 2016) makes Focus3D a suitable choice for scanning the Gomantong Cave.

The scan data were pre-processed in FAROScene v.5.5.0 software package (http://www2.faro.com/downloads/training/Software). Since points scanned at each setup are stored in different scanner Cartesian coordinates, the initial task was to align individual scans into one point cloud referenced to a single Cartesian coordinate system. For high quality registration, automatic target-based point correspondence which employs the iterative closest point (ICP) algorithm (Besl and McKay, 1992) was used. The system automatically detects at least three points from the artificial targets strategically placed to be visible in successive adjacent scans to compute accurate transformation parameters. The quality of registration was further improved by activating auxiliary sensors (inclinometer, compass and altimeter) to speed-up the process of individual scan correlation. Thereafter, the point cloud was filtered using a distance threshold of 200 m to automatically eliminate isolated points considered as noise whereas points that belong to features outside the cave were manually deleted. Finally, 99 intensity images of the registered scans were exported, from which seven were randomly selected for the development and validation of the method.
METHODOLOGY

The research was executed through a step-by-step workflow presented in Fig. 2. A brief description of the data processing and analysis employed follows.

**Optimizing segmentation**

**Taguchi method**

Dr. Genichi Taguchi developed statistical methods to reduce variation in factors that undermine consistency in a product or process through a robust design of experiments to improve the quality of manufactured goods (Rao et al., 2008; Chou et al., 2009; Thaduri et al., 2013). The method determines optimum parameters by manipulating variable factor levels, and estimates their effects on the response mean and variance of the process performance characteristics. In Taguchi methods, the experiment is designed using an orthogonal array such that within each pair of columns representing independent variables, each combination of levels occurs an equal number of times (Rao et al., 2008; Pradhan et al., 2015). By doing so, the effects of all the factors can be balanced and, thus, give a relative value that represents the effects of a level compared with other levels of the same factor. The advantage of Taguchi’s orthogonal array design of experiment (DOE) is that it considerably minimizes the number of experiments by employing fractional factorial design (Chou et al., 2009). Taguchi’s Signal-to-Noise ratios, which are log functions of desired output, provide a measure of optimization through constraints and with variables that need to be minimized or maximized. Analysis of the combined experimental result and optimization using S/N ratio produce the best statistical prediction of optimum process (or segmentation) parameters.

**Objective function**

Even though correct selection of segmentation parameters is important, and the power of Taguchi methods optimize this process, it does not automatically result in high-precision target detection because the segmentation process depends not only on these parameters, but also on correct grouping of image spectral relative their spatial arrangement (autocorrelation). As mentioned earlier, the objective function exploits the combination of spatial autocorrelation and variance indices to select the ideal parameters to evaluate segmentation quality (Espindola et al., 2006; Gao et al., 2011). Spatial autocorrelation indicates the level of distinctiveness between regions (heterogeneity) while the variance indicator expresses the uniqueness of the pixels in individual segments. So, segmentation is of good quality when intrasegment homogeneity and intersegment heterogeneity conditions are strictly maximized. Fundamentally, experiments with the highest Plateau objective function (POF) value indicate best performance and, therefore, a mark of strength of the quality. This can be true where only a single variable factor like scale is considered to affect the result (Espindola et al., 2006; Gao et al., 2011). But where multiple variable factors influence the result, the use of objective function may not likely yield the best result.

Operationally, the objective function \( F \), fuses the within-segment variance \( \nu \) measure and the between-segment autocorrelation Moran’s I index \( I \) (Espindola et al., 2006) as given by:

\[
F(\nu, I) = F(\nu) + F(I)
\]  

where \( F(\nu) \) and \( F(I) \) are normalization functions as

\[
F(x) = \frac{X_{\text{max}} - X_i}{X_{\text{max}} - X_{\text{min}}}
\]  

The first element of objective function, mean and variance of the segments was computed using the laser intensity value of sampled regions.

\[
\nu = \frac{\sum_{i=1}^{n} a_i \nu_i}{\sum_{i=1}^{n} a_i}
\]

Where \( a_i \) and \( \nu_i \) are the area and intrasegment variance the respective segment \( i \). And the second component, Moran’s I index expressed as

\[
I = \frac{n}{S_0} \sum_{i=1}^{n} \sum_{j=1}^{n} W_{ij} z_i z_j
\]

where \( z_i \) is the deviation of the brightness value of the object \( i \) from its mean \( (x_i - \bar{x}) \), \( w_{ij} \) is the spatial weight between objects \( i \) and \( j \), which is 1 for adjacent regions or 0 otherwise, \( n \) is the total number of objects, and \( S_0 \) is the aggregate of all spatial weights.

\[
S_0 = \sum_{i=1}^{n} \sum_{j=1}^{n} W_{ij}
\]
Robust Hybrid optimization strategy

The basis of this hybrid robust design is the awareness that the quality of the objective function is still subject to the quality of the scale, shape, and compactness parameter variation. And achieving this by trial-and-error approach can introduce errors that may affect the quality of the resulting optimized parameter (Pradhan et al., 2015). This necessitates the idea of integrating statistical (Taguchi method) and spatial (objective function) optimization methods in a single processing step to model optimal parameters that guarantee best quality segments. Sensitivity of segmentation to pixel neighborhood as the bases for feature grouping and the role of scale, shape, and compactness parameters on the quality of segmentation and classification are the core fundamentals of this approach. To accomplish the hybrid optimization strategy, the following core steps were implemented.

a) Evaluate process objectives to understand variables that control the desired result.
b) Determine the parameters and the range of values that directly influence the quality of the process.
c) Based on the number of parameters and variable level, design experiments to determine number runs.
d) Use the values of the parameter level combinations to compute the POF for each experiment.
e) Combine results of steps c and d to analyze and predict the optimum segmentation parameters using Taguchi’s S/N ratio.
f) Implementation – the final follow-up experiment performed to verify the result.

Unlike the standard full factorial design, the orthogonal array examines pairs of combinations with each factor level weighted equally so that each factor is assessed independently. The advantage of the Taguchi method is that it significantly reduces the number of experiments and minimizes cost (Moosavi et al., 2014). The initial requirement to use the orthogonal arrays is to identify the number of factors affecting the process and the levels of variation. For region-growing segmentation in eCognition, only three factors; scale, shape, and compactness that control segmentation were used with five varying conditioning levels (Table 1). After careful consideration of the size of the cave birds with some tests, it was discovered that the effective scale range should be between 10 and 30 to avoid over- and under-segmentation. Again, since shape and compactness factors depend on color density and smoothness, same values were assigned to both factors at each level.

Unlike in standard factorial designs that yield $5^3$ (125) number of experiments for this combination, the proposed Taguchi orthogonal array experimental design table provides only 25. This means that only 25 experiments (one-fourth of the full factorial design) is capable of producing equally weighted factor levels across the design. The Taguchi orthogonal array design was implemented in Minitab v.17 statistical software (Minitab17, 2016). Based on parameter level combinations in the orthogonal arrays, 25 segmentation tests were executed and the corresponding POF were computer for each test. For consistency, the experimental segments were extracted from the subset image with fair representation of birds and cave wall. Thereafter, analysis of the signal-to-noise (S/N) ratio was used to model the optimal segmentation parameters. To arrive at this, the effect each variable has on the output was determined by calculating the S/N ratio for each experiment conducted using the mean value, $y_i$, and $s_i$ variance expressed as

$$SN_j = 10\log_{10} \frac{\bar{y}_j}{s_j^2} \quad (6)$$

where

$$\bar{y}_j = \frac{1}{N_j} \sum_{u=1}^{N_j} y_{i,u} \quad (7)$$

$$s_j^2 = \frac{1}{N_j-1} \sum_{u=1}^{N_j} (y_{i,u} - \bar{y}_j)^2 \quad (8)$$

In equations (7) and (8), $i$ is the experiment number, $u$ is the trial number, and $N_i$ is the number of trials for experiment $i$. Our objective is to maximize the performance characteristic, so, the 'higher is better' option was used for the calculation of the S/N ratio based on the following equation:

$$SN_j = -\log_{10} \left[ \frac{1}{N_j} \sum_{u=1}^{N_j} \frac{1}{\bar{y}_u^2} \right] \quad (9)$$

After that the S/N ratio has been computed for each experiment, the average of the S/N value for each factor and level is derived and the result is exported as a table and a graph.

Segmentation and classification processes

Test

The widely used multiresolution segmentation algorithm was employed on a subset image to test the efficiency of the proposed method and the resulting image object was classified to extract bird nest polygons. The modeled optimal scale, shape, and compactness parameter that maximizes peak plateau was utilized to segment the image using the bottom-up region growing technique (Danneels et al., 2007; Blaschke, 2010). The parameters exhibit an utmost ability to delineate objects as small as the size of a bird (Fig. 3), measuring, at most, about 50 square centimeter in area, a departure from the natural physical surface where features of

<table>
<thead>
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<th>Parameters</th>
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<tr>
<td>Scale</td>
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<tr>
<td>Shape</td>
<td>0.05 0.2 0.4 0.7 0.9</td>
</tr>
<tr>
<td>Compactness</td>
<td>0.05 0.2 0.4 0.7 0.9</td>
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Table 1. Segmentation factors and their levels used for optimization.

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interest occupy large areas at varying spatial scales. Before the classification process, spectral segmentation was performed (Fig. 3B) to further merge contiguous regions of similar physionomy, especially for the rock surface that appears over-segmented due to the scale factor considered for bird detection.

Feature discrimination was accomplished through a combination of spectral and geometric properties associated with the segments. In the intensity image, nests stand out because the spectral characteristics are darker than the cave wall and the geometric characteristics (shape and size) are quite small (centimeter level). This information was useful for the development of a rule set (a knowledge-based expert system) that was transferred to a process in eCognition to automatically detect and classify the image objects using a combination of segmentation and classification algorithms. From the result, features of interest (bird nests) were extracted. Complete darkness in caves make it impossible to use photographic images to compare the results, therefore, the decision was made to use image section that facilitates direct manual counting of the detected/undetected nests.

Validation
To empirically proof the effectiveness of the proposed approach, the developed rule-set was tested on the full scan intensity image to concurrently segment and classify the image objects into two feature classes, nests and cave-wall. The data-driven thresholding operation employs both the spectral and geometric characteristics (Gao et al., 2011; Tumuhairwe, 2011) of the diagnostic features for the classification process. A subclass, shadow, was added to cave-wall class to understand how low contrast between cave wall and nest class affects detection. The classified object layers were later transferred to ArcGIS10.2 for further analysis. Prior to feature extraction, the precision of detection was statistically evaluated using a binary classifier, Receiver Operating Characteristic (ROC) curve analysis. Finally, the nest class was extracted, followed by a clean-up exercise to eliminate image objects wrongly detected as bird nest using pixel area thresholding.

Accuracy assessment
From computer-vision point of view, the task of detecting objects from an image is far from trivial, and may give wrong results if executed imprecisely. Unlike the standard remote sensing image classification of the terrain surface with many land use/cover classes, the physical topography of a cave is substantially made up of rock that forms the wall, and only secondarily bats, birds, reptiles, and occasionally an underground river. So, a binary classification was employed to evaluate the classification results using receiver operating characteristic (ROC) curve (Powers, 2011). ROC is an excellent tool that is increasingly used in machine learning and data mining research of this nature for diagnostic performance evaluation. Again, since the two classes are not symmetric, rather than overall accuracy, the relative proportion of the different types of errors is of interest. Details of the basic ROC analysis can be found in Hanley & McNeil (1982), Flach et al. (2011), and Powers (2011).

A Supervised learning method was employed by collecting random sample points representing the classes as test data to evaluate the precision of the probabilistic observations. Metz (1978) suggested that meaningful qualitative conclusions can be reached with a total dataset of 100 samples. Here, we used 200 samples: 100 birds; 60 cave-wall features; and 40 shadow areas. The distribution of samples, for cave wall and shadow areas (both of which are considered as the same class) was done to cater for bias in the sensitivity/specificity performance. With the spatial join function in ArcTool box, the sampled test data and the corresponding properties of the feature class layers (the diagnostic data) were retrieved in a single table for use in the statistical MedCalc software (MedCalc16.0, 2016). First, an independent evaluation of the intensity-based classifications was performed. Thereafter, accuracy of the combined spectral and geometric properties-based classification result was examined against the diagnostic data to assess their respective response to detectability using the area under ROC curve indices.

RELIABILITY TESTING
The consistency test is essential for analyzing the reliability of a method or process. Knowing how consistent the results of different studies are is
useful for determining the credibility of the method that produced them. For this reason, the method was implemented using six images randomly selected from the cave. The ROC curve analysis of the six trials was processed simultaneously to test the statistical significance of the difference between the areas under ROC curves and to obtain their quantitative results for further analysis. The quantitative outcome from the compared ROC curves was used as input parameters for the reliability test. In this case, decision on whether the method is credible or not was investigated using two measures; i) estimate the overall effects of interest and ii) statistical test for heterogeneity.

Estimating overall effects

The effect of interest here is the area under ROC curves. A numerical estimate of the overall effects was achieved by taking the weighted average of the estimated effect from the respective study (Petrie et al., 2003). The overall effect is expressed as:

$$\hat{\theta} = \frac{\sum \hat{\theta}_i w_i}{\sum w_i} \quad (10)$$

where $\hat{\theta}_i$ is the estimated effect and $w_i$ the weight of the $i^{th}$ study. Usually the reciprocals of the standard errors are allocated as weights; thus, smaller standard errors get more weight in the calculation of the pooled effect size. The overall pooled effect is determined quantitatively using the fixed-effects and random-effects estimation methods. Fixed-effects estimator believes that there is a common true effect in each study which is constant to all the studies; hence, what is reported is an estimate of the magnitude of the common effect. Conversely, the random-effects method assumes that the true effects vary between studies and the estimated effect is the weighted average of all the effects obtained from all the trials (Petrie et al., 2003; Borenstein et al., 2009). But unlike the fixed-effects, the random-effects estimator takes into account both the random variation and the heterogeneity of the standard error across all the trials. As a rule, therefore, results of the two models will be similar if there is no heterogeneity.

Testing for heterogeneity

Investigating the null hypothesis that all the studies are evaluating a uniform effect of interest is usually verified on a standard scale of the test statistic, Cochran’s Q (Petrie et al., 2003; Higgins et al., 2003) computed by taking the sum of squares of the deviation of the individual estimated effect from the overall effect.

$$Q = \sum w_i (\hat{\theta}_i - \hat{\theta})^2 \quad (11)$$

In general, the magnitude of the test statistic is decided on the basis of a P value which is compared to a chi-square distribution with k-1 degree of freedom. Q test has been widely used for testing heterogeneity; however, it is known to be weak at identifying true heterogeneity among studies and the inability to describe reasonably the extent of intrinsic heterogeneity (Higgins et al., 2003). So, in addition to Q-test, the degree of disagreement in the studies’ results was quantified using the inconsistency $I^2$ test (Higgins et al., 2003). $I^2$ is computed using the results obtained from the Cochran’s test statistic (Equation 11) except that it is expressed as a percentage of total variation across studies that results from true heterogeneity rather than probability (Higgins et al., 2003); as given in the expression:

$$I^2 = 100% \times \frac{(Q - df)}{Q} \quad (12)$$

where $Q$ is the Cochran’s heterogeneity and $df$ is the degree of freedom. The advantage of $I^2$ over Q-test is that it is independent on the number of experiments and takes into consideration inherent heterogeneity (Higgins et al., 2003; Borenstein et al., 2009).

RESULTS AND DISCUSSION

Parameter optimization results

Table 2 presents the Taguchi orthogonal array and POF values computed based on scale, shape, and compactness level combinations. Minitab conventionally code factor levels as integer, depending on the number of levels. For instance, numbers 1, 2... 5 under each factor represents the unit vector (refer to Table 1).

From the table, the highest POF value (1.46) was generated with parameter combinations of experiment 19. According to the logic of objective function, it means that scale, shape, and compactness factors at levels 4, 4, and 2 (i.e., 25, 0.7, and 0.2), respectively.

<table>
<thead>
<tr>
<th>RUN</th>
<th>SCALE</th>
<th>SHAPE</th>
<th>COMPACTNESS</th>
<th>POF</th>
</tr>
</thead>
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<tr>
<td>Test-1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.11</td>
</tr>
<tr>
<td>Test-2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1.09</td>
</tr>
<tr>
<td>Test-3</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>1.00</td>
</tr>
<tr>
<td>Test-4</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>1.17</td>
</tr>
<tr>
<td>Test-5</td>
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<td>5</td>
<td>5</td>
<td>1.26</td>
</tr>
<tr>
<td>Test-6</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0.97</td>
</tr>
<tr>
<td>Test-7</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>0.96</td>
</tr>
<tr>
<td>Test-8</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>1.03</td>
</tr>
<tr>
<td>Test-9</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>1.21</td>
</tr>
<tr>
<td>Test-10</td>
<td>2</td>
<td>5</td>
<td>1</td>
<td>1.19</td>
</tr>
<tr>
<td>Test-11</td>
<td>3</td>
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<td>0.89</td>
</tr>
<tr>
<td>Test-12</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>0.85</td>
</tr>
<tr>
<td>Test-13</td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>1.24</td>
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<tr>
<td>Test-14</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>1.11</td>
</tr>
<tr>
<td>Test-15</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>0.60</td>
</tr>
<tr>
<td>Test-16</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>1.09</td>
</tr>
<tr>
<td>Test-17</td>
<td>4</td>
<td>2</td>
<td>5</td>
<td>1.11</td>
</tr>
<tr>
<td>Test-18</td>
<td>4</td>
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<td>1</td>
<td>0.80</td>
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<td>4</td>
<td>2</td>
<td>1.46</td>
</tr>
<tr>
<td>Test-20</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>0.51</td>
</tr>
<tr>
<td>Test-21</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>0.99</td>
</tr>
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<td>Test-22</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>0.83</td>
</tr>
<tr>
<td>Test-23</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>1.02</td>
</tr>
<tr>
<td>Test-24</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>1.02</td>
</tr>
<tr>
<td>Test-25</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>0.15</td>
</tr>
</tbody>
</table>
will be considered. But a closer look at the table reveals POF with values close to the highest POF in a pattern that correlates with the analyzed signal-to-noise values. For example, in column for scale factor the POF values for level 1 have closely the same high values. The same trend can be observed in all rows where 4 appeared in the shape column, likewise in all rows with 5 in compactness column. The hybrid strategy simultaneously weighs the orthogonal vectors and POF to compute the S/N ratios. This results in the predictive optimal factor levels that are suitable for segmentation. Table 3 and Fig. 4 show the S/N ratio values and mean effect plots.

Table 3. Response table for signal to noise ratios (larger is better).

<table>
<thead>
<tr>
<th>Level</th>
<th>SCALE</th>
<th>SHAPE</th>
<th>COMPACTNESS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.01205</td>
<td>0.05714</td>
<td>-0.02912</td>
</tr>
<tr>
<td>2</td>
<td>0.5713</td>
<td>-0.34412</td>
<td>-0.11493</td>
</tr>
<tr>
<td>3</td>
<td>-0.83372</td>
<td>0.08019</td>
<td>-1.40909</td>
</tr>
<tr>
<td>4</td>
<td>-0.58264</td>
<td>1.48495</td>
<td>-3.13898</td>
</tr>
<tr>
<td>5</td>
<td>-3.5766</td>
<td>-4.68778</td>
<td>1.2825</td>
</tr>
<tr>
<td>Delta</td>
<td>4.58864</td>
<td>6.17274</td>
<td>4.42148</td>
</tr>
<tr>
<td>Rank</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

In the graph, the factor level is in the x-axis while the mean S/N ratio is in the y-axis. The level with highest average S/N ratios (Table 3) indicates maximum performance and, ultimately, the one that meets the optimum condition to produce best quality segments. Therefore, the scale, shape, and compactness factors are best at levels 1, 4, and 5 respectively; representing vector combinations of 10, 0.7 and 0.9 (refer to Table 2). Fig. 4 displays this graphically as an indicative of the “Larger is better” option which guarantees maximizing the output values. It can be observed that the new combinations are not just a selection from the experiments based on the POF index, as in the works of Gao et al. (2011), Martha et al. (2011), and Moosavi et al. (2014), but a completely new synergistic combination of factors that guarantees the best mix that satisfies optimally inter-segment heterogeneity, intra-segment homogeneity, and segment quality for the target object. Aside optimization, the factors are quantitatively ranked according to their level of significance. Here, shape ranks as the best contributor, followed by scale and, lastly, compactness. The ranking further supports our argument that keeping other factors constant aside scale can reduce the quality expected with the objective function.

**Classification results**

**Test result**

The knowledge-based expert system is a valuable resource for feature extraction. The selected classes were extracted on the basis of the strength of laser intensity and segment area. The method is efficient with nearly 100 percent success in detecting nests. In the test image, a total number of 610 objects were detected as birds (Fig. 5). However, visual observation and counting indicates that the method over-approximated birds by 9% of the actual number of birds in the image. It efficiently discriminates shadow from rock projections and fractures that share similar intensity value with birds but characteristically differ in shape indices (area, elongation, etc.). An accurate population count was achieved semi-automatically by eliminating instances in which dark crevices on the cave wall are detected as birds using area pixel threshold and manual editing. The actual number of nests detected in the image subset is 556, equivalent to 91% of the detected polygons.

**Validation result**

Figure 6 shows the detected birds from the whole intensity image. It can be observed that the nests are precisely detected in their roosting position, high on the cave ceiling. The result demonstrates that our method can sufficiently detect nests in areas of low contrast caused by dark surface background as much as it does in a highly contrasting background. Spectral ambiguities that may have arisen from low contrast between the cave wall and birds were correctly resolved by taking object geometry into consideration rather than relying solely on intensity values of detection. Furthermore, the sensitivity of the method to measure spatial autocorrelation and variance and to identify and group objects based on their spectral relationship and form using a correct mix of segmentation parameters improves feature detectability.

The general spatial arrangement observed visually was supported using global Moran’s I. Positive spatial autocorrelation with Moran’s I of 0.47 indicates spatial distribution that is neither randomly distributed nor clustered in agreement with the findings of McFarlane et al. (2015). In addition, the patterns in which swiftlets choose roosting locations show that the cave morphology has a strong influence. This was confirmed through visual and cross-sectional analysis of the 3D point cloud in CloudCompare. It was discovered that roosting
locations are more concentrated around rock joints, rough and concave-like surfaces with slope varying between gentle and steep slope (3° – 35°). After clean-up operations, the centroid point of the polygons was generated making a total number of 7,306 nests detected from the single image scene.

**Accuracy assessment results**

Results of the classification accuracy assessment are presented in Fig. 7 and Table 4. The graph of sensitivity-specificity plots provide visual and statistical understanding of how well the method is able to discriminate between nests and cave wall. Interactively, the sensitivity measures how good the classification is at picking out segments that are truly bird (otherwise called the true positive), while the specificity indicates ability of the classification to correctly identify non-nest segments (true negative) (Flach et al., 2011). Assessment with brightness value shows that the laser return intensity value is highly sensitive to detecting nests with 94% sensitivity. However, apparent overlap between bird and cave wall classes cause the curve to intercept the diagonal line (Fig. 7A), supported statistically with a specificity value of 57%. The implication of this is that there is a greater suspicion of the possibility to choose an individual from the negative class as true positive if only the intensity value is used.

Accuracy measurement of the brightness value-based classification yielded area under the ROC curve (AUC) of 0.59 (P=0.03). The drift around the null hypothesis (AUC=0.5) where the variable cannot distinguish between two classes validates the conclusion that laser intensity values alone have marginal prospect to differentiate between birds and cave wall. Comparatively, the use of spectral and area properties in the classification process results in better detection capability with sensitivity and specificity values of 87% and 98% respectively (Fig. 7B). Despite a slight decrease in sensitivity as a result of shadow effect and low contrast, the strength of the area factor to detect non-bird objects neutralized the negative effect it could have had on the final accuracy (Table 4). Again, area under the ROC curve of 0.93 and p-value of <0.0001 was achieved at 95% confidence level. Since the P is by far smaller than 0.05, it is valid to conclude that the area under the ROC curve is significantly different from 0.5 (the null hypothesis), confirming that our proposed method was able to distinguish between cave wall and birds with high accuracy.

**Reliability test results**

Reliability estimation requires analyzing the quantitative results of the individual trial to test for the homogeneity (null hypothesis). The initial task was to obtain the necessary variables for consistency test by examining the ROC curves of the six experiments concurrently. It can be seen that the ROC curves of the six experimental studies approach the upper left corner of the plot (Fig. 8); this is interpreted to mean a high overall accuracy similar to the result obtained previously for the
accuracy assessment. The calculated P values for each pair of the area under the compared ROC curves range between 0.093 and 1.0, comparatively greater than the standard 5% (P<0.05) cut-off. Meaning that the estimated area under the ROC curves at 95% exact Binomial confidence interval (Philip, 2011) do not exhibit a significant difference.

The test for statistical significance did established that a relationship exists; however, accepting with completely 100% assurance that a relationship exists between the variables without controlling the sources of error increases the chance of taking wrong conclusions if the probability of being wrong is not estimated. For instance, bias assumption in research design, research methodology, sampling error, human mistakes, etc. (Borenstein et al., 2009) may affect the reliability of the findings. So, analysis of the overall effects and tests for heterogeneity does not only provide statistical measure of significance, but also, the extent of the relationship.

Results of the six trials, with 95% confidence interval, and the overall effect under the fixed and random effect models is shown in the forest plot (Fig. 9). In the figure, it can be seen that the all horizontal lines that define effect of the respective study at 95% confidence intervals overlap properly pictorially revealing agreement between the quantities. Similarly, the diamond marker symbolizes the summary effects. The position of the marker indicates the pooled effects size while the width which is the approximate 95% confidence interval given as

$$\theta \pm 1.96\sqrt{\frac{1}{\sum w_i}}$$

where $\omega_i$ being the inverse of the standard error of the individual trial (Petrie et al., 2003; Borenstein et al., 2009; Philip 2011). Both the fixed and random effect models have equal effect size of 0.980 (Table 5). On the basis of the result of these two models, it suffices to state that the outcome of the six trials are compatible, that is there is no heterogeneity. Nevertheless, being compatible does not mean that the results are consistent.

The test of heterogeneity with the two prominent statistical tools, Cochran’s Q and inconsistency $I^2$ (Table 5), provide a measure and degree of reliability. The Q-test yields a p-value of 0.384, commonly interpreted as being non-significant based on the suggested threshold of p<0.1 due to its low sensitivity to true heterogeneity (Higgins et al., 2003). The result implies that the null hypothesis of homogeneity is valid and therefore cannot be rejected, nonetheless, it does not provide evidence that there is no heterogeneity at all. So, the extent of the effect of heterogeneity was
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quantified using a measure of inconsistency. The value of $I^2$ shows a minor degree of inconsistency ($I^2 = 5.10\%$) among the trials that results from true heterogeneity. Practically, the result of the inconsistent test is also not significant enough to reject the null hypothesis. Consequently, the summary of all statistical tests point to the same conclusion that the test of homogeneity is not significant and therefore the null hypothesis (that all the studies observe the same effects) can be accepted. Ultimately, it is valid to conclude that the method that produced consistent effects across the six experimental trials is reliable.

Following the reliability test, the detected nests in each scan scene were also counted. The results obtained are: 4,143; 3,188; 3,881; 1,618; 3,526; and 2,297 in that order for image one to six. In all, a total number of 25,959 nests were counted from the seven images used to test this method.

Fig. 9. Area under the ROC curve of the six experimental trials at 95% confidence interval and the overall effects. The horizontal lines shows the coverage of the confidence interval with square markers that vary in size according to the weights assigned.

Table 5. Estimated overall effects and test for heterogeneity.

<table>
<thead>
<tr>
<th>Study</th>
<th>ROC area</th>
<th>Standard error</th>
<th>95% CI</th>
<th>Test for heterogeneity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test1</td>
<td>0.970</td>
<td>0.0121</td>
<td>0.946 to 0.994</td>
<td>Q = 5.2687</td>
</tr>
<tr>
<td>Test2</td>
<td>0.970</td>
<td>0.0120</td>
<td>0.946 to 0.994</td>
<td>DF = 5</td>
</tr>
<tr>
<td>Test3</td>
<td>0.990</td>
<td>0.00707</td>
<td>0.976 to 1.000</td>
<td>Significance level = P = 0.3840</td>
</tr>
<tr>
<td>Test4</td>
<td>0.965</td>
<td>0.0129</td>
<td>0.940 to 0.990</td>
<td>$I^2$ (inconsistency) = 5.10%</td>
</tr>
<tr>
<td>Test5</td>
<td>0.985</td>
<td>0.00863</td>
<td>0.968 to 1.000</td>
<td>95% CI for $I^2$ = 0.00 to 76.61</td>
</tr>
<tr>
<td>Test6</td>
<td>0.975</td>
<td>0.0111</td>
<td>0.953 to 0.997</td>
<td></td>
</tr>
<tr>
<td>Total (fixed effects)</td>
<td>0.980</td>
<td>0.00404</td>
<td>0.972 to 0.988</td>
<td></td>
</tr>
<tr>
<td>Total (random effects)</td>
<td>0.980</td>
<td>0.00412</td>
<td>0.972 to 0.988</td>
<td></td>
</tr>
</tbody>
</table>

CONCLUSIONS

Processing large volumes of point clouds currently faces enormous challenges that limit the use of xyz-point dataset for bird population inventory to quite very small areas. However, laser intensity images from high-resolution scanning offer potentially adequate feature characteristics to effectively extract information using remote sensing image analysis techniques. Our method demonstrates resourcefulness as an effective tool to suitably detect nests over large areas with high precision, and therefore expedites counting to the tune of tens of thousands of birds. The method can precisely detect nests in large number across wide areas for ecological studies, population census, and conservation management planning, but cannot be used to differentiate between species of birds. Contrary to the use of 3D point datasets for population surveys, this method is computationally efficient, timely, and may be useful in different caves for the same purpose.

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REFERENCES

Iosif Viehmann - a lifetime dedicated to cave and karst

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Iosif (Pepi) Viehmann, a former karst scientist in the “Emil Racoviţă” Institute of Speleology (Cluj, Romania), passed away on August 6, 2016 at age 90. Born on September 1, 1925 and raised in Cluj, his childhood, and especially his adolescence, were marked by turbulent historical events on the eve of World War II. Between 1946 and 1950, Pepi attended the Faculty of Natural Sciences at the „Regele Ferdinand I” University (now „Babeş-Bolyai” University) in Cluj. During his first year in college, he had the chance to meet and be inspired by Emil Racoviţă, who founded the world’s first Speleological Institute in Cluj in 1920. From Racoviţă, Pepi inherited the skills to develop scientific and popularization conferences peppered with charming stories and illustrated with slide projections covering many karst- and cave-related topics.

After graduation, Pepi spent five years as a Natural Science teacher at the Pedagogical School in Năsăud. During his tenure, he teamed up with another local teacher (L. Birte) and discovered the Tăușoare Cave (Rodnei Mountains) (Viehmann & Şerban, 1963; Viehmann et al., 1964b). Subsequently, in 1958, Pepi returned to Cluj to join the research group at the Speleological Institute. He dedicated more than 50 years to the exploration and study of several karst regions in Romania; the karst of the Apuseni and Rodnei mountains were closest to his heart.

The text below is a modified version of a note I wrote in September 2000 when celebrating our colleague and friend Pepi Viehmann on his 75th birthday. I realize now that this note best characterizes Pepi Viehmann’s activity, which identifies with the post-war beginnings of scientific speleology in Romania. His activity, longer than half a century, has been highlighted by many achievements in both exploration and publication. He was a member of teams that made exceptional cave discoveries and explorations during the ’50s and ’60s. Along with Mihai Şerban, Marcian Bleahu, Emilian Cristea, Dan Coman, Theodor Rusu, Corneliu Pleşa, Gheorghe Racoviţă, and Valentin Crăciun, he surveyed caves such as Pojarul Poliţei, Vârtop, Cetăţile Ponorului, Șesuri, Căput, Vadu-Crisului, Neagră, Gemânaţa, Tăușoare, Igeheabul lui Zalion, Iza, and Urşilor, to name only a few (Viehmann, 1967, 1963; Bleahu & Viehmann, 1963; Viehmann et al., 1964a, 1979, 1980). However, a brief examination of the references included in four different editions of Peșteri din România (Caves of Romania) reveals Pepi Viehmann’s name on numerous cave maps, indicating his significant contribution and assiduous field activity.

From a scientific point of view, Pepi Viehmann published more than a hundred papers in Romanian and foreign journals. Among these are monographs concerning karren and stream whirlpools (Viehmann, 1959a, 1964), as well as a series of studies on the genesis and morphology of cave pearls (Viehmann, 1957, 1959b, 1993). He is the main author of the
Pepi Viehmann's name is also linked to the sensational discoveries of the prehistoric human footprints in the clay of Ciur-Izboae Cave (Rusu et al., 1969; Webb et al., 2014) and in the hardened moonmilk of the Vârtop Cave (Casa de Piatră) (Viehmann, 1975b; Viehmann et al., 1982, 1996; Onac et al., 2005). He is also known for his thorough observations on the cave bear traces and their cohabitation with the prehistoric humans (Viehmann, 1987).

An excellent pedagogue and good mate during field campaigns, he led several series of students through the fascinating world of caves with professionalism. He shared his love of speleology with the help of charming slideshows and unforgettable caving camps. He helped anyone seeking advice, and was loved and appreciated by all. To his merit, Pepi not only discovered some outstanding Romanian caves, but he also continuously discovered people, who in turn discover and study new caves.

The boundless love and passion for the magnificent world of karst was the secret of his youth and power of work. All these characteristics made Pepi Viehmann a continuous stimulus for the young generation in the Speleological Institute and beyond. Pepi's death has deprived the karst community, jazz fans, and many others of his enthusiasm and charm, and he will be very much missed by his many friends and colleagues. He is survived by his wife, Alina, and his children, Radu and Cristina.

Selected books, chapter, edited (co-edited) volumes, and papers by Iosif Viehmann:

Şerban M. & Viehmann I., 1987 - Tendances de long terme dans la dynamique des formations de glace de la Grotte de Șcarisoara (Monts de Bihor). Theoretical and Applied Karstology, 3: 143-163.
Viehmann I., 1959a - Contributions à la connaissance de la génése des marmites. Speleologie, 1,3: 145-175.

Fig. 2. In October 2012, at age 87, Pepi returned to Pojarul Poliței Cave (photo: Ch. Ciubotărescu).