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New records of guano-associated minerals from caves in northwestern Borneo

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Abstract: Recent studies of ancient bat guano deposits in the caves of Gunung Mulu National Park and Niah National Park, Sarawak, Malaysia, have resulted in noteworthy records of phosphate minerals from these environments, including variscite, nano-particulate silica, fluorapatite, and niter.

Keywords: Niah, Gunung Mulu, Niter, Fluorapatite, Variscite

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INTRODUCTION

In tropical caves, some insectivorous bat species can form very large colonies that deposit huge quantities of guano over extended periods of time. These accumulations can reach tens of thousands of cubic meters in volume (Peck & Kukalova-Peck, 1981), 10 m or more in depth, and incorporate material dating back more than 50,000 years (Bird et al., 2007). In wet or moist conditions, guano decomposition can be rapid and the products of decomposition are often removed by percolating water. Conversely, under very dry conditions, bat guano may be preserved with little or no diagenesis over thousands of years (e.g., Wurster et al., 2010a). Over appropriate timescales, these deposits may generate a range of diagenetic nitrate, phosphate and sulfate minerals within a stratified, organic-rich sequence (Shahack-Gross et al., 2004; Pogson et al., 2014). Here we report on two phosphatic mineral species of distinctive appearance associated with bat guano diagenesis in caves of northwestern Borneo.

MATERIALS AND METHODS

Samples were taken from two caves in Sarawak, northwestern Borneo: Racer Cave (4.056 N, 114.827 E), Mulu National Park, and Niah Cave (3.816 N; 113.781 E), Niah National Park. Mineral composition and morphology was identified using X-ray diffraction (XRD; Attard Laboratories, San Diego, CA, US), and scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) at the Nano-imaging Facility of Carleton University, Ottawa, Canada.

RESULTS

Racer Cave (Variscite and nano-particulate Silica)

Racer Cave preserves several exposures of a distinctive, pink-colored mineral that has developed as an efflorescence, in association with a white powdery material, dating back more than 50,000 years (Bird et al., 2007). The pink phosphate exists as a thin crust (generally less than 1 mm in thickness) on the white material which proved to be silica (details below).

XRD spectral analysis on the pink mineral and automatic software identification suggests that it is a complex phosphate, dominated by variscite, and niter.

Fig. 1. Racer Cave phosphate: the pink material, revealed when the surface crust is peeled back, is variscite; the white powdery material is nano-particulate silica; and the pale-brown underlying material is guano. A) Macro view; B) General view.
AlPO₄·2H₂O, but with some gibbsite, Al(OH)₃, and berlinite, AlPO₄ (Fig. 2, Table 1). The semi-quantitative result indicates approximately 76% variscite, 13% gibbsite, and 11% berlinite. Note that the software did not identify any quartz in this material. The berlinite peak at 26.70º 2θ, standing out clearly from the two gibbsite peaks to either side, cannot be confused with a quartz peak, expected at 26.65º 2θ, and this material also does not have the other quartz peaks expected at 20.85, 36.54, and 40.29º 2θ (Morris et al., 1981).

At the micro-scale, the variscite crust is revealed to have an external morphology that is botryoidal, with globules of ~100-300 microns diameter (Fig. 3A, B). EDS on the surface of the crust confirmed that it is composed of variscite. The orthorhombic dipyramidal crystal structure of variscite (http://webmineral.com/data/Variscite.shtml) is here modified by the packing of crystallites (Fig. 3C). Internally, each globule can be seen to be made up of radiating blades

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(Fig. 4A, B) in what appears to be a druzy or spherulitic structure. Some of these show modifications that suggest stages in the alteration/erosion of variscite. Viewed from above, some blade edges are corroded and inter-crystallite spaces concomitantly increased (Fig. 5A); internally, especially towards the centers of the spherules, the bladed/platy structures show more-or-less chattering of the edges, in the vicinity of randomly-shaped powdery detritus that occupies the inter-blade spaces (Fig. 5B, C) and appears to be replacing the corroded variscite. This powder, which could not be pinpointed for a confident EDS analysis, is most likely the gibbsite shown by XRD analysis or, perhaps, berlinite.

The white material underneath the red variscite layer gave a clear XRD peak (Fig. 6, Table 2) of quartz, $\text{SiO}_2$, with some components of berlinite, nitratine, $\text{NaNO}_3$, calcite, $\text{CaCO}_3$, and chrysotile, $\text{Mg}_3[\text{Si}_2\text{O}_5](\text{OH})_4$. Note that in this case (in contrast to the variscite sample above) the software did identify quartz, and, although it also identified berlinite, here the peaks centered on 21.02 and 26.79° 2θ perhaps cannot be clearly distinguished from the quartz peaks at 20.85 and 26.65° 2θ.

SEM analysis showed that the material is largely made up of a loose powder (Fig. 7). EDS analysis on this yielded only O and Si, in proportions consistent with pure silica. The powder consists of tiny particles (Fig. 7A), some 100 nm in size (at the limit of resolution of our SEM). In places they appear to be organized into strings or domains (Fig. 7B, C) suggesting a colloidal structure (or potentially simply an artifact of drying). Although the SEM images are similar to many published of amorphous silica (e.g., Musić et al., 2011), the sharp XRD peak precludes both colloidal and amorphous silica. Therefore we identify this material as nano-particulate silica.

In summary, this Racer Cave deposit at the top of the guano is made up of an upper layer of red botryoidal/spherulitic variscite, some of which is slightly corroded/ altered to gibbsite or berlinite, and an underlying layer of white nano-particulate silica.

**Niah Cave (Fluorapatite)**

Niah Cave contains extensive and deep deposits of stratified bat guano, dating through and beyond the radiocarbon age range of >40,000 years (Dykes, 2007). An exposure of the guano sequence, a vertical face created by guano mining, probably within the...
past 100 years, has developed a distinctive blue/green efflorescence (Fig. 8). XRD analysis (Fig. 9, Table 3) demonstrates that the material is fluorapatite, Ca$_5$(PO$_4$)$_3F$, along with gypsum, and niter, KNO$_3$. The bright blue color comes from the fluorapatite, the other components being white.

SEM analysis shows a fine powder overlying and interspersed with tabular/platy/foliated crystals (Fig. 10). EDS analysis of the powder, composed mainly of O, P, and Ca, is consistent with the composition of fluorapatite but with very small components of clay minerals or silicate minerals, indicated by tiny amounts of Na, Al, Si, Cl, and Fe. The powder lies between and over the crystals (Fig. 10A, B). The fluorapatite powder is very delicate, burning very soon after the electron beam hits it, and transforming into amorphous clouds. The images at higher power (e.g., Fig. 11A) are necessarily of low quality because they had to be taken as rapidly as possible. The powder looks like nano-particles in most images, ~500 nm long and ~200 nm in diameter; they are revealed as six-sided crystals only in some of the highest magnification images. The arrangement of the nano-crystals has a suggestion of order, such as in biofilm, with domains of attached nano-crystals in strings and curved pouches like alveoli (Fig. 10C).

The large platy crystals proved to be gypsum, with EDS analysis showing O, S, and Ca as the only components, in proportions consistent with gypsum. The micro-scale morphology of the crystals (tabular crystals, breaking up into flakes, Fig. 10C) mirrors macro-scale crystals of gypsum or anhydrite blades fragmenting at the edges into delicate fibers (e.g., they

Table 2. XRD data for white powder underneath variscite layer in Racer Cave.

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Fig. 7. A, B, and C) Three SEM images of the white powder, nanoparticulate silica, underneath the variscite layer in Racer Cave.
are very similar to the gypsum/anhydrite crystals depicted in Oliveira, 2013, or in Rauh & Thuro, 2006, or in http://publications.iodp.org).

This sample showed ample evidence of bacterial colonization of the surfaces of much of the gypsum. Rod-shaped bacteria (bacilli) can be seen occupying rod-shaped depressions in the surface of the crystal (Fig. 11A-C). It is not clear from the SEM images whether the bacilli are directly involved in the precipitation of the fluorapatite.

No niter was detected in the surfaces visible to SEM.

**DISCUSSION**

Variscite has been reported from multiple caves, and is associated with guano decomposition (Hill & Forti, 1997; Onac et al., 2004). Acidic, phosphate-rich solutions released from the guano by microbial action react with aluminum-rich clays present as underlying fluvial sediments or cave dust, to produce the hydrated aluminum phosphate. The Racer Cave sample shows no evidence of bacteria under SEM, suggesting that the variscite develops from simple chemical/evaporation processes. Absent the bat colony, strong airflow generates evaporation and a chromatographic, upward-wicking effect in the drying guano mass. The result is the sequential deposition of minerals in order of solubility, the less soluble silica, followed by the more soluble variscite. The alteration to gibbsite would then be a secondary process. The physical evidence of alteration and replacement of variscite suggests that the deposit may be short-lived.

Berlinite is a rare mineral in cave contexts, known only from Cioclovina Cave in Romania (Onac & White, 2003; Onac & Effenberger, 2007). In metamorphic and synthetic contexts, berlinite is a high-temperature mineral developing at temperatures above 550°C (Onac et al., 2007), leading these authors to infer spontaneous combustion of the guano deposit in Cioclovina Cave. The presence of berlinite as an efflorescence in the moist tropical environment of Racer Cave is incompatible with the combustion hypothesis. While our XRD analysis is strongly suggestive of the presence of berlinite in Racer Cave (and therefore the second reported occurrence of this mineral in a cave situation), the evidence is perhaps not conclusive. However, we argue that the absence of evidence for burning does not necessarily preclude the presence of berlinite. We suggest that in the Racer Cave situation, berlinite is almost certainly the product of microbial biochemical reactions on the surface of the parent variscite crystals, transforming to berlinite by loss of water. Similarly, we interpret the Racer Cave gibbsite, previously reported in association with guano in Green Cave, Malaysia (Laverty, 1982) to be a decomposition product of variscite. This interpretation is consistent with the observations of Banfield et al. (1999) who report that

**Table 3. XRD data for Niah Cave mineral.**

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**Table 3. XRD data for Niah Cave mineral.**

![Figure 9. XRD spectra, Niah Cave fluorapatite-gypsum-niter.](image-url)
microbes contribute to enhanced dissolution of insoluble secondary phosphates, possibly by release of organic acids. Banfield et al. (1999) also report that variscite is microbially precipitated. Redox conditions are important: Wisawapipat et al. (2017) observed that gibbsite was transformed into variscite during soil reduction, and back-transformed during soil re-oxidation. These varying conditions can be expected to occur during the transition from water-saturated, anaerobic guano beneath an active roost to dry, more aerobic conditions in relict guano deposits and the efflorescence of phosphate minerals into a fully aerobic cave atmosphere.

Silica has been reported (as quartz) from a number of bat guano deposits in peninsula Malaysia, Borneo, and the Philippines (Wurster et al., 2015). Amorphous silica was reported by Pogson et al. (2014) from the Jenolan caves, Australia, where it was attributed to the breakdown of guano. No publications could be found reporting nano-particulate silica in caves, although many studies have been done of such material in other natural systems. Tobler et al. (2009) report that silica nano-particulate formation in natural systems generally starts with silica polymerization and nucleation, followed by particle growth and “ripening”, and, finally, particle aggregation. The maximum particle size is about 8 nm. They also report that in most natural waters nano-particles do not form because aggregation tends to occur before the ripening stage.

The ultimate source of this silica is unknown, although, noting that other accounts of amorphous silica in the literature all relate to biogenic precursors such as cow dung, or corn husks, e.g., Rani et al. (2014), it is likely to be simply part of the geochemical cycling. Silica is a normal component of plant tissue (especially grasses), occurring in the form of hydrated condensates of orthosilicic acid, Si(OH)₄, linked to biomolecules (Smis et al., 2014). Most plants have up to 2% Si (by dry weight) but Smis et al. (2014) report a maximum of 7.8% in horsetails. This plant-sourced silica would be passed through the food chain up to and through the bats, and thereby disseminated in the guano.

Fluorapatite is known from several other cave localities, e.g., Slaughter Canyon Cave, New Mexico (Hill & Forti, 1997), and Lighthouse Cave, Bahamas (Onac et al., 2001), where it is invariably associated with guano or fossil bone deposits. The origin of the fluorine in bat guano has not been previously investigated, but it has been reported as a significant component of ornithogenic soils such as those associated with penguin rookeries (Tatur, 1987), where it complexes with calcium phosphates, and as a constituent of Peruvian bird guano (Hutchinson, 1950). Fluorine is not homeostatically regulated.
in mammals (Buzalaf and Whitford, 2011) but is excreted primarily in urine (Massman, 1981) and absorbed into bone by substitution of F− for OH− in the hydroxyapatite matrix (Bertonia et al., 1988). Mammalian bone can be a significant accumulator of environmental fluorine; in humans, fluorine content of bone increases linearly with age at a rate of 0.08 ± 0.01 mg F/kg Ca per year (McNeil et al., 2016). Long-lived mammals such as bats (e.g., Tadarida brasiliensis, a sister species to Chaerephon plicatus at Niah and Mulu, lifespan ~10 years; Brunet-Rossinelli & Austad, 2004; Weigl, 2005) have ample opportunity to accumulate fluorine. Some plants are known accumulators of fluorine, e.g., tea (Camellia sinensis), where levels of 1243 ± 573 mg F/kg dried, freshly-harvested leaves of tea have been recorded (Lu et al., 2004). Fluorine in plant tissues would be passed to herbivorous insects. Fluorine concentrations in insect chitin have not been explicitly studied, but can be expected to be low since the ions would not be retained by the N-polysaccharide matrix. However, fluorine in insect tissues would be absorbed onto bat bone and excreted in bat urine. The appearance of fluorapatite in the post-mining excavation of old, dry guano at Niah, long after exposure to significant quantities of bat urine, suggests that the original source is bioaccumulated fluorine in the abundant bat bone of the deposit.

The presence of niter in the Niah guano deposit is expected. Fresh Free-tailed bat (Chaerephon plicatus) guano from Deer cave, Mulu contains 7.6% nitrogen, which is progressively released in acidic solution by microbial decomposition (McFarlane et al., 2017). Although at least 11 nitrate mineral species are known from guano-associated cave deposits (Hill & Forti, 1994), most are highly deliquescent and only niter can crystallize at relative humidities of 85% or higher (Hill, 1981) and are thus likely to occur in caves of the moist tropics.

ACKNOWLEDGEMENTS

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Moonmilk as a human and veterinary medicine: evidence of past artisan mining in caves of the Austrian Alps

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Abstract: The use of moonmilk for medical and other purposes in the Alps is documented since the 16th century. This article reviews speleological reports and sparse historical accounts about the extraction of moonmilk from 18 caves in the Eastern Alps of Austria in an artisan mining style. One such example from a cave in Tyrol is documented in detail, where moonmilk was mined until the beginning of the 20th century and which, due to its remote location, uniquely preserved traces of both the mining and processing style.

Keywords: speleothem, moonmilk, mining, medical use, history, Alps, Austria
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INTRODUCTION

One of the most widespread types of speleothems in caves worldwide is moonmilk. This white, water-rich material can be mineralogically complex (Hill & Forti, 1997), but mostly consists of microscopic fibres of calcite (e.g., Borsato et al., 2000; Richter et al., 2008; Cailleau et al., 2009). Recent studies have shown that moonmilk contains a wide variety of microorganisms including bacteria, fungi and archaea (e.g., Barton & Jurado, 2007; Engel et al., 2013; Reitschuler et al., 2015, 2016; Maciejewska et al., 2016). These microbial communities are thought to play a key role in carbonate precipitation (Blyth & Frisia, 2008; Maciejewska et al., 2017).

Another aspect that sets moonmilk apart from other types of cave deposits is its use as a veterinary and human medicine in former centuries. One of the oldest historical documents is Konrad Gesner’s report (1555) about Montmilchloch near Lucerne, Switzerland, where moonmilk was extracted by locals and used as a remedy. Scattered reports exist about this practice in other regions of Central Europe and in particular in the Alps (e.g., Shaw, 1992). Moonmilk at that time was known as Lac lunae or Nihilum album, although dozens of other synonyms exist (Heller, 1966). Nihilum album is in fact the old pharmaceutical term of zinc oxide (e.g., Hahnemann, 1798; Richter, 1832; Moll, 1839). Its colour and powdery appearance are similar to (dehydrated) moonmilk. Zinc oxide is known for its antibacterial and antifungal properties (e.g., Sawai & Vijayaraghavan, 2008) and, for hundreds of years, was widely used to treat a variety of skin conditions and eye diseases (e.g., Lonitzer, 1578) until the discovery of antibiotics in the first half of the 20th century. On the other hand, it is still unclear to what extent moonmilk has pharmaceutical effects. In fact, most previous authors have supposed that it has little or no such effect and was an intended or unintended substitute for zinc oxide (e.g., Kyrlé, 1923; Binder, 1963; Trimmel, 1968; Reinbacher, 1994).

Interestingly, despite a large number of mostly concise reports mentioning the use (or abuse) of moonmilk, little systematic research has been done, the etymology of this peculiar term being a notable exception (Reinbacher, 1994, 1995). Here, I review the sparse accounts and hints about mining activities for moonmilk in caves of the Eastern Alps of Austria and document one such cave which, due to its difficult access, uniquely preserved traces of former moonmilk extraction including relics of the primitive tools and techniques used.

MOONMILK CAVES IN THE ALPS

There are hundreds if not thousands of caves in the Alps containing moonmilk, either as an actively forming, i.e. soft deposit, and/or as (partly) dehydrated, hard or crumbly crusts coating cave floors, walls and ceilings or other speleothems. Moonmilk deposits are commonly between a few millimetres and several centimetres thick, but reach up to about 1 m in places (Pavuza, pers. comm., 2018). These formations occur...
in a variety of geometries, from planar deposits to mammillary and stalactitic forms. The vast majority of these deposits consists of calcite. A systematic study in the Southern Alps (Borsato, 1996) showed that moonmilk is most abundant in caves between about 1,400 m a.s.l. and the upper limit of the timberline (i.e., close to 2,000 m a.s.l.). Moonmilk is rare in the twilight zone close to the entrance, and locally also forms subaqueously in shallow pools (so-called cottonballs).

The German expression for moonmilk in the western part of the Alps (mainly Switzerland) is Montmilch, while the term Bergmilch is common in the eastern part (Austria). The latter term is etymologically rather young and first appeared at the end of the 18th century (Heller, 1966, p. 56; Reinbacher, 1995, p. 19). In previous centuries, the synonym Nix was widely used and is still in use in Austria. This word is derived from Nihilum album and appears in historical accounts since the 17th century (Heller, 1966, p. 56; Reinbacher, 1995, p. 19). In previous centuries, the synonym Nix was widely used and is still in use in Austria. This word is derived from Nihilum album and appears in historical accounts since the 17th century.

HISTORICAL REPORTS ABOUT MOONMILK MINING IN AUSTRIAN CAVES

Figure 1 provides an overview of caves in the Austrian Alps whose (German) names indicate the presence of moonmilk. Highlighted are those sites where either written reports or in-situ observations of moonmilk mining exist. Below I summarize the available information about those caves where mining evidence is rather compelling, starting with the sites in the west and grouped according to the Austrian provinces, whereby the westernmost site and the only one in the province of Tyrol (Nixofen, 1264/9) will be discussed separately in the next section. The number in parentheses behind the cave name is the cave number based on the Austrian cave register.

Salzburg

Nixloch (1331/17) is a small, 32 m-long cave near Weißbach bei Lofer, whose entrance lies at 800 m a.s.l. Czoernig-Czernhausen (1926, p. 126), Waldner (1942, p. 3) and Klappacher & Knapczyk (1977, p. 181) reported that the moonmilk deposits from this cave were used by locals as animal medicine.

Scheukofen (1335/4), a 1,400 m-long cave that opens at 740 m a.s.l. in the Salzach Valley on the eastern flank of Hagenegbirge, is an important site where moonmilk, present in the side chambers of the large entrance hall, was mined until the 19th century (Klappacher & Knapczyk, 1979, p. 143). The report from AD 1650 by the administrator at the guardianship court in Werfen mentioned above is not just the oldest known official document about primitive mining of moonmilk in the Eastern Alps; in spite of its shortness, it also gives some hints...
about the style of these activities. Importantly, the cave and its moonmilk deposits were apparently unknown to the authorities who became suspicious as a result of folk stories, e.g. about treasures in this cave guarded by mountain trolls. On behalf of the archiepiscopal counsellor, the administrator made inquiries about this cave. He learned that crowds of people, both citizens and foreigners, knew about the cave and had entered it (some of them secretly). They removed a white substance from the cave and sold it to the local pharmacist (Vierthaler, 1816, p. 181).

The document even reports how much money these people made per sold pound: the pound moonmilk was sold for 4 Kreuzer. A common way to compare historical prices to present-day currencies is to take a common commodity for which there are records of prices over the centuries. E.g. in 1650 the price for one pound (half a kilogram) of beef in Vienna was about 3 Kreuzer (http://www.habsburger.net/en/glossary/what-could-viennese-buy-their-kreuzer-and-gulden). In the 17th century 1.4 litres of wine costed between 10 and 16 Kreuzer in Linz (Rumpl, 1962, p. 339).

While no details are given about the style of mining in Scheukofen, it is interesting that the administrator had trouble finding people who were ready to provide information about the cave and its location. Several witnesses disappeared or pretended not to know the whereabouts of this cave (Klapptacher & Knapietzky, 1979, p. 147). Only an old miner told the administrator that the last time he visited the cave was some fifty years ago, when he searched for ore but only found two skulls. The overall impression this report from the 17th century conveys is that several people knew about the cave and its moonmilk but that this information was kept secret.

Schotterloch (1528/2) is a short, only 12 m-long cave at 830 m a.s.l. east of Fuschl. Waldner (1942, p. 8) reported that locals were still visiting this cave to obtain moonmilk in the 1940s, which was being used by farmers to cure intestinal diseases in cattle. Interestingly, there is no mention of these activities in other speleological accounts, e.g. Czernig-Czernhausen (1926, p. 47) or Klappacher (1992, p. 338).

Another moonmilk-bearing cave is Nixloch (1532/1) located 2.5 km northeast of Schotterloch on the Drachenwand (1,100 m a.s.l.). While neither Czernig-Czernhausen (1926, p. 45-46) nor Waldner (1942, p. 3) mentioned traces of mining, Klappacher (1992, p. 346) stated that moonmilk was extracted from this cave for centuries.

Upper Austria

Nixlucke im Annerlgraben (1567/14) is a 150 m-long cave located west of Ebensee, whose entrance opens at 755 m a.s.l. Franz Kraus, an eminent Austrian speleologist and author of the first book on cave science published in German (Kraus, 1894), visited this cave in 1879 guided by an old man whom he referred to as Nixgräber (“moonmilk digger”). Three other men joined them; one of them was a licenced local mountain guide. It is telling that this person had heard about the cave but did not know its location. Kraus described the difficult access to this cave, its narrow entrance and the presence of a wooden ladder which was installed by the Nixgräber seven years earlier (Kraus, 1880, p. 79).

Kreidelucke (1682/2) is a well-known cave rich in moonmilk, located near Hinterstoder. It has a total length of 1,042 m and opens at 580 m a.s.l. Hauenschild (1866, p. 361-362) organised an expedition into the cave and described traces of moonmilk extraction, including deep holes left by mining, steps carved into the moonmilk, and old wooden ladders and inscriptions. He mentioned that moonmilk was sold also to cattle dealers who added it to the forage to make farm animals, in particular horses, look stronger. Gressel et al. (1951) studied the cave and its sediments and noticed clear traces of moonmilk mining. They were told by an informant that moonmilk was used until recent times as a raw material for the production of chamotte.

Nixlucke (1664/15) is a cave on the northern side of Sengsengebirge that opens at 1,470 m a.s.l. The walls of this 36 m-long cave are partly covered by moonmilk and show clear cut marks that were created during the former extraction of this material (Weichenberger, 2000, p. 134).

Lower Austria

Southeast of Göstling, Krähenloch (1815/91) opens at 760 m a.s.l. Hartmann & Hartmann (1985, p. 125) reported traces of moonmilk extraction in this 100 m-long cave.

Galmelioch (1816/4), 226 m long and located at 1,346 m a.s.l. WNW of Mariazell was also visited in former times in order to extract moonmilk. Waldner (1942, p. 5-6) reported that this cave was still being visited by people looking for moonmilk and locals told him that moonmilk was commonly used to clean cutlery. Hartmann & Hartmann (1985, p. 172) mentioned the presence of wooden ladders as evidence of the primitive mining activity.

WSW of Frankenfels is another former Nixbergwerk (“moonmilk mine”), Mariannenhöhle (1836/18, 437 m, 636 m a.s.l.). In addition to clear traces of moonmilk extraction, Waldner (1942, p. 4) reported black characters made by the diggers using torches, whereby crossed strokes and scissors-like signs were the most common ones.

Nixhöhle (1836/20) is a show cave located WSW of Frankenfels that is 1,410 m long and opens at 556 m a.s.l. According to locals this cave was visited by “moonmilk diggers” who carried the material to Mariazell and probably sold it there (Waldner, 1942, p. 4). Hartmann & Hartmann (1982, p. 74) also mentioned traces of moonmilk mining, and the use to 1 m deep cuts in the moonmilk deposit were later made in the course of the development to a show cave (Pauza, pers. comm., 2018).

The eponymous Nixhöhle (1834/9, 73 m long, 695 m a.s.l.) SSW of Türnitz was a moonmilk mine whose thick deposits show clear traces of extraction of this material (Waldner, 1942, p. 4; Hartmann & Hartmann, 1982, p. 37).

Stadelbauernhöhle (1866/12) is 70 m long and its entrance opens at 860 m a.s.l. south of the village
Traisen. Waldner (1942, p. 5) reported abundant traces of mining activity and found remnants of old mining tools on the floor. Interestingly, the local farmers did not know about this cave and its moonmilk deposits, and nobody was able to provide information about the cave. Waldner did not report the name of the cave, but it is undoubtedly Stadelbauernhöhle (cf. Hartmann & Hartmann, 1982, p. 129).

Another cave where moonmilk was found and used in the past is Nixofen (1867/10), a 34 m-long cave located at 840 m a.s.l., NNW of Gutenstein. Hartmann & Hartmann (1982, p. 160) referred to it as an old Nixbergwerk.

The easternmost cave with clear traces of moonmilk extraction is Hermannshöhle (2871/7) at Kirchberg am Wechsel. This 4.4 km-long labyrinth cave has long been known and opens at 620 m a.s.l. Jäger (1873, p. 250) reported that tests had shown that the dazzling white speleothems (which include moonmilk) can be used in the production of various things including soda water, paper and Gutta-percha. He quoted a price of 3 Gulden per 50 kg which is quite inexpensive when compared to other products and services at that time (Mrkos, 1997). Although there are traces of moonmilk mining at Weiße Kluft and near the Teich (Ilming, 1973) as well as above Dietrichshalle (Plan, pers. comm., 2018), the extracted volume was apparently small. Topitz (1974, p. 200) reported that local farmers used this moonmilk to prepare eyewashes.

**Carinthia**

The only site currently known in the southern part of Austria where moonmilk was extracted is Nixlucke (2753/1) near Klippitztörl. According to an old report, local farmers frequently went into this sub-horizontal 250 m-long cave, whose entrance is located at 1,545 m a.s.l., and obtained moonmilk that they used as veterinary medicine (Reiner, 1857, p.145). Several inscriptions can be found in the cave, some dating back to the 18th century. According to Waldner (1942, p. 3) this cave was visited more frequently in past centuries than in recent times.

**Bavaria (Germany)**

One site close to the Austrian border SSW of Salzburg is Nixloch bei Hallthurm (1339/39), which opens at 723 m a.s.l. and is 163 m long. According to Waldner (1942, p. 2), this cave was still visited by “moonmilk diggers” during the first half of the 20th century.

**CASE STUDY: NIXOFEN**

The only cave where moonmilk was extracted in the western part of Austria and probably one of the best-preserved sites in the Austrian Alps is Nixofen (1264/9) in Brandenberg in Tyrol (Fig. 1). It opens at 1,283 m a.s.l. on a steep north-facing slope (Fig. 2) that can only be accessed during snow- and ice-free conditions. Due to its remote location it is rarely visited, even by local cavers. The cave developed along the intersection of a fault and a bedding plane in thick-beded Triassic limestone and has a simple, slanted geometry with the rear part being the highest (Fig. 3). Moonmilk is abundant in the middle and rear parts of the cave and comprises soft, sheet-like deposits typically 10-20 cm in thickness and moonmilk stalactites and columns that reach up to 2 m in length and up to about half a meter in diameter (Fig. 4A).
This Nixbergwerk is unique in the sense that it contains well-preserved traces of moonmilk mining as well as the remnants of primitive tools and devices used for mining and processing of moonmilk. Mining traces are found primarily in the rear part of the cave and show short linear features indicating that some sort of hoe was used to chop off moonmilk (Figs. 4B-4D). The miners proceeded in a rather systematic manner and mined the moonmilk from below, working their way up the slope in the rear part of the cave. Close inspection of the hoe marks shows that they have since been overgrown by new moonmilk forming a layer up to about half a centimetre in thickness (Fig. 4E). The innermost part of the cave is still fairly pristine and contains white moonmilk up to 40 cm thick, indicating that the operation had ceased before all the moonmilk was extracted. The 10-20 cm thick moonmilk deposit on the inclined slope of the cave was the primary target of the miners, but at some places they also tried thick stalactites as shown by hoe marks (Fig. 4F). These moonmilk stalactites consist of partly dehydrated moonmilk that was apparently more difficult to mine than the soft variety. On the steep slope the miners made narrow horizontal terraces up to a few meters long in order to better access the moonmilk and because moonmilk-covered slopes are slippery.

Wooden tools are still preserved in the middle part of the cave (Fig. 5A) and in two places there are also rotten logs and wooden sticks that were obviously transported into the cave. The tools comprise (a) a wooden barrow (mortar trough) to be carried by two people, which was apparently used to transport the wet moonmilk to the flat middle part of the cave where it was processed, (b) a wooden tray, again probably used to transport moonmilk (has meanwhile

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**Fig. 4.** A) Moonmilk columns and stalactites in the rear part of Nixofen. The column in the centre is almost 2 m tall; B) Sheet-like moonmilk (view upslope), which has largely been extracted except for the uppermost part. Width of image about 4 m; C) Close-up of slope just beneath (B) showing hoe marks at the base of the former moonmilk deposit and traces of old wood fragments. Width of image about 0.5 mm; D) Lateral hoe marks produced by a tool with a round edge. The entire deposit was mined exposing the bedrock underneath (white area in the foreground). Glove for scale; E) Close-up of (D) showing newly formed moonmilk covering the hoe marks. Width of image about 20 cm; F) Hoe marks at the base of a moonmilk stalactite partly overgrown by new moonmilk. Width of image about 0.7 m.
disappeared), (c) a spade, (d) a wooden mallet and (e) a wooden ladder (Fig. 5B). Previously, an old hobnail leather boot was also found there. Interestingly, the shoe was rather small (about European size 37, i.e. about 23 cm long: Krejci et al., 1982). Of particular interest are the remains of a primitive facility to process mined moonmilk. A breakdown block with a flat inclined surface was used to dry moonmilk formed into hand-sized balls. For this purpose, vertical holes were drilled into the rock in order for short wooden sticks to hold horizontal wooden sticks that acted as shelves. This wooden structure is still preserved in the cave and the fact that these sticks are not rotten demonstrates that this spot was well selected by the miners to dry moonmilk. A photograph taken in 1977 still shows several moonmilk balls on these shelves (Fig. 5C) indicating that the mine was abandoned in a somewhat disorganized manner. The moonmilk balls have since been removed by occasional visitors and a few can still be found in private collections (Fig. 5D).

No historical documents exist about this Nixbergwerk and it was apparently forgotten. Georg Mutschlechner, the doyen of the mining history in Tyrol who had searched for past mining activity in Brandenberg did not mention this cave (Mutschlechner, 1975). According to Georg Auer (Brandenberg, pers. comm., 2017), the critical information for the re-discovery came from an old local (Josef Hintner, born in 1887) who saw the cave at the age of 14, i.e. at the beginning of the 20th century, when it was still in operation. In his old days he still remembered how to reach the cave and in 1977 Georg Auer and four other locals found it after a long search and took the first photographs.

The style of moonmilk mining in Nixofen suggests a multi-annual operation restricted to the summer/fall season when this site could be accessed safely. Very few people probably knew about this activity and the authorities had no information about it. Due to the lack of written and oral documentation we can only speculate that the miners worked in this Nixbergwerk for short intervals, possibly slept in the cave, processed a batch of moonmilk balls and then left the cave with rucksacks full of dried moonmilk balls. The final product was most likely sold to a pharmacy. Back in the 19th century, the closest one was located in Rattenberg in the Inn Valley to the south of the cave, which is approximately a 20 km-long hike (depending on the route).

The amount of moonmilk extracted from Nixofen is difficult to assess. Using the conservative assumption of an average thickness of the deposit of 10-15 cm, the cave survey suggests that between 4 and 7 m$^3$ of wet moonmilk was mined in total. Given the small dimensions of the drying facility (and the unknown time required to dehydrate the moonmilk so that it could be transported and sold - likely months), this volume of mined moonmilk suggests that the Nixbergwerk was in (seasonal) operation for decades. The precise timing and reason for its abandonment are unknown, but the available information and in-situ observations point to an unplanned end early during the 20th century, possibly related to the fact that most men were drafted into the Austrian army during WWI and many never returned.

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Fig. 5. A) View from the middle part of the cave towards the entrance. Wooden tools lean on the left slope and remains of the drying facility can be seen on the right side; B) Primitive tools left by the moonmilk miners; C) View of the drying facility showing a series of ball-shaped moonmilk chunks left by the miners. Width of image about 2 m. Photograph taken in 1977 (courtesy G. Auer); D) One of the very few remaining (and meanwhile completely dehydrated) original moonmilk balls.
DISCUSSION AND CONCLUSIONS

The picture that emerged from the study of Nixofen is in many ways consistent with the scattered and scarce information available from other sites in Austria:

- Extraction of moonmilk was common practice and the majority of these Nixbergwerke (17) existed in the eastern segment of the Northern Calcareous Alps (Fig. 1). Only one site is currently known in the southern part of Austria.
- All mines were small operations involving probably less than a handful of informed people.
- This artisan-type of mining used primitive, but nonetheless effective techniques of moonmilk extraction.
- The wet moonmilk had to be dried and this was accomplished by forming hand-sized balls and letting them dry on wooden racks. Interestingly, according to an old encyclopedia, Nihilum album (the true zinc oxide) was also sold as balls in past centuries (Hübner, 1721, p. 874).
- No official maps, reports or photographs exist about the work in these Nixbergwerke and all reports indicate that these activities were intentionally kept secret (e.g., Waldner, 1942, p. 2)
- As has been suggested previously (e.g., Topitz, 1974), experienced prospectors (termed Nixsucher or Nixgräber) apparently searched for moonmilk deposits in caves in the Eastern Alps and started these operations.
- Nixbergwerke were most likely worked during the snow-free season only.
- The timing of the onset of moonmilk mining in the Alps is still hidden in the darkness, but certainly goes back at least half a millennium (cf. Gesner, 1555). The oldest reliable report from Austria is one hundred years younger (Scheukofen, 1650).
- Nixbergwerke in Austria were in operation until the first half of the 20th century. This is in contrast to Shaw (1992, p. 224) who concluded that the medical use of moonmilk ceased about the middle of the 18th century in the western world. Due to the lack of historical data it can only be speculated that the abandonment of the Austrian sites was at least in part related to the advent of antibiotics, which are much more efficient than the previously used zinc oxide (and moonmilk).
- The use of moonmilk, however, was not restricted to human medical purposes; it was widely applied also to cure animal diseases. In addition, people used it for a variety of other purposes (e.g., Mattes, 2015, p. 108).

OUTLOOK

For a long time it has been supposed that dried moonmilk was used as an essentially illegal substitute for zinc oxide – with little or no pharmaceutical effect. Already Gesner (1555, p. 66) and later Kappeler (1767; reprint 1967; see also Jans, 1983) ridiculed that superstitious people use moonmilk to cure any sort of disease. On the other hand, the Swiss pharmacist Sidler (1939/40, p. 228), who studied the use of moonmilk, concluded by quoting a local physician, Hans Portmann, that it would be interesting to examine moonmilk closely, because our ancestors were known to be sensitive observers.

Although quackery, superstition and placebo were certainly involved in the century-long business of moonmilk as a medical product, it is interesting to note that recent microbiological studies suggest that moonmilk might “effectively treat various infectious diseases thanks to the presence of a highly diverse population of prolific antimicrobial producing Streptomyces, and thus may indeed constitute a promising reservoir of potentially novel active natural compounds” (Maciejewska et al., 2016, p. 1-2).

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Replication and reinsertion of stalagmites sampled for paleoclimatic purposes

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Abstract: Sampling stalagmites for paleoclimatic study can enter into conflict with preserving the beauty and integrity of caves. To minimize this impact, a variety of sampling strategies have been used by researchers aware of cave-conservation issues. Based on our experience in two caves (El Soplao and La Buenita, Cantabria, N Spain), we propose to apply molding and casting laboratory techniques to create replicas of stalagmites, placing the replicas back in the original cave locations so that the impact of sampling to the cave is severely reduced. We provide detailed descriptions of the molding and casting methods, which vary depending on stalagmite size. For relatively small specimens (less than ~35 cm tall), we use a single-piece mold and two jackets. For larger stalagmites (~40-70 cm tall), we use a two-piece mold and two jackets. In a first casting step, we obtain a master piece in dental plaster that is preserved. In a subsequent casting step, we use epoxy resin to generate the replica that will be placed in the cave. We use extra-hard plaster coated with epoxy resin to fix the replicas to their original substrates. Both the epoxy resin and plaster are carefully dyed to match the original surface texture and color of the sampled stalagmites. Once in place, the stalagmite replicas are almost indistinguishable from the natural specimens.

Keywords: speleothem sampling, stalagmite reinsertion, molding and casting, paleoclimatology, geoethics

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INTRODUCTION

Stalagmites can preserve valuable paleoclimate information with high temporal resolution compared to other proxy records (e.g., marine or lacustrine sediment cores), and provide important paleoclimate information for most continental regions (Fleitmann & Spötl, 2008; Fairchild & Baker, 2012). Also, stalagmites can be precisely dated using U-series methods. Therefore, stalagmites are increasingly being used for paleoclimatic research, as there is a need to understand the climatic past in order to evaluate the magnitude, causes and consequences of the present day climate change.

The paleoclimatic study of a stalagmite normally requires its removal from the cave for petrographic and geochemical analysis, hence disturbing to some extent the integrity of the cave and potentially creating a conflict between scientific sampling and cave conservation (Springer, 2012; Truebe, 2013).

The paleoclimatic interpretation of stalagmite records is not straightforward, partly because they may be influenced by local factors that overlap with climatic controls. Therefore, some stalagmites may not be ideal for paleoclimatic study. Even in the case of stalagmites showing textures consistent with the preservation of paleoclimatic signals, it is advisable to obtain at least two coeval stalagmite records from the same cave or region to test their sensitivity to local versus climatic factors (i.e., a replication test; Dorale et al., 2002; Dorale & Liu, 2009). In addition, the number of sampled stalamites may increase if they contain abundant detrital material, which complicates U-Th dating (Hellstrom, 2006), or specimens are affected by diagenetic alteration.

Therefore it is clear that most paleoclimatic studies based on stalagmites normally require sampling several specimens. Because the number of stalagmites in a cave is finite, sampling conflicts with cave conservation, especially for caves containing few speleothems. Therefore, the sampling strategy must be selective and trying to reach a compromise between the scientific goal and cave conservation issues. For instance, the shape and diameter of stalagmites may provide clues about their usefulness for paleoclimatic studies. This allows a screening,
and commonly narrows the search down to those with constant diameters of about 11 cm or more (Dreybrodt & Romanov, 2008; Dreybrodt & Scholz, 2011).

To minimize the impact of stalagmite sampling, researchers have developed a variety of strategies. Frappier (2008) designed a screening strategy aimed to select paleoclimate-sensitive stalagmites. For instance, this author recommends the preferential sampling of already broken specimens due to natural causes (collapses), accidents, or modern or historical vandalism. This approach was followed by Domínguez-Villar et al. (2009) and Baldini et al. (2015), among others. Another strategy is coring the central part of stalagmites, either in the cave (Brook et al., 2006; Verheyden et al., 2006; Yang et al., 2007) or in the laboratory, in the latter case placing the drilled specimens back in their original location in the cave (Dorale et al., 1992). In any case, patching the drill holes is simple. Details of these methods are rarely published, with the notable exception of Spötl & Mattey (2012).

Drilling stalagmites minimizes the visual impact of sampling. However it has some disadvantages. The best paleoclimatic records are those obtained from the axis of maximum extension (Dreybrodt & Scholz, 2011). The growth axis of a stalagmite can change in time due to drip displacement. Therefore is almost impossible to anticipate if a given drill core will follow the growth axis in deeper parts of the stalagmite. Also, the small diameter of the cores only permits a limited view of the general structure of the stalagmite, especially when compared to longitudinal sections of the entire specimen. However, actually most coring is normally not done vertically (in order to obtain a complete profile) but horizontally near the base, simply to get a basal age of the specimen (Spötl & Mattey, 2012).

In this paper we propose applying molding and casting laboratory techniques to create replicas of stalagmites sampled for paleoclimatic purposes, placing the replicas in the original cave locations so that the impact of sampling is severely reduced. For stalagmites that were actively growing when sampled, the deposition of new CaCO₃ layers will likely make the replicas virtually indistinguishable from the natural specimens within a few years. The main objective of this article is to provide a detailed description of the methods. We report our experience in two caves (El Soplao and La Buenita, Cantabria, N Spain). Both caves are profusely decorated with speleothems and they were discovered during mining activities, which have resulted in considerable damage.

**PREVIOUS WORK**

To date, most replicas of stalagmites have been produced in prehistoric cave-art settings, where certain parts of caves with paintings have been reproduced entirely to preserve the fragile cave environments while allowing public display of the reproductions (Altamira Cave, Cantabria, Spain: Laheras et al., 2002 and Chauvet Cave, Ardèche, France: Pigeaud, 2014). Also, speleothem replicas have been produced to restore vandalized caves, such as Vatnshellir (Snæfellsnes, Iceland), where 37 replicas were generated to replace broken specimens (Stefánsson, 2010).

In the case of stalagmites sampled for scientific purposes, replicas are rarely considered and, when employed, technical details are typically not provided (Spötl & Boch, 2012). Muñoz-García (2007) elaborated polyurethane-resin replicas of several stalagmites sampled for paleoclimatic purposes in Cobre Cave (N Spain). Vaks et al. (2013) replaced an active stalagmite by a ceramic replica in Okhotnitsya Cave (Siberia, Russia), but no technical details about the replication process were provided. Truebe et al. (2011) elaborated a replica of a stalagmite sampled for a paleoclimatic study using a mixture of cement and crushed marble and temporarily placed a replica back in the cave (Kartchner Caverns, Arizona, USA) to determine whether the materials were suitable for the cave environment. Baeza & Durán (2015) describe the replication of a peculiar speleothem from Las Maravillas Cave (Huelva, Spain). The original speleothem remained in the cave, and the replica was made for preserving its shape from possible future alteration. Finally, D. Tremaine and C. Scott-Smith created reproductions of stalagmites used for paleoclimatic research from Hollow Ridge Cave (Florida, USA), using polyurethane-resin molds and a cement-glass mixture for casting (Florida State University, 2011).

**EL SOPLAO AND LA BUENITA CAVES**

El Soplao Cave is located in the Arnero Sierra (Cantabria, N Spain; Fig. 1). It contains ~23 km of surveyed passages, developed in Aptian dolostone hosting Mississippi-Valley-type Pb-Zn deposits. With no known natural entrances, El Soplao Cave was discovered during mining operations in 1908. El Soplao contains abundant calcite and aragonite speleothems (Gázquez et al., 2012; Rossi & Lozano, 2016), including outstanding helictites and anthodites, which prompted the development of the westernmost section of El Soplao as a show-cave in 2005. U-series dating (Rossi et al., 2016) indicates that aragonite and calcite stalagmites and flowstones have grown intermittently in the caves at least for the last 1.5 Ma. El Soplao is particularly noteworthy for its unique ferromanganesestromatolites (Rossi et al., 2010), formed in water-
table canyons during the early Pleistocene as revealed by $^{234}$U-$^{238}$U and paleomagnetic dating (Rossi et al., 2016). The stromatolites contain zaccagnaita-3R, a new polytype of the hydrotalcite group (Lozano et al., 2012) and unusually well preserved Mn-oxidizing microbes (Lozano & Rossi, 2012). La Buenita Cave is located in the same region (Fig. 1), develop in the same Aptian dolostone formation as El Soplao was also discovered during mining operations.

### SAMPLED STALAGMITES

Four stalagmites were sampled for paleoclimatic purposes in El Soplao (La Sirena passage) and one stalagmite in La Buenita (Table 1). La Buenita is not open to tourism, and La Sirena passage in El Soplao is located relatively far from the show-cave section. Three of the sampled stalagmites were located under active drips. The stalagmites consist of calcite passing laterally into flowstone. In the case of El Soplao, the speleothems grew on sand, gravel and clay with intercalated manganese speleostromatolites (Rossi et al., 2010). In both caves, the stalagmites were extracted using a portable diamond saw equipped with a 2-mm-thick diamond blade. After performing typically one of two low-angle basal cuts, the stalagmite was removed with the aid of a hammer and a broad and flat chisel. This procedure minimizes the loss of material from the stalagmite base, so that placing back the replica is facilitated.

### MOLDING AND CASTING

Molding and casting procedures are slightly different depending on the stalagmite size, as detailed below. Also, resin pouring becomes more difficult with increasing mold size. For relatively small specimens (less than ~40 cm tall), we use a single-piece silicone mold and two jackets, the resin being poured directly into the mold. For larger stalagmites (~40-70 cm tall), we use a two-piece mold and two jackets, the resin being introduced into the mold using a spatula and a brush. The precise molding and casting procedure for each case is described below.

#### Case 1: Small stalagmites

First, the stalagmite is placed vertically on a block of non-sulphure plasticine (Fig. 2A; Fig. 3A). Then, a layer of room-temperature-vulcanizing silicone (Down Corning 3481) is applied on the specimen with the aid of a brush (Fig. 2A; Fig. 3B). During this step, silicone viscosity exerts a major control on the quality of the replica. If the silicone is too fluid, it could penetrate into pores of the specimen, complicating demoulding. If the silicone is too viscous, bubbles may form, decreasing the quality of the replica. Optimal viscosity levels are achieved by adding 7% wt. of curing Agent 3481-F.

After applying a second layer of thixotropic silicone (Fig. 3C) and immediately before curing, a nylon network is fixed to the silicone to increase its stability (Fig. 2B; Fig. 3D). Then, a third, 1-cm thick, blue-dyed thixotropic silicone layer is applied. When all silicone layers are cured, the plasticine basal stand is removed (Fig. 3E).

<table>
<thead>
<tr>
<th>Stalagmite ID</th>
<th>Drip activity</th>
<th>Height (cm)</th>
<th>Basal diameter (cm)</th>
<th>Mold type</th>
<th>Emplacement date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soplao-1</td>
<td>Inactive</td>
<td>71</td>
<td>23</td>
<td>2-piece</td>
<td>June 2017</td>
</tr>
<tr>
<td>Soplao-2</td>
<td>Inactive</td>
<td>34</td>
<td>11</td>
<td>1-piece</td>
<td>March 2012</td>
</tr>
<tr>
<td>Soplao-3</td>
<td>Active</td>
<td>21</td>
<td>18</td>
<td>1-piece</td>
<td>March 2012</td>
</tr>
<tr>
<td>Soplao-4</td>
<td>Active</td>
<td>17</td>
<td>11</td>
<td>1-piece</td>
<td>June 2017</td>
</tr>
<tr>
<td>Buenita-1</td>
<td>Active</td>
<td>40</td>
<td>12</td>
<td>2-piece</td>
<td>June 2017</td>
</tr>
</tbody>
</table>
Once cured, the silicone mold can be easily deformed. Therefore, pouring resin or plaster into the mold will likely result in deformed replicas. To avoid this, two rigid jackets plaster (low hardness, type II; Alamo 70) are prepared to be fixed to the mold. To prepare the first jacket, the silicone-covered stalagmite is placed horizontally over a thick section of polyethylene foam, which is previously prepared to fit the specimen by removing material from its central part (Fig. 3F). Plasticine is used to improve the fit between the silicone and the polyethylene foam (Fig. 2C). The jacket is fixed to the silicone mold by using swallowtail keys made of plasticine. To improve stability, an additional piece of polyethylene foam is adjusted to the base of the stalagmite (Fig. 3F). Finally, to prevent plaster from penetrating the surface of the silicone mold, the latter is wrapped in polyethylene film. Following hardening of the first plaster jacket, both the silicone-covered specimen and the jacket are flipped together (Fig. 3H), so that a second plaster jacket can be prepared, similar to the first one (Fig. 3I).

A first casting is performed using high-hardness type-IV dental plaster (a 50% mixture of Hebodur and Arquero). The resulting master replica is preserved, so that a new mold can be prepared in case the first mold is damaged. A second casting provides the replica to be placed in the cave. For this casting, we used epoxy resin (Fetadit 55/63), charged with silica powder. To reduce replica weight and cost prior to pouring the resin we fixed a thick polyethylene rod into the axis of the mold with the aid of a thin wooden stick (Fig. 2G; Fig. 3K-L). This procedure also prevents unwanted increases in temperature during resin curing.

To obtain a color as close as possible to that in the surface of the original specimen, we first perform several tests by combining dyes, obtaining several

![Diagram](image-url)
fragments of colored resin. To achieve best results, we do not apply the chosen dyes on the finished replica, but on the internal part of the silicone mold, before introducing the epoxy resin of the casting. Thus the dye penetrates the resin, permanently coloring the selected sectors. To make sure that the resulting color is right, we previously did a series of color tests using a silicone mold divided into several hollow spaces (5 x 5 cm section and 1 cm deep). We impregnate the bottom of each space with the selected mixture of dyes and introduce the epoxy resin. After curing, we check the surface color obtained. The application of the pigment to the silicone can be done in dry or wet conditions. However, we recommend the wet application because it produces a glossy aspect in the finished replica very similar to most of the original specimens.

**Case 2: Large stalagmites**

For larger stalagmites (~40-70 cm tall) inserting polyethylene rods in the mold axis before resin pouring is more delicate, as it becomes more difficult to prevent the rods from touching the mold walls. Therefore, in these cases it is advisable to elaborate a two-piece instead of a single-piece mold so that it is easier to safely insert polyethylene rods in the mold and the volume of resin used is minimized. Also, as the silicone molds become larger they are more prone to break during casting.

First, a cavity is carved into a thick piece of polyethylene foam to fit one longitudinal half of the stalagmite. A layer of plasticine is then applied on the surface of the polyethylene foam, and a groove is carved in the plasticine near the edges of the stalagmite. This groove is the link between the two silicone molds and prevents possible spills during casting. Plasticine swallowtail keys are prepared to obtain a good fit between the future jacket and the specimen (Fig. 4A; Fig. 3N) and to improve stability when both are placed in a vertical position. The exposed half of the specimen is covered by three layers of silicone-bearing nylon network, similar to case 1 (Fig. 4B; Fig. 3O). A barrier of paper-covered polyethylene foam sheets is then glued to the base of the polyethylene foam. Finally, the first jacket is obtained by covering the silicone-covered stalagmite with low-hardness plaster (Fig. 4C; Fig. 3P).

Following hardening of the first plaster jacket, both the silicone-covered specimen and the jacket are flipped together, removing the basal piece of polyethylene foam in order to expose the other half of the stalagmite. Again, plasticine swallowtail keys are prepared (Fig. 4D). Before covering the corresponding half of the stalagmite with silicone, a release agent (black soap) must be applied to the first mold to prevent the molds from sticking together. After applying the silicone layers (Fig. 3Q) and wrapping with polyethylene film, a barrier of polyethylene foam is spread around the set. This prevents plaster spills during the elaboration of the second jacket (Fig. 4E-F; Fig. 3R). Once the plaster of the second jacket is hard, the polyethylene foam barriers are removed (Fig. 4G) and the set is opened (Fig. 4H) to release the stalagmite (Fig. 4I; Fig. 3S).

Similar to case 1, a first casting is performed using type-IV dental plaster to obtain a master replica, and the replica is obtained by means of a second casting using dyed epoxy resin (Fetadit 55/63). This is done by applying a ~0.5 mm thick resin layer to the inner parts of each mold, with the aid of a brush (Fig. 3T-U). Then, ~6% cellulose is added to the epoxy resin, which is applied over the epoxy layer using a spatula (layer thickness: 1-2 cm). The purpose of adding cellulose is to regulate the transparency of the epoxy resin. Then, both molds and their respective jackets are assembled together. After resin curing, a light-weight and hollow replica is obtained, ready to be placed into the cave (Fig. 3V). The procedure to obtain the final color of the replicas is similar to that described for case 1.
ANCHORING OF STALAGMITE REPLICA TO THE ORIGINAL SUBSTRATES IN THE CAVES

After cleaning and drying the substrate of the stalagmite, the replica is placed to match the original orientation using images taken before extracting the specimens and the impact location of the drops falling from the corresponding stalactite. To fix the replica to the cave floor, we used high hardness plaster (Arquero; type IV) coated with epoxy resin. We use an umbrella to avoid the impact of drops during plaster and resin hardening. In the case of relatively flat substrates, the insertion of the replicas is relatively easy (Fig. 5A-D) and simply involves dyeing the plaster to match the appropriate color.

However, the insertion on inclined substrates, such as for stalagmite Soplao-1, is more difficult. This stalagmite passed laterally into a thin flowstone overlying unconsolidated detrital sediments (Fig. 5E), which was partially broken during stalagmite extraction. Therefore, a layer of dyed plaster, resembling the original flowstone, was applied during the insertion of the replica (Fig. 5F).

LONG-TERM STABILITY OF THE REPLICA

The long-term stability of the materials used in the replicas is of great importance because they can deteriorate easily under cave conditions (Werker, 2006ab; Werker & Hildreth-Werker, 2006). The epoxy resin we have used for both casting and covering the insertion plaster is manufactured locally, so it does not appear in the lists published by Werker (2006a) or Werker & Hildreth-Werker (2006). This resin is of relatively good quality and we have used it for more than two decades in diverse restoration projects with excellent results and durability. However, its long-term stability inside the caves has not been described so far. After almost six years in the cave, replicas Soplao-2 and Soplao-3 (emplaced in March, 2012) have not experienced any obvious signs of alteration, suggesting that the used epoxy is rather stable in the cave environment.

Regarding the plaster used for anchoring the replicas to the substrate, we initially tested its stability in the laboratory by submerging a ~125-cm$^3$ piece in deionized water for one year in an isolated environment. After this time the conductivity of the water barely increased, implying no significant dissolution of the plaster. Therefore this material seemed adequate to fix the replicas to the substrate. However, tests at the El Soplao Cave showed that the plaster partially disintegrated rapidly (in a few months) when dripwater hit it directly, but much slower when it was at the base of the stalagmite. We solved the problem by coating the base of the stalagmite with a layer of epoxy resin, which effectively waterproofed the plaster. This epoxy resin is the same used in the casting of the replicas.

In the replicas, we have only used inorganic dyes that should be relatively inert in the cave environment: iron oxi-hydroxides (light yellow to dark brown), titanium oxide (white) and graphite (black). Furthermore, these dyes are not exotic in the caves we are dealing with: in El Soplao Cave, titanium and iron oxides are abundant in detrital sediments, speleo-stromatolites, and cave walls (Rossi et al., 2010; Lozano et al., 2012), and coal fragments are locally present in the host rock (García et al., 2007).

Given the significant anthropogenic influence in the caves associated with mining during the 20th century (García et al., 2007), we did not sterilized the replicas before placing them in the caves. Even though, so far we have not observed any perceptible microbial disturbances on the surfaces of the replicas that have remained in the cave for ~6 years.

For the replicas of stalagmites that were actively growing when sampled (3), it is reasonable to assume that the deposition of new CaCO$_3$ layers will further stabilize the replicas in the cave environment. Two of these replicas (Soplao-4 and Buenita-1) were placed in the caves in June 2017, so significant layers of recent calcite are probably not developed yet on their surfaces. The remaining “active” replica was placed in 2012, but recent calcite precipitation is prevented by the presence of a drip-counting device on its surface. Therefore, we have no information yet on how modern calcite is adhering to and growing over the replicas. However, in drip sites of El Soplao Cave characterized by relatively high CaCO$_3$ supersaturations (saturation index for calcite around 0.8-1.2: Rossi & Lozano, 2016), obvious crusts of recent calcite are covering.

Fig. 5. Replicas installed in La Buenita Cave (A) and in La Sirena Passage (El Soplao Cave) (B, C, D, E, F). A) Replica of the Buenita-1 stalagmite; B-C-D) Replicas of Soplao-4, Soplao-3 and Soplao-2 stalagmite, respectively; E) Soplao-1 stalagmite before sampling; F) Replica of Soplao-1 stalagmite placed in its original location.
stalagmite surfaces that were restored with epoxy putty after localized sampling (Rossi & Lozano, 2016). Such crusts have developed in less than two years, suggesting that the epoxy replicas located under active drips will be eventually covered by calcite too.

CONCLUSIONS

Elaborating replicas of stalagmites sampled for paleoclimatic studies is an effective means to reconcile scientific research and cave conservation. Once in place, the stalagmite replicas are almost indistinguishable from the natural specimens. In the case of originally active stalagmites, the impact of sampling will be likely erased in a few years, depending on the rate of calcite deposition.

The molding and casting methods vary depending on stalagmite size. For relatively small specimens (less than ~35 cm tall), we use a single-piece mold and two jackets. For larger stalagmites (~40-70 cm tall), we use a two-piece mold and two jackets.

To reduce replica weight and costs, and to prevent unwanted temperature increases, for relatively small specimens we introduce polyethylene rods into mold axes during casting. For larger specimens, we use techniques to produce hollow casts.

We use extra-hard plaster coated with epoxy resin to fix the replicas to their original substrates. Both the epoxy resin and plaster are carefully dyed to match the original surface texture and color of the sampled stalagmites.

The epoxy used to elaborate and emplace the replicas, as well as the dyes used, are apparently stable in the cave environment, at least for periods of at least six years.

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The investigation of the internal structure of calcite crystals is a new focus in speleothem science, especially in the range of crystallization temperatures close to 0°C. Recently found calcite spars from Zinnbergschacht Cave of the Franconian Alb (SE Germany) are ideal for multi-method investigation. The elongated calcites (up to 6 cm in length) with three to six lateral faces and basal triangular faces at the ends are observed in collapse-zones in the cave. \(^{230}\)Th/U-ages of 38.9 ka suggest formation during the periglacial Weichselian, between the Scandinavian and Alpine Glaciations. The \(\delta^{18}O\) and \(\delta^{13}C\) values of the calcite spars vary from -11.18 to -16.11‰ V-PDB and from -4.78 to -6.13‰ V-PDB, respectively. The exceptionally low \(\delta^{18}O\) values of these calcites appear to be due to precipitation in pools on ice. The values deviate considerably from those of conventional interglacial speleothems (\(\delta^{18}O = -7.21\) to \(-7.55\)‰, \(\delta^{13}C = -9.77\) to \(-10.86\)‰) and also from true Weichselian cryogenic calcites (composite spherulites and rhombohedral chains with \(\delta^{18}O = -15.06\) to \(-18.04\)‰ and \(\delta^{13}C = -3.52\) to \(-4.13\)‰). The \(\delta^{18}O\) values of the latter calcites is typical of cryogenesis of calcites with extensive oxygen isotope fractionation (preferred incorporation of \(^{18}O\) into the co-occurring ice). Thus, the \(\delta^{18}O\) values of the calcites suggest cold conditions up to the beginning of cryogenesis. Cathodoluminescence (CL) and backscattered electrons (BSE) indicate the distribution of impurities within the calcite spars as pigmented triangles surrounded by clear calcite, with a higher density of the triangles in the outer areas. Three hierarchies of triangles can be distinguished by BSE, documenting a filigreed primary structure of the spars. Electron backscatter diffraction (EBSD) reveals a divergent orientation of the triangular subcrystals from the center to the outer corners of the calcites. Thus, their internal structure reflects an example of fascicular optic fibrous calcites (FOFC), frequently discussed in carbonate petrology.

Abstract:

Composition and morphology of cave minerals are highly diverse due to variation in host rock and physicochemical conditions within the system (Hill & Forti, 1997). The last 20 years of research have primarily focused on calcite and aragonite stalagmites due to their potential as climate archives (e.g., Fairchild & Baker, 2012). Due to the frequent occurrence of calcite mineralogy in speleothems, their crystallography and chemical composition is of particular importance. The calcite crystallographic composition (Goldschmidt, 1913) and diversity (Onac, 1997) are well known. In calcitic speleothems, the most common crystal type is the columnar - so called normal, length-fast, palisade calcite (Folk & Assereto, 1976, Kendall & Broughton, 1978). Recently, Riechelmann et al. (2014) investigated speleothem calcite crystals precipitated on watchglass surfaces from various caves with calcareous and dolomitic host rock and correlated crystal morphology and Mg/Ca composition of the drip water.

Keywords:

Weichselian, cryogenic calcites, fascicular optic fibrous calcite, electron backscatter diffraction, Franconian Alb

Citation:


INTRODUCTION

Composition and morphology of cave minerals are highly diverse due to variation in host rock and physicochemical conditions within the system (Hill & Forti, 1997). The last 20 years of research have primarily focused on calcite and aragonite stalagmites due to their potential as climate archives (e.g., Fairchild & Baker, 2012).

Due to the frequent occurrence of calcite mineralogy in speleothems, their crystallography and chemical composition is of particular importance. The calcite crystallographic composition (Goldschmidt, 1913) and diversity (Onac, 1997) are well known. In calcitic speleothems, the most common crystal type is the columnar - so called normal, length-fast, palisade calcite (Folk & Assereto, 1976, Kendall & Broughton, 1978). Recently, Riechelmann et al. (2014) investigated speleothem calcite crystals precipitated on watchglass surfaces from various caves with calcareous and dolomitic host rock and correlated crystal morphology and Mg/Ca composition of the drip water.
In this pilot study, we present unusual, elongated calcite spar with a size of up to 6 cm from a recently discovered cave in the Jurassic dolomites of SE Germany. In addition to the abnormal morphology, the discussion focuses on the unusual internal structure of the crystals and their $\delta^{13}$C and $\delta^{18}$O values. This research is important due to the possibility to compare the stable isotope values of these calcites with convetional interglacial speleothems, such as stalagmites, as well as cryogenic calcites, such as spherulitic components (Richter et al., 2013), which occur in the same cave.

A special type of spherulitic calcite components are composite spherulitic calcites shaped like braided hair (“Zopfsinter” sensu Erlemeyer et al., 1992 and “braided sinter” sensu Richter & Riechelmann, 2008). Due to the negative $\delta^{18}$O values (up to -20‰ V-PDB), this characteristic particle type in former ice caves is a main type of coarse cryogenic calcites (Richter et al., 2013).

The results of this pilot study must be put in context with recent articles discussing cold water and cryogenic calcites (e.g. Žák et al., 2012, 2018; Richter et al., 2013). First, it was important to identify the different calcites as cryogenic crystals (strongly negative $\delta^{18}$O values). Furthermore, considering the $^{230}$Th/U-ages and the typical depth of the cryogenic calcites in caves, it was possible to reconstruct a minimum thickness of permafrost in former periglacial areas (Žák et al., 2012). It is further important to decipher the crystallization path of single crystals as an evidence for the process of cryogenesis. Here, according to preliminary $\delta^{18}$O values, an increasing cryogenesis in calcitic fibers of spherulites could be demonstrated (Richter & Riechelmann, 2008). Additionally, cathodoluminescence (CL) spectrography often reveals an incorporation of rare earth elements, such as dysprosium and samarium, in the central parts of the cryocalcites (e.g. Richter et al., 2008), a possible effect of concentration due to melting of frozen soil above the cave.

CAVE SETTING AND SAMPLING LOCATIONS

Zinnbergschacht Cave (No. HFA-A 205 in the register of assessment) is located 40 km ENE of Nuremberg (Fig. 1), near the show cave Maximiliansgrotte, and 20 km SE of the famous Zoolithen Cave. Upper Jurassic dolomites (Franconian Dolomite) comprise the host rock of the cave area (Meyer, 1972). The dimension of Zinnbergschacht Cave is currently investigated by the speleogroup “Höhle und Karst e. V. Nürnberg”.

Within the 400 m long cave, calcitic crystals and aggregates were found 30-60 m SE to SSE of the entrance (Fig. 2), approximately 40 m below surface. The (now closed) entrance is formed by a 9 m shaft, followed by mainly flat galleries.

The host rock near the entrance is pure dolomite, whereas the partly calcitized dolomites in deeper galleries contain centimeter to decimeter-sized white silicious concretions, which are well developed in the Canyon site (Fig. 2). Over 2 m of centimeter to meter-sized blocks of the host rock with a reddish brown loamy matrix filled the gallery. These collapse breccias are well developed around two depressions (I and II in Fig. 2), which may have formed from the dissolution of carbonates beneath the cave as in the case of dolines or from melting cave ice during late/postglacial time as in the case of sols (see discussion).

The investigated calcites occur in the areas around the two depressions in association with the collapse breccias (mostly between blocks) (Fig. 2). Crystals from the three studied locations are characterized as follows: Loc. A - translucent elongated crystals and aggregates with a size of up to 5 cm in the Alter Sulzbacher (Figs. 3b, 4) - in some places translucent crystals with whitish translucent overgrowths (Figs. 3c, 4a, and 5b); Loc. B - whitish composite spherulitic calcites with a size of up to 3 cm in diameter in the Canyon (Fig. 5c); Loc. C - whitish elongated crystals (Fig. 5a) and aggregates with a size of up to 2 cm as well as whitish rhombohedral chains and composite spherulitic calcites with a size of up to 1 cm in the Petersdom. The genesis of these calcite speleothems (crystals and aggregates) seems to be very young (Upper Pleistocene to Holocene) as indicated by the lacking lithification of most speleothem particles found in association with broken blocks or on loamy sediments. According to published articles on cryogenic calcite genesis (e.g. Žák et al., 2012, 2018),
Weichselian coldwater calcite spars from Zinnbergschacht Cave

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Fig. 2. Sampling locations (A-C) in Zinnbergschacht Cave.

X-ray diffraction of the different carbonate phases was performed using a Pananalytical MPD diffractometer. Ground samples were measured in a diffraction angle range of 26-38° using quartz powder as an internal standard. Each $d_{104}$ value of the rhombohedral carbonates was identified to determine the Ca/Mg distribution (Füchtbauer & Richter, 1988).

Cathodoluminescence (CL) investigations utilized a “hot-cathode” CL microscope HC1-LM (Neuser et al., 1995). CL spectra were recorded with a digitally controlled EG&G spectrograph attached to a peltier cooled PIXIS CCD camera by Princeton Instruments (for details, see Hoffmann et al., 2016).

Fluorescence microscopy was applied using a Leica DM4500P microscope equipped with a mercury short-arc reflector lamp, and coupled with a Leica EL6000 compact light source (Hoffmann et al., 2016).

For EBSD analyses, thin sections were chemomechanically etched with colloidal silica on an atomic scale (Massonne & Neuser, 2005) and coated by a thin carbon layer. All coated thin sections were analyzed using backscattered-electrons (BSE) in high contrast mode. Crystallographic orientation of the calcite spars was determined by EBSD (Nordlys, OXFORD, Instruments). The data acquisition and analysis was performed using the software packages AzTec and Channel5 by Oxford Instruments. EBSD analyses were performed at a beam energy of 20 kV, an aperture of 60 µm, a working distance of 25 mm, and a tilt angle of 70°.

The $\delta^{13}$C and $\delta^{18}$O values of the carbonates was determined using a delta 5 mass spectrometer (Finnigan MAT) and calibrated with V-PDB (Standards: CO-1 and CO-8). The 1σ-reproducibility of the measurements is 0.04‰ V-PDB for $\delta^{13}$C, and 0.08‰ V-PDB for $\delta^{18}$O.

Samples for $^{230}$Th/U-dating were prepared and analysed at the Max Planck Institute for Chemistry, Mainz. Chemical separation of U and Th isotopes was performed as described by Scholz & Hoffmann (2008) and Yang et al. (2015). Uranium and Th isotopes were analysed using a Nu Plasma MC-ICP-MS. Analytical details are described by Obert et al. (2016). Details about the calibration of the mixed U-Th spike are given by Gibert et al. (2016). To account for the potential effects of detrital contamination, all ages

METHODS

Samples were cleaned in an ultrasonic bath in order to remove fine-grained clastic spelean sediments. Images of crystals and aggregates were taken using a Canon EOS 50 D. The morphology was further documented in detail using a high-resolution field emission scanning electron microscope (HR-FESEM type LEO/ZEISS 1530 Gemini).

The crystals and aggregates crystallized in pools on ice. Subsequent to the melting of the ice, they accumulated at the bottom of the cave.

Fig. 3. Views of location A (Alter Sulzbacher): a) overview; b) large aggregates of Weichselian calcites (arrow); c) aggregates of translucent calcites with whitish ends.

Fig. 4. Translucent calcitic aggregates of location A: a) longitudinal view of a cm-sized calcite spar with milky ends (red arrows) and smaller crystals at the lower right (blue arrow); b) view of a cm-sized calcite spar with triangular end faces (green arrow) and filling of small aggregates within the hollow spars to the right (black arrow).
were corrected assuming an average upper continental crust $^{232}\text{Th}/^{238}\text{U}$ weight ratio of 3.8 for the detritus and $^{230}\text{Th}$, $^{234}\text{U}$, and $^{238}\text{U}$ in secular equilibrium. All activity ratios were calculated using the half-lives from Cheng et al. (2000), and all ages are reported at the 2$\sigma$-level.

RESULTS

Here we focus on the elongated calcite spars, whereas observations of other speleothem components are only marginally presented. Such considerations are important for the subchapters concerning crystal morphology, x-ray diffraction and stable isotope values.

Crystal Morphology

The millimeter- to centimeter-sized calcites of location A and, less frequently, location C are mostly parts of aggregates, whereas single crystals are more rare (Figs. 4 and 5a). The crystallographic characteristics and the position of the transect for thin section analyses are shown in Fig. 6. The elongated crystals contain three or six triangular shaped side faces with basal triangular faces at the end (Figs. 4-7), often containing one large triangular channel. The side faces are stepped, whereas basal faces are rough to fragmentary (Fig. 4). Due to preferential crystallization at the edges between side and basal faces, the diameter of the elongated crystals is larger at the ends than in the middle portion and often results in funnel-like ends of the crystals (Fig. 4b).

Notably, elongated crystals with completely intact faces are rare. In general, crystals are of filigreed composition with indications of high porosity. Thus, degradation of these crystals is widespread, and small movements can result in re-deposition of skeletal crystals (Fig. 3c).

Fig. 5. Whitish calcitic speleothem components: a) elongated calcites of location C; b) whitish spherulithic overgrowths on translucent calcites of location A; c) whitish spherulithic calcite of location B.

The other components of the calcites are similar to other cryogenic calcites found in several German caves (e.g. Richter et al., 2013). Composite spherulitic components (dominantly at location B, Fig. 5c, rare at location C) are composed of spheroidal aggregates of fibrous calcites, with steep rhombohedral faces at the end. Rhombohedral chains are rare and were only found at location C.

Coloration seems to be important with regard to the formation of the speleothem components. The translucent spars are sometimes more whitish (richer in gas/fluid inclusions) at the crystal ends only, and some crystals have white coatings of spherulitic calcites. We conclude that the white calcites must be younger than the translucent crystals, and the white ends of the crystals, the white coatings of spherulitic calcites as well as the white spherulitic components must be younger than the translucent crystals.

X-ray diffraction (XRD)

The $d_{(104)}$ values of the investigated calcite spars vary between 3.031 and 3.028Å ($n = 5$) indicating a $\text{MgCO}_3$ content of 1.3 to 2.4 mol-% in the lattice. This non-stoichiometric composition is not surprising because of the dolomitic host rock ($d_{(104)} = 2.888 - 2.905\text{Å}; n = 10$). For comparison, other calcites ($n = 5$ for each type) were also analysed. Spherulitic calcites (location B) produced $d_{(104)}$-values between 3.023 and 3.021Å (4.0 to 4.7 mol.% $\text{MgCO}_3$), whereas dolomite host rock calcites were nearly stoichiometric (3.035 - 3.032Å; <0.1 to 1.0 mol-% $\text{MgCO}_3$).

Cathodoluminescence

The calcite spars show an intrinsic CL as most calcitic speleothems (Richter et al. 2002). This blue CL colour is indicative of very low (or no) Mn$^{2+}$ content in the calcite lattice (<10 ppm according to PIXE (Proton Induced X-ray Emission) measurements) (Habermann et al., 1998, 1999). However, it is possible to distinguish...
between dark blue and pale blue sub-crystal areas within the CL pattern of the calcites (Fig. 8a). The dark blue CL corresponds to pure calcite, whereas the areas showing a lighter blue CL are rich in sub-microscopical pigmentation, which can deflect the crystal lattice and result in the lighter coloration (Richter et al. 2002).

Furthermore, the CL-distribution of dark and pale blue areas is common throughout the sample type. Pale blue triangular areas of sub-crystals are generally rich in inclusions and are joined by triangular shaped, dark transparent blue calcite (Figs. 7 and 8). Mn$^{2+}$-activated calcite is visible only in the very thin zones of the cm-sized crystals due to the cleavage planes (Fig. 7b).

**Backscattered electron (BSE) imaging**

High resolution BSE-images acquired with the HR-FESEM display dark and bright calcite phases (Figs. 8b, c and 9).

Thin sections cut perpendicular to the c-axis of the elongated calcites reveal a complex, dark framework of triangular sub-crystals (2-20 µm in size). The space between the dark portions is filled by bright calcite. The darker areas show dark triangles and laths in transmitted light as well as light domains in BSE. The translucent areas mostly display light areas in transmitted light, and dark triangles and laths in BSE (compare Table 1). Brighter areas in BSE are more likely to be composed of near-stoichiometric CaCO$_3$ with higher average atomic masses (mainly Ca), whereas darker areas in BSE correspond to lower average atomic masses (Hoffmann et al., 2016). The reason for lower average atomic masses in calcites may be organic material or small solid and fluid inclusions.

There are three hierarchies of crystal habit concerning the cm-sized crystals: 1. Triangles with lateral faces up to 500 µm; 2. triangles with lateral faces between 100 and 10 µm; 3. triangles with lateral faces below 10 µm (Figs. 6, 8, and 9).

**Fluorescence microscopy**

The fluorescence images of thin sections cut perpendicular to the c-axis of the calcite spars show a complex framework of triangular figures resembling the patterns seen in CL and BSE. Pigmented areas are lighter green in fluorescence, while translucent areas are characterized by lower fluorescence. According to Wanamaker et al. (2009) and Hoffmann et al. (2016), the brighter portions reflect areas, which incorporate organic matter or crystal defects (such as inclusions) within the crystals.
Electron backscatter diffraction (EBSD) mapping

The crystallographic orientation of the calcite spars was studied in a section perpendicular to the long axis of the crystal. The orientation of the c-axis in the center deviates from the orientation of the outermost edges by 9°. To visualize the directions and deviations of the c-axes, a rainbow color code was applied, which ranges from red to blue from the center towards the edges of the calcite (Figs. 7c and d). The resulting pattern is a central area with three regions towards the side planes of the crystal showing a similar orientation of the c-axis (red). The deviation angle increases towards the corners of the triangle and gains a maximum value of 9° (blue). EBSD mapping reveals a divergent orientation of the c-axes within the calcite crystal. The central point of the grain is more or less a single crystal, with the strongest divergence observed in the most distal points from the center.

δ¹³C and δ¹⁸O values

Mass spectrometric analyses of the translucent cm-sized calcite spars show δ¹³C values between -4.78 and -6.13‰, and δ¹⁸O values ranging from -11.18 and -16.11‰ (Fig. 10). The δ¹⁸O values of the calcites correspond to the largest values of cryogenic calcites from Zoolithen Cave (10 km NW of Zinnbergschacht Cave), but are lower than the values of conventional stalagmites (Fig. 10). Mixed calcitic components with translucent cores (cm-sized calcites and parts of them) and whitish overgrowths (Fig. 5b) show δ¹³C (-4.42 to -4.79‰) and δ¹⁸O values (-15.51 to -16.26‰) plotting at one end member of the calcite spars, with negative δ¹⁸O and more positive δ¹³C values. The whitish rhomboedral chains of locality C exhibit more negative δ¹⁸O values (-17.69 to -18.04‰) compared to the composition of the investigated calcites. The
composite spherulites of locality B are characterized by \( \delta^{18}O \) values from -15.06 to -15.94‰ and \( \delta^{13}C \)-values from -3.59 to -4.13‰ (Fig. 10).

\[ 230^{\text{Th}}/U \text{-dating} \]

Ages between 33.4 and 38.9 ka were determined by \( 230^{\text{Th}}/U \)-dating, thus confirming a growth period of approximately 5.5 ka during the Late Weichselian (Table 2). Since this time span is characterized by a succession of several stadial and interstadial periods (Fig. 11), it is likely that several cryogenic calcite generations developed and are present within the system today. This is particularly important for the oldest translucent crystals (38.9 ka), which may contain many growth phases.

**DISCUSSION**

The character of the elongated calcite spars of Zinnbergschacht Cave is similar to the “type E” carbonate (sample I), which precipitated from dolomite host rocks in the monitored Zoolithen Cave in close proximity (20 km north, Riechelmann et al., 2014). The development of these deposits is typical for drip waters with high Mg/Ca ratios.

The stepped and rough faces of the calcite spars coincide with the composition of the contained sub-crystals. The internal structure of the cm-sized spars is dominated by crystals with prismatic or near-prismatic side faces. This distribution is illustrated by pigmented fibers with relatively fast crystallization and clear cement in between. The pattern type of the sub-crystal is very systematic, as documented in BSE images. The center of the crystals is more transparent than the outer portions, with differential rates of crystallization resulting in the funnel-type ends of the elongated crystals. The different rates of crystallization seem to be causal for a more prismatic or near prismatic shape of the fibers, with only three triangular side faces in the central parts of the calcites, and very steep rhombohedra with six elongated side faces towards the exterior. The microscopic features of the sub-crystals are summarized in Table 1.

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**Table 2.** \( 230^{\text{Th}}/U \)-data of calcite crystals from the three studied locations

<table>
<thead>
<tr>
<th>No.</th>
<th>Notation</th>
<th>( \text{[^{238}U]} ) [µg/g]</th>
<th>( \text{[^{232}Th]} ) [ng/g]</th>
<th>( \text{[^{234}U/^{238}U]} )</th>
<th>( \text{[^{230}Th/^{238}Th]} )</th>
<th>( \text{[^{230}Th/^{232}Th]} )</th>
<th>Age uncorrected [ka]</th>
<th>Age corrected [ka]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cm-sized calcite</td>
<td>0.1965±0.0012</td>
<td>5.327±0.051</td>
<td>2.6758±0.0079</td>
<td>0.8337±0.0046</td>
<td>94.01±0.85</td>
<td>39.16±0.28</td>
<td>38.89±0.31</td>
</tr>
<tr>
<td></td>
<td>Alter Sulzbacher</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>2</td>
<td>spherulitic calcite</td>
<td>0.5669±0.0032</td>
<td>9.159±0.090</td>
<td>2.5973±0.0054</td>
<td>0.7078±0.0036</td>
<td>133.9±1.3</td>
<td>33.61±0.21</td>
<td>33.44±0.22</td>
</tr>
<tr>
<td></td>
<td>loc. B Canyon</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>rhombohedra chain</td>
<td>0.1846±0.0012</td>
<td>23.54±0.23</td>
<td>3.817±0.011</td>
<td>1.206±0.013</td>
<td>28.90±0.38</td>
<td>39.52±0.51</td>
<td>38.63±0.79</td>
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<tr>
<td></td>
<td>loc. C Petersdom</td>
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</table>
The distribution of the sub-crystals in the calcite spars becomes evident by EBSD mapping. From the middle of the elongated crystals towards the edges, an increasingly three-radial divergence of the sub-crystals is observed. For this reason, thin sections parallel to the c-axis show an undulose extinction of fascicular-optic fibrous calcite (FOFC, Kendall, 1985) with crossed polars. The cause of this pattern is currently unknown. The Mg-content as an exclusive cause seems unlikely because of stoichiometric calcites found in the Malachitdom Cave of NW Germany, which show similar FOFC patterns (Richter & Riechelmann, 2008). However, two pools in Zoolithen Cave (20 km north) have produced comparable Mg-containing calcites, but with a different orientation of sub-crystals (FOFC versus radiaxial fibrous calcite (RFC) - Richter et al., 2015). In the latter case, the only difference between the two rimstone pools is the saturation index of the drip water. In the pool containing RFC, the saturation index (SI) is 0.87, while the pool containing FOFC has a SI of 0.54. The change in the textural character of calcites during growth is another aspect, with the rate of crystallization probably being an important factor. The crystallization of calcites begins with very steep rhombohedral faces and a fast growth rate, while the final growth phases exhibit less steep rhombohedra and a slower growth rate. Further work is needed to explain this phenomenon. In summary, there are no hints for an original crystallization of hydrated carbonate phases and later recrystallization to calcite. Due to the typical habit of calcite in dolomite caves, we consider the observed features as primary.

The δ^{18}O values of the calcites are more negative than those of Holocene stalagmites due to cold water conditions up to the start of freezing of the pool water with the corresponding oxygen isotope fractionation. During freezing, the co-precipitated calcites expose more negative δ^{18}O values (Souchez & Jouzel, 1984; Žák et al., 2012). There is no exact boundary between coldwater calcite and cryogenic calcite within the cm-sized calcites. Thus, we assume a very slow transition during crystallization. The mixed speleothem components with translucent cores and whitish spherulitic overgrowths show more negative δ^{18}O values than the cm-sized calcites with a translucent composition. This is due to the final stage of crystallization of fibrous calcites in freezing water pools. In summary, the speleothem components of locality A (Alter Sulzbacher) formed within in a pool containing ice during interstadial 8 during the Weichselian (Fig. 11). Žák et al. (2012) assume several intervals of growth and melting of permafrost (up 100 m thick) during the periglacial time span between the Scandinavian and Alpine glacial areas (10-70 ka before present). In this context, it is possible to form pools on ice in caves that subsequently froze and crystallize coarse grained cryogenic calcites. Different pools are assumed for the cryogenic calcites of localities A to C within Zinnbergschacht Cave due to the various types and ages of the calcite grains (A/C versus B - see Table 2).

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The activity of saccharolytic enzymes in Collembola is associated with species affinity for caves

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Abstract: The activity of enzymes associated with digestion can reflect food availability and feeding preferences of invertebrates in a particular habitat. Caves are mostly nutrient-poor habitats lacking primary production. In the present study the enzymatic activity of cellulases, trehalases and chitinases was measured in eight collembolan species differently associated with the cave environment: the troglobionts (obligate cave species) Pseudacherontides spelaeus and Protaphorura janosik; the eutroglophiles Ceratophysella denticulata, Folsomia candida and Heteromurus nitidus; the subtroglophiles Hypogastrura aequepilosa and Orthonychiurus rectopapillatus; and the trogloxene (not associated with caves) Megaphorura arctica. Qualitative enzymatic patterns and quantitative differences in species activity were considered in terms of the taxonomic, feeding and ecological classifications of Collembola. Activity of the tested enzymes was confirmed in all species. Cellulolytic and chitinolytic activity seemed to play a crucial role for the discrimination of guilds within all categories. An increasing trend of chitinolytic activity was observed in Collembola associated with the subterranean environment and deeper soil layers, while cellulolytic activity decreased towards more adapted cave forms. Variability in enzymatic activity in cave-dwelling species indicated food specialization across sub- and eutroglophiles and troglobionts, respectively. The results of this study point out that enzymatic activity varies between groups of the cave fauna with different degrees of association to subterranean habitats (cave guilds).

Keywords: enzymatic activity, cellulase, trehalase, chitinase, Collembola, cave guild

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INTRODUCTION

Feeding ecology among soil Collembola reflects the high heterogeneity of the soil environment (Chahartaghi et al., 2005). Collembola are generally classified as decomposers (Rusek, 1998), feeding unselectively on a wide variety of food materials. However, the trophic niches of species vary among different collembolan orders and families, indicating a pronounced phylogenetic signal that supports the trophic-niche conservativism hypothesis (Potapov et al., 2016).

Food resources in caves are depleted when compared with surface ecosystems, which obtain energy and organic carbon from photosynthesis by green plants. Microbial communities represent the base of the food pyramid in caves (Camassa, 2004), and they rely almost entirely on surface organic resources actively or passively transported into the caves. The most available food sources in caves are animal cadavers, faeces, animal eggs and plant debris (leaf litter, rotten wood). Generally, Collembola that occupy environments with both plant and fungal food sources available are able to alternate herbivory and fungivory (Endlweber et al., 2009).

While collembolan communities diverge vertically in above-ground ecosystems, their horizontal distribution is distinct in caves. Trogloxenes and subterranean troglobionts are abundant in entrance zones illuminated during the day (euphotic zone) and characterized by the...
presence of algae, bryophytes and phanerogams, while eutroglrophiles and troglobionts live permanently in an environment devoid of fresh plant food sources (Culver and Pipan, 2009; Kovač et al., 2014).

Food composition in Collembola has been studied using various techniques, such as analyses of gut content (e.g. Ponge, 2000; Castaño-Meneses et al., 2004; Fiera, 2014), activity of digestive enzymes (Zinkler, 1971; Urbášek & Rusek, 1994; Berg, 2004), lipid composition (Ruess et al., 2007) or stable isotopes (e.g. Ruess et al., 2004; Chahartaghia et al., 2005; Endlweber et al., 2009; Potapov et al., 2016). Each method has its own limitations, and a combination of several approaches seems to be the proper way to better understand feeding ecology and to refine the functional ecological classification of soil mesofauna.

Regarding the usual interpretation of digestive enzymes, the presence of cellulase activity in the digestive tract of an animal suggests the ability to digest algae and plant materials and indicates herbivory. On the other hand, the presence of trehalase activity indicates the ability to digest the cell contents but not the cell walls of lichens and microbes, while chitinases enable digestion of the hyphae contents and cell walls of fungi (Siepel & DeRuiter-Dijkman, 1993; Berg et al., 2004). Along with digestion, potential non-digestive roles of enzymes also appear. Invertebrate chitinases can subserve arthropod molting (Merzendorfer & Zimoch, 2003), defense/immunity and pathogenicity (Arakane & Muthukrishnan, 2010). Trehalose is a haemolymph transport sugar of insects having a role in the regulation of food intake and stress protection (Thompson, 2003). In Collembola, trehalase has an important role as a cryoprotective agent as well (Sinclair & Sjursen, 2001), and its activity can vary seasonally, especially in arctic species.

Moreover, an association is assumed between the specific enzymatic activity of Collembola and their vertical stratification in the soil profile (life form) (Urbášek & Rusek, 1994). Vertical migration induced by seasonal changes in habitats and subsequent seasonal alternation of food sources is common in some collembolan species (e.g. Hishi et al., 2007).

The level of enzymatic activity depends on the type of food consumed prior to sampling and phase of the life cycle (Berg et al., 2004). Moreover, the activity of some enzymes may significantly differ in the same species at two different sites (Berg et al., 2004).

The high morphological and ecological heterogeneity of Collembola has resulted in the proposal of different types of ecological classifications. The traditional classification of life forms is based on the general body plan (Gisin, 1943; Ruess, 2007), while enzymatic activity and the stable isotope ratio have been considered in more recent studies (Berg et al., 2004; Potapov et al., 2016). Based on qualitative differences in enzymatic equipment, several functional groups were defined in soil mesofauna (Siepel & DeRuiter-Dijkman, 1993), representing functional variability within the group. Berg et al. (2004) found qualitative differences in cellulolytic, trehalolytic and chitinolytic equipment between collembolan species and sorted them into 4–5 principal feeding guilds (Siepel & DeRuiter-Dijkman, 1993).

It is expected that enzymatic equipment may also differ considerably between cave-dwelling animals, depending on their nutritional ecology, but these studies have not been conducted yet.

The present study is one of the first attempts aimed at analyzing enzymatic activity in subterranean arthropods, specifically qualitative and quantitative differences in the activity of trehalase, cellulase and chitinases between Collembola with different affinity to the cave environment (cave guilds). We hypothesized that non-cave-adapted species living in an environment with more heterogeneous food sources would have higher activity of some enzymes, especially cellulase, compared to obligate cave species.

**MATERIALS AND METHODS**

**Tested species and categorical variables**

Specimens were collected in the field together with substrate and organic material from collection sites and transported to the laboratory (Table 1). Seven collembolan species with various affinity to cave habitats were examined: *Ceratophysella denticulata*, *Folsomia candida*, *Heteromurus nitidus*, *Hypogastrura aequipilosa*, *Orthonychiurus rectopapillatus*, *Protaphorura janosik* and *Pseudacherontides speleaeus*. For comparison, *Megaphorura arctica* was tested as an example of a species that does not have any association with the cave environment; therefore, we consider it as “trogloxene”. For practical purposes, we use the term “cave guild” to designate groups of species with different affinities to the cave environment, namely trogloxenes, subtroglrophiles, eutroglrophiles and troglobionts (Sket, 2008). Soil life forms after Ruess (2007) and feeding guilds after Potapov et al. (2016) were used as other categorical variables to reveal potential trophic niche specialization. Body length, number of eyes and total protein content were used as functional traits, with the body weight as a covariable.

**Evaluation of enzymatic activity**

Cellulase, trehalase and chitinase (glucosaminidase, chitobiase, endo-chitinase) activity was measured in eight collembolan species (see above). From 8 to 15 individuals of each species were weighed and washed with 0.6% cholic acid. Each group of collembolans was placed into a small glass mortar filled with 0.25 ml of Britton-Robinson (BR) buffer (pH = 6) and pulverised with a glass pestle until no body parts were recognizable. The mixture was transferred to a plastic Eppendorf tube placed in crushed ice. The mortar and pestle were rinsed with 0.25 ml of BR buffer, and this volume was added to the Eppendorf tube. The homogenate was centrifugated at 6,000 rpm/7 min., and the supernatant was stored at -18°C until used for enzyme activity assay. The activity of each enzyme was measured in 4-6 replications. In *P. janosik*, enzymatic activity in specimens from laboratory culture was compared with those collected in the field and pulverised immediately after transporting to the laboratory.
Table 1. Collembola species and their life forms (LF) after Rusek (2007), cave forms (CF) after Sket (2008) and feeding guilds (FG) after Potapov et al. (2016).

<table>
<thead>
<tr>
<th>Species</th>
<th>Abb.</th>
<th>Family</th>
<th>LF</th>
<th>CF</th>
<th>FG</th>
<th>bl</th>
<th>S</th>
<th>FH</th>
<th>Original locality</th>
<th>RC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceratophysella denticulata (Bagnall, 1941)</td>
<td>Cde</td>
<td>Hypogastruridae</td>
<td>Bb1</td>
<td>EU</td>
<td>EPAM</td>
<td>1.8</td>
<td>L</td>
<td>A</td>
<td>Čertova diera Cave (SK)</td>
<td>s, y</td>
</tr>
<tr>
<td>Folsomia candida (Willemin, 1902)</td>
<td>Fca</td>
<td>Isotomidae</td>
<td>Bc2a</td>
<td>EU</td>
<td>HEM</td>
<td>1.7</td>
<td>L</td>
<td>A</td>
<td>Domica Cave (SK)</td>
<td>s, y</td>
</tr>
<tr>
<td>Heteromurus nitidus (Templeton, 1835)</td>
<td>Hni</td>
<td>Entomobryidae</td>
<td>Bc1a</td>
<td>EU</td>
<td>HEM</td>
<td>3.0</td>
<td>L</td>
<td>A</td>
<td>Domica Cave (SK)</td>
<td>s, y</td>
</tr>
<tr>
<td>Hypogastrura aequipes (Stach, 1949)</td>
<td>Haq</td>
<td>Hypogastruridae</td>
<td>Bb1</td>
<td>SUB</td>
<td>EPAM</td>
<td>2.0</td>
<td>L</td>
<td>A</td>
<td>Čertova diera Cave (SK)</td>
<td>s, y</td>
</tr>
<tr>
<td>Megaphorura arctica Tulberg, 1876</td>
<td>Mar</td>
<td>Onychiuridae</td>
<td>Bc1b</td>
<td>TX</td>
<td>EPPM</td>
<td>3.5</td>
<td>L</td>
<td>N</td>
<td>Svalbard, soil, moss under bird cliffs (N)</td>
<td>s, -</td>
</tr>
<tr>
<td>Orthonychiurus rectapapillatus (Stach, 1933)</td>
<td>Ore</td>
<td>Onychiuridae</td>
<td>Bc1b</td>
<td>SUB</td>
<td>EUM</td>
<td>1.3</td>
<td>L</td>
<td>A</td>
<td>Domica Cave (SK)</td>
<td>s, y</td>
</tr>
<tr>
<td>Protophorura janosik Weiner, 1990</td>
<td>Pja</td>
<td>Onychiuridae</td>
<td>Bc1b</td>
<td>TGB</td>
<td>EUM</td>
<td>3.8</td>
<td>F</td>
<td>N</td>
<td>Jaskyňa pod Spišskou Cave (SK)</td>
<td>-</td>
</tr>
<tr>
<td>Protophorura janosik Weiner, 1990</td>
<td>Pja2</td>
<td>Onychiuridae</td>
<td>Bc1b</td>
<td>TGB</td>
<td>EUM</td>
<td>3.8</td>
<td>L</td>
<td>A</td>
<td>Jaskyňa pod Spišskou Cave (SK)</td>
<td>s, y</td>
</tr>
<tr>
<td>Protophorura janosik Weiner, 1990</td>
<td>Pja4</td>
<td>Onychiuridae</td>
<td>Bc1b</td>
<td>TGB</td>
<td>EUM</td>
<td>3.8</td>
<td>F</td>
<td>N</td>
<td>Duča Cave (SK)</td>
<td>-</td>
</tr>
<tr>
<td>Pseudacherontides spelaeus (Ionesco, 1922)</td>
<td>Psp</td>
<td>Hypogastruridae</td>
<td>Bc2b</td>
<td>TGB</td>
<td>EUM</td>
<td>0.7</td>
<td>L</td>
<td>N</td>
<td>Fănaţe Cave (RO), guano</td>
<td>g, -</td>
</tr>
</tbody>
</table>


Cellulase and trehalase activity was measured by quantifying the rate of glucose production from substrates. For samples, 39 μl of homogenate (supernatant) were pipetted into 1.5 ml plastic Eppendorf tubes filled with 39 μl of substrate solution: carboxymethyl-cellulose (0.02g/ml) or trehalose (0.06 g/ml). Two technical replications for each sample were tested. A homogenate blank containing homogenate and 260 μl of trichloracetic acid (TCA) and substrate blanks containing 1 μl of substrate were incubated together with the sample for 24 h at 37°C. Before incubation, a drop of tolune was added as a bactericide. After incubation, 260 μl of TCA was added to the sample and 39 μl of incubated substrate blank solution was added to the homogenate blank and mixed. Samples and blanks were centrifugated at 6,000 rpm/7 min., and 50 μl of supernatant of samples and blanks were added into separated wells in a microtitration plate filled with 250 μl of GLU GOD 250 Lachema reagent to determine the glucose concentration. The microtitration plate was incubated for 20 min at 37°C. Then the absorbance was measured at 495 nm using a microplate reader (Synergy 2, BIOTEK). A glucose solution was used for the calibration.

Chitinase activity (glucosaminidase, chitinbiase, endo-chitinase) was measured fluorometrically by quantifying the amount of transformed specific fluorogenic substrates 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (MUNAGA), 4-Methylumbelliferyl N,N′-diacetyl-β-D-chitobiose (MUCHB) and 4-Methylumbelliferylβ-D-N,N′,N″-triacyctichitotriose, respectively (MUCHT), using the Chitinase Assay Kit, Fluorimetric (Sigma-Aldrich CS1030). For blanks, 100 μl of diluted (1:79 by BR buffer) substrate stock solutions of MUNAGA, MUCHB and MUCHT were pipetted onto a black microtitration plate. For samples, 90 μl of the same diluted substrate solutions and 10 μl of homogenate were used for each of the 3 substrates. The microtitration plate was incubated for 60 min at 37°C. After incubation, 200 μl of 0.4 M sodium carbonate (Na₂CO₃) was added into each hollow. Fluorescence was then measured using a microplate reader (Synergy 2, BIOTEK, Excitation = 360/40, Emission = 460/40); 4-Methylumbelliferone solution was used for calibration.

The content of total soluble proteins was determined in each homogenate by adding 100 μl of the homogenate to 100 μl of Bradford reagent (Sigma, B6916) and measuring the absorbance at 595 nm (Synergy 2, BIOTEK) after incubation at room temperature for 5-45 min. A solution of bovine serum albumin (Sigma, P 7656) was used as the protein standard for calibration.

Statistical analyses

For statistical evaluation of differences in the enzymatic activities among species One-Way ANOVA with HSD post-hoc comparisons were used (software Statistica v6.0). Relationships between body mass and total soluble protein content and between both these parameters and enzymatic activity were tested using multidimensional regression (Statistica v6.0).

The variability of the enzymatic activity was analyzed by applying direct analysis with linear response associated using the CANOCO 5.03 multivariate software package. Constrained partial RDA was performed with species mean body weight as the covariable. We assessed the proportion of the variability explained by different taxonomical categories, morphological traits and environmental
variables. The forward selection method (FW) showed variables explaining the highest proportion of enzyme variability in a given species. The significance of the environmental variables and categories was specified using the Monte Carlo test with 9999 randomizations.

**RESULTS**

The presence of the tested enzymes (trehalase, cellulase and chitinases) from the whole-body homogenates was confirmed in all species under study.

**Enzymatic activity in relation to mean body mass and total soluble protein content**

Collembola species differed significantly in their mean body mass (One-way ANOVA, \(F(7,75) = 62.42, p < 0.001\)), with *P. spelaeus* as the smallest species and *M. arctica* and *P. janosik* as the largest ones (Table 2).

The content of the soluble proteins in one milligram of homogenate increased with decreasing mean body mass of the Collembola groups used for the homogenizations (\(r = 0.33\), ANOVA, \(F(1,48) = 5.87, p = 0.02\)). Considering mean body mass of species, the highest concentration of proteins was measured in the smallest species, *P. spelaeus*, and the lowest in *H. nitidus*. Differences in the content of soluble proteins among species were significant (ANOVA, \(F(7, 43) = 4.5265, p < 0.001\)). The mean value of this parameter differed significantly from *H. nitidus*, *F. candida*, *M. arctica*, *O. rectopapillatus* and *P. janosik* (HSD post-hoc comparison, \(p < 0.03\), Table 2).

However, the differences in enzymatic activity among species did not simply follow the protein content pattern. The species means of mass-specific enzyme activity of trehalase and chitinase showed increasing trends, with an increasing mean total soluble protein content \((r \text{ from 0.02 to 0.56})\), although linear regression models were not significant (ANOVA, \(p < 0.19\)). Therefore, we preferred to compare enzymatic activities based on protein-specific values. In contrast to other enzymes, the mass-specific activity of cellulase increased insignificantly with a decreasing of the body protein content \((r = -0.33\).

The interspecific differences in protein-specific trehalolytic activity were not significant (ANOVA, \(F(7, 33) = 1.97, p = 0.089\)), with the highest activity observed in *H. nitidus*. Trehalolytic activity in this species differed insignificantly from the lowest activity measured in *M. arctica* and *C. denticulata* (HSD post-hoc comparison, \(p < 0.19\), Table 2). The values expressed in mass-specific units showed a similar pattern, with the exception of the overestimated value observed in *P. spelaeus* (Table 2).

Cellulolytic activity showed significant interspecific differences for both mass-specific and protein-specific values, with the highest activity measured in *M. arctica* (ANOVA, \(F(7, 32) = 4.31, p = 0.0019\) and *F(7, 32) = 4.2951, \(p = 0.0019\)). Activity of the protein-specific cellulase in *M. arctica* was significantly higher compared to *H. aequepilosa*, *F. candida*, *P. spelaeus* and *P. janosik* (HSD post-hoc comparisons, \(p < 0.035\), Table 2).

Chitinolytic activity expressed in protein-specific units displayed significant differences between species. *O. rectopapillatus* and *H. aequepilosa* showed the highest activity of glucosaminidase compared to other species and significantly higher levels of all chitinolytic enzymes (ANOVA, \(F(7, 35) = 5.58, p = 0.0002\), HSD post-hoc comparison, \(p < 0.05\)).

**Table 2. Mean body mass, total soluble protein content and enzymatic activities of Collembola species (whole body homogenates). Enzymatic activities expressed as mass-specific \((U \cdot g^{-1})\) and protein-specific \((U \cdot gP^{-1})\). U = unit of enzyme activity defined as \(\mu M\text{ol.min}^{-1}\). Means ± standard errors are presented; small letters indicate significant differences among means at \(p = 0.05\).**

<table>
<thead>
<tr>
<th>Species</th>
<th>Body mass</th>
<th>Proteins</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu g. ind.^{-1})</td>
<td>(mg. g^{-1})</td>
<td>Trehalase</td>
</tr>
<tr>
<td><em>P. spelaeus</em></td>
<td>17.98 ± 0.78 c</td>
<td>13.84 ± 2.97 b</td>
<td>0.431 ± 0.364</td>
</tr>
<tr>
<td><em>F. candida</em></td>
<td>127.86 ± 15.84 c</td>
<td>4.37 ± 1.41 a</td>
<td>0.067 ± 0.023</td>
</tr>
<tr>
<td><em>C. denticulata</em></td>
<td>133.91 ± 13.45 bc</td>
<td>7.66 ± 1.67 ab</td>
<td>0.109 ± 0.049</td>
</tr>
<tr>
<td><em>O. rectopapillatus</em></td>
<td>134.37 ± 10.71 bc</td>
<td>5.22 ± 0.31 a</td>
<td>0.282 ± 0.172</td>
</tr>
<tr>
<td><em>H. aequepilosa</em></td>
<td>127.28 ± 6.15 bc</td>
<td>7.43 ± 1.48 ab</td>
<td>0.186 ± 0.060</td>
</tr>
<tr>
<td><em>H. nitidus</em></td>
<td>253.95 ± 32.46 b</td>
<td>4.00 ± 0.43 a</td>
<td>0.029 ± 0.056</td>
</tr>
<tr>
<td><em>P. janosik</em></td>
<td>590.58 ± 105.97 a</td>
<td>4.54 ± 0.53 a</td>
<td>0.255 ± 0.087</td>
</tr>
<tr>
<td><em>M. arctica</em></td>
<td>620.27 ± 12.59 a</td>
<td>4.77 ± 0.89 a</td>
<td>0.065 ± 0.002</td>
</tr>
</tbody>
</table>

**Enzyme activity**

<table>
<thead>
<tr>
<th>Glucosaminidase</th>
<th>Chitobiase</th>
<th>Endochitinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>(U \cdot g^{-1})</td>
<td>(U \cdot gP^{-1})</td>
<td>(U \cdot g^{-1})</td>
</tr>
<tr>
<td><em>P. spelaeus</em></td>
<td>0.955 ± 0.921</td>
<td>0.062 ± 0.055 b</td>
</tr>
<tr>
<td><em>F. candida</em></td>
<td>0.156 ± 0.118</td>
<td>0.036 ± 0.027 b</td>
</tr>
<tr>
<td><em>C. denticulata</em></td>
<td>9.712 ± 6.092</td>
<td>0.087 ± 0.018 b</td>
</tr>
<tr>
<td><em>O. rectopapillatus</em></td>
<td>2.035 ± 0.343</td>
<td>0.414 ± 0.086 a</td>
</tr>
<tr>
<td><em>H. aequepilosa</em></td>
<td>1.840 ± 0.266</td>
<td>0.273 ± 0.071 a</td>
</tr>
<tr>
<td><em>H. nitidus</em></td>
<td>0.644 ± 0.101</td>
<td>0.161 ± 0.023 b</td>
</tr>
<tr>
<td><em>P. janosik</em></td>
<td>0.766 ± 0.279</td>
<td>0.183 ± 0.060 b</td>
</tr>
<tr>
<td><em>M. arctica</em></td>
<td>0.602 ± 0.176</td>
<td>0.126 ± 0.037 b</td>
</tr>
</tbody>
</table>
The activity of chitobiases in *O. rectopapillatus* was higher than in other species, with an exception of *H. aequipilosa* and *P. spelaeus* (ANOVA, F(7, 35) = 3.77, p = 0.0038, HSD post-hoc comparison, p < 0.05), while the endochitinase activity in this species was higher than in all other species (ANOVA, F(7, 35) = 7.0734, p = 0.00003, HSD post-hoc comparison, p < 0.002).

**Enzymatic activity and Collembola categories**

Considering the taxonomical categories, the highest proportion of enzymatic variability was explained at the species level (Table 3). The degree of association with caves (cave guilds) was more important for the collembolan enzymatic equipment than feeding guilds or life forms (Table 4).

Direct RDA ordination (Fig. 1) placed *F. candida* and *P. spelaeus* in the same quadrant of the ordination space, contrary to *M. arctica* and *O. rectopapillatus*. All populations of *P. janosik* had similar enzymatic activity and did not overlap with any other species. The presence or absence of certain enzymes in these species was important for their classification into certain guilds and life forms.

Table 3. Enzymatic variability (partial RDA) explained by systematic categories. The significance of the first canonical axis was tested. F – Fisher statistics, P – significance level, ND – not determined.

<table>
<thead>
<tr>
<th>Categories</th>
<th>explained variability %</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Order</td>
<td>4.6</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Family</td>
<td>19.3</td>
<td>9.6</td>
<td>0.025</td>
</tr>
<tr>
<td>Species</td>
<td>53.0</td>
<td>26.0</td>
<td>0.001</td>
</tr>
<tr>
<td>Order + Family + Species</td>
<td>53.8</td>
<td>18.6</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 4. Enzymatic variability (partial RDA) explained by ecological categories. The significance of the first canonical axis was tested. F – Fisher statistics, P – significance level.

<table>
<thead>
<tr>
<th>Categories</th>
<th>explained variability %</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feeding guilds</td>
<td>25.1</td>
<td>10.2</td>
<td>0.006</td>
</tr>
<tr>
<td>Life forms</td>
<td>14.5</td>
<td>5.7</td>
<td>0.009</td>
</tr>
<tr>
<td>Cave forms</td>
<td>35.7</td>
<td>14.9</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 5. Enzymatic variability explained by categorical variables and functional traits; variables that explain the highest proportion of enzymatic variability using the forward selection method (FW) are marked. F – Fisher statistics, P – significance level, P (adj) – significance with Holm correction P-values in bold are significant. Significance based on Monte Carlo tests with 9999 randomizations. Covariable: body weight; analysis: interactive forward selection with covariate.

<table>
<thead>
<tr>
<th>Categorical variables</th>
<th>Explained variability %</th>
<th>F</th>
<th>P</th>
<th>P(adj)</th>
<th>FW selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cave dwelling – troglobine</td>
<td>16.3</td>
<td>3.9</td>
<td>0.019</td>
<td>0.235</td>
<td>✓</td>
</tr>
<tr>
<td>Cave dwelling – subtrogliph</td>
<td>54.8</td>
<td>16.6</td>
<td>0.000</td>
<td><strong>0.001</strong></td>
<td>✓</td>
</tr>
<tr>
<td>Cave dwelling – eutrogliph</td>
<td>16.9</td>
<td>4.1</td>
<td>0.014</td>
<td>0.183</td>
<td></td>
</tr>
<tr>
<td>Cave dwelling – troglodine</td>
<td>10.6</td>
<td>2.5</td>
<td>0.070</td>
<td>0.630</td>
<td></td>
</tr>
<tr>
<td>Life form – Bc1</td>
<td>2.3</td>
<td>0.5</td>
<td>0.660</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Life form – Bc1a</td>
<td>5.6</td>
<td>1.3</td>
<td>0.251</td>
<td>1</td>
<td></td>
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<tr>
<td>Life form – Bc1b</td>
<td>28.3</td>
<td>7.3</td>
<td>0.001</td>
<td><strong>0.025</strong></td>
<td></td>
</tr>
<tr>
<td>Life form – Bc2a</td>
<td>8.8</td>
<td>2.0</td>
<td>0.098</td>
<td>0.778</td>
<td></td>
</tr>
<tr>
<td>Life form – Bc2b</td>
<td>8.4</td>
<td>1.9</td>
<td>0.110</td>
<td>0.778</td>
<td></td>
</tr>
<tr>
<td>Feeding guilds – epam</td>
<td>2.3</td>
<td>0.5</td>
<td>0.664</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Feeding guilds – eppm</td>
<td>16.3</td>
<td>3.9</td>
<td>0.022</td>
<td>0.242</td>
<td></td>
</tr>
<tr>
<td>Feeding guilds – hem</td>
<td>9.2</td>
<td>2.1</td>
<td>0.097</td>
<td>0.778</td>
<td></td>
</tr>
<tr>
<td>Feeding guilds – eum</td>
<td>27.4</td>
<td>5.8</td>
<td>0.002</td>
<td><strong>0.039</strong></td>
<td></td>
</tr>
</tbody>
</table>

### DISCUSSION

**Enzymatic equipment in cave-dwelling Collembola**

The presence or absence of certain enzymes associated with digestion can reflect the ability of animals to utilize various food sources. The proportions of digestive enzymes may indicate what amount of different food sources may be potentially digested by the animal; however, the potential and actual diet may differ. Quantitative proportions of digestive enzymes may indicate what amount of different food sources may be potentially digested by the animal; however, the potential and actual diet may differ. Quantitative proportions of different enzymes may indicate what amount of different food sources may be potentially digested by the animal; however, the potential and actual diet may differ.
enzyme activity may respond to the actual food more sensitively than qualitative ones.

In the present study, the species collected in caves were examined to reveal their enzymatic equipment in relation to specific conditions of subterranean habitats. Urbášek & Rusek (1994) found only slight differences in the enzymatic activity between Collembola life forms. Similarly, we confirmed the presence of all tested enzymes in body homogenates of the species involved in this study. The enzymatic equipment corresponded to herbo-fungivorous grazers, the most prevalent collembolan feeding guild (Berg et al., 2004). Although all species examined were qualitatively uniform in digestive enzymes, the activities of enzymes exploiting the same substrate quantitatively differed between species. The quantitative differences of enzymatic activities did not simply follow concentrations of soluble proteins; thus, an effect of the genetically fixed infraspecific proportions of certain digestive enzymes is indicated. This may reflect food specialization determined by evolutionary adaptation or non-genetic regulation of synthesis or activation of different enzymes.

We observed significant differences in cellulolytic and chitinolytic activity between the cave species, confirming the key importance of these enzymes in the indication of their feeding habits. The majority of species displayed very low cellulolytic activity compared to other enzymes. The increasing trend of cellulase activity towards trogloxenes and subtroglophiles confirmed our hypothesis. Thus, the degradation of fresh plants and wood is non-relevant for collembolans occupying a deeper cave environment, which is primarily oligotrophic (Culver & Pipan, 2009). Along with cellulase produced in the midgut or by salivary glands, some insects contain cellulases released by symbiotic bacteria (Fisher et al., 2013) or may have horizontally transferred genes for cellulases involved in carbohydrate metabolism and cellulose degradation (Faddeeva-Vakhrusheva et al., 2016). In general, it is difficult to specify whether the enzymes responsible for the breakdown of food items originate from the animal itself or are excreted in the alimentary tract by the microflora when deriving the enzymatic activity from whole-body homogenates (Berg et al., 2004; Fisher et al., 2013). An endogenous cellulolytic protein has been confirmed in only one collembolan species, Cryptopygus antarcticus (Song et al., 2017).

Rearing conditions can influence the enzymatic equipment as well, especially quantitatively. Despite the fact that most of the species examined in the present study came from a laboratory culture feeding with yeast, significant interspecific differences were observed. This suggests that these differences are not associated with the current physiological responses in
relation to artificial food but reflect the species-specific enzymatic equipment. Two natural populations of *P. janosik* did not display any differences from one another in enzymatic activity. In contrast, a distinct pattern in the activity of digestive enzymes was found in comparing natural populations with a long-term laboratory culture reared on substrate from the sampling locality and feeding on yeast. Thus, the enzymatic equipment of the laboratory population characterized by high activity of chitinases probably reflects the artificial yeast-enriched diet. However, only one sample from the culture was available; thus, the increasing trend towards chitinolytic activity in the population feeding on yeast could not be statistically supported. The influence of the above-mentioned factors on enzymatic equipment of the cave collembolans requires further study. Unfortunately, the difficult access to subterranean environments and mostly dispersed distribution of Collembola specimens in caves make the collection of the material for study of digestive enzymes highly demanding.

**Enzymatic activity of Collembola and affinity to cave environment (cave guild)**

Trogloxene species *M. arctica* is classified as a euedaphobic life form (Rusek, 2007) and an epigeic plant and microorganisms consumer (Potapov et al., 2016). We observed high cellulolytic activity and very low chitinolytic activity in this trogloxene species, which is in accordance with findings of Hodkinson et al. (1994) that it feeds predominantly on living and dead bryophytes, detritus and algal cells.

As to the troglophiles, both subtroglophile species, *O. rectopapillatus* and *H. aequepilosa*, differed in the proportion of enzyme activities, indicating variability of feeding preferences inside this cave guild. Generally, subtroglophiles were established as secondary decomposers based on analyses of stable isotopes (Chahartaghia et al., 2005), grazing on saprophytic, endomycorrhizal and ectomycorrhizal fungi. This corresponds to the relatively high activity of chitinases in *O. rectopapillatus*. On the other hand, we registered low activity of all tested enzymes in *H. aequepilosa*, which is classified as an animal and microorganisms consumer (Potapov et al., 2016). Subtroglophiles appear to be food generalists rather than specialists (Maraun et al., 2003). This is likely the case of the guild occupying the cave entrances, the habitat enriched by heterogenous dead organic material colonized with subtroglophiles.

The category “eutroglophile” is represented in our study by *F. candida* and *H. nitidus*, both classified also as hemiedaphic microorganism consumers, and *C. denticulata*, also classified as an epigeic plant and animal consumer and fungal feeding secondary decomposer (Ruess et al., 2007; Chahartaghia et al., 2005; Potapov et al., 2016). Such variability in feeding preferences in this cave guild is indicated by
Enzymatic activity in cave-adapted fauna in evolutionary approach

The edaphic lifestyle was proposed as the evolutionary ancestral state of Collembola (D’Haese, 2002). Considering the evolution of cave-adapted fauna from preadapted relatives (Holsinger, 2000) and the lack of primary production in caves, we assumed a shift of enzymatic activity from cellulases in edaphic forms towards chitinases in obligate cave forms. On the other hand, incidental and relatively poor food sources in caves may suggest the evolutionary pressure to maintain the ability to utilize various sources of energy. It is also conceivable that diversification of species generally leads to the colonization of various food niches during their evolution and is associated with more rapid changes in enzyme activities compared to body or mouthparts morphology. In the present study, higher variability was observed at the species level compared to families. This suggests that affiliation to higher taxonomical categories, and thus similar mouthparts morphology as well as general body plan, does not fully designate species to an equal ecological role. This is supported by different enzymatic activities found in species within the same family. The above-mentioned disproportions between enzymatic activity and available categorizations point to the existence of food-specialists, regardless of the life-form or cave guild.

Enzymatic activity as an adaptive feature in obligate (troglobiotic) cave Collembola is still poorly known. It is necessary to examine a wider spectrum of such species and several populations within each cave-adapted form to investigate interspecific differences in enzymatic equipment as well as differences within the same species. Moreover, the molecular approach, especially identification of genes responsible for production or activation of particular enzymes, could explain the mechanisms leading to different physiology of digestion in subterranean Collembola.

CONCLUSIONS

Activity of all tested enzymes was confirmed across all cave guilds, probably as a result of the evolutionary pressure to maintain the ability to utilize various food sources. The quantitative differences of enzymatic activities did not simply follow concentrations of soluble proteins; thus, an effect of the genetically fixed infraspecific proportions of certain digestive enzymes is indicated. Cellulolytic and chitinolytic activity showed a key role in the indication of species feeding habits. Different enzymatic activity was found within the same family, supporting the statement that the ecological role of the species is not determined by its taxonomical category. Quantitative interspecific differences were not associated with the current physiological responses in relation to artificial food, thus reflecting the species-specific enzymatic equipment. The study confirmed our hypothesis that there is a shift of enzymatic activity from cellulases in edaphic forms towards chitinases in obligate cave forms of Collembola.

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Co-authorship analysis of the speleothem proxy-climate community: working together to tackle the big problems

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Abstract: Understanding the environmental context of speleothem palaeo-climate proxies is fundamental to their interpretation. We analyse four methodological approaches to accomplish this: stalactite discharge analysis, proxy/process tracer studies, discharge modelling, and geophysics. Datamining produced citation data sets that reflected these methodological sub-disciplines. Social network analysis is used to examine co-authorship within and between these sub-disciplines, and between the joint methodological community and the broader speleothem proxy climate community. Members of the sub-disciplines have become more connected to one another over time, and to members of the other sub-disciplines. High degrees of connectivity between and within communities allows for the rapid and efficient adoption of new ideas and methods, and will enable the community to effectively tackle emerging complex problems.

Keywords: paleoclimatology, co-authorship, speleothem

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INTRODUCTION

The speleothem-derived palaeo-climate proxy community (SPCCPC) seeks to improve understanding of past climates through the interpretation of climate sensitive proxies in stalagmites. This necessitates better understanding of the environmental context, including climate impacts (from the micro-scale (cave environment) to the macro-scale (large weather systems and climate patterns)) and groundwater hydrology (McDermott, 2004; Fairchild et al., 2006a; Lachniet, 2009; Fairchild & Baker, 2012). We analysed four methodological approaches to achieve this, which are broadly grouped into; stalactite discharge analysis, proxy/process tracer studies (hereinafter "tracer studies"), discharge modelling, and geophysics (Table 1). Nonetheless, the SPCPC faces ongoing challenges related to the common assumption of stationarity in how the proxy data respond to changes in climate, i.e. that the proxies will exhibit the same behaviour in response to changes in climate over time (Gedalof, 2002; Jones & Mann, 2004; Bradley et al., 2010; Baker et al., 2013; Moerman et al., 2014).

As systems-based approaches have become prevalent in the environmental sciences, many disciplines have advocated inter-, trans- and multi-disciplinarity (Steele & Stier, 2000; Klein, 2008; Bark et al., 2016). Choi and Pak, (2006) note that while ‘inter-,’ ‘trans-,’ and ‘multi-disciplinarity’ are commonly used terms, they are poorly defined, applied ambiguously, and used interchangeably. Here, we use the term “inter-disciplinary” to refer to any instance of co-authorship between scientists from different disciplines, where co-authorship is defined as collaboration between unique authors in a published piece of work.

Social network analysis methods have been developed to measure collaborative behaviour. These methods are based on the study of social structure using graph theory, and originate in sociology (Scott, 1988). Co-authorship networks have been used to represent acquaintance-ship and research collaboration effort, and thereby the sharing of ideas (Newman, 2001, 2004; Huang & Chang, 2011). Co-authorship social network analysis (C-SNA) has been used for strategic planning of research and development (Morel et al., 2009), to investigate the relationship between co-authorship and h-index (McCarty et al., 2013), to study inter-disciplinarity (Huang et al., 2011), and to investigate the structure of different fields of study (Grossman, 2002; Newman, 2004; Zare-Farashbandi

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et al., 2014). At the individual level, McCarty et al. (2013) showed that scientific impact as measured by the h-index increases when authors collaborate with as many co-authors as possible.

We use C-SNA to investigate co-authorship within methodological sub-disciplines of the SPCPC, between these sub-disciplines, and across the whole community from 1996 to 2017. This has implications for the capability of the community to identify and address emerging complex problems. We address three specific questions:

1) Have the populations of the methodological sub-disciplines become more connected over time?
2) Have the methodological sub-disciplines become more connected to one another? Is there a trend towards inter-disciplinarity?
3) Has the whole speleothem palaeo-climate community moved towards inter-disciplinarity?

Table 1. Strengths and weaknesses of methods used to contextualise stalagmite proxy climate records.

<table>
<thead>
<tr>
<th>Method</th>
<th>Strengths</th>
<th>Weaknesses</th>
<th>Key Applications</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stalactite discharge analysis</td>
<td>Common method; Rugged technology; capable of long-term and removal deployments; information about physical (drip) hydrology.</td>
<td>Without tracer data can’t quantify impact of subsurface processes on proxy record.</td>
<td>Commonly used to support palaeo-climate reconstructions. Includes discharge response to precipitation, effective infiltration, etc.</td>
<td>Baldini et al., 2006; Fairchild et al., 2006; Geurts &amp; Deflandre, 1998; Hu et al., 2008; Mahmud et al., 2016; Markowska et al., 2015</td>
</tr>
<tr>
<td>Proxy/Process tracer studies</td>
<td>Quantitative results: event times, mixing, transmission time.</td>
<td>Can be conservative; false breakthrough signatures; approvals and social licence for using artificial tracers; natural variability of stable isotopes; karst complexity complicates signal.</td>
<td>Landscape-scale karst hydrology and small scale karst drip hydrology; tracer studies – including stable isotopes, radio isotopes, stable isotope concentrations and processes such as source–precipitation–infiltration–drip discharge; dissolution processes and disequilibrium/kinetic isotope fractionation.</td>
<td>Bottrell &amp; Atkins, 1992; Bradley et al., 2010; Callow et al., 2014; Cuthbert et al., 2014; Fairchild et al., 2006b; Friedrich &amp; Smart, 1982; Fuller et al., 2008; Gunn, 1974; Jex et al., 2012; Kogovsek &amp; Petric, 2014; Poulain et al., 2015; Treble et al., 2013, 2005</td>
</tr>
<tr>
<td>Discharge modelling</td>
<td>Extend limited observational records; infer subsurface processes that affect dripwater behaviour and chemistry.</td>
<td>Simplify reality, may not capture complexity of the physical system; not always physically-based.</td>
<td>Commonly used in association with drip monitoring and tracer experiments, recently a greater emphasis on modelling isotopic values in dripwaters.</td>
<td>Arbel et al., 2010; Bradley et al., 2010; Cuthbert et al., 2014; Fairchild, et al., 2006b; Tooth &amp; Fairchild, 2003; Treble et al., 2013; Wackerbarth et al., 2010</td>
</tr>
<tr>
<td>Geophysics</td>
<td>Non-invasive, high spatial resolution (incl. 3D); image physical structure.</td>
<td>Cannot image at the pore-scale; limited resolution at depth; artefacts caused by (for example) large cavities limit resolution.</td>
<td>Limited applications at stalagmite-scale settings.</td>
<td>Al-fares et al., 2002; Campbell et al., 2017; Carrière et al., 2013; Roth &amp; Nyquist, 2003; Valois et al., 2010; van Schoor, 2002; Zhou et al., 2000</td>
</tr>
</tbody>
</table>

**METHODS**

We apply CSNA to citation data obtained by data mining the Web of Science – Core Collection (26th of May 2017) using keywords (Morel et al., 2009). Keyword searches were applied for the four identified methodological approaches; stalactite discharge analysis (“speleothem AND hydrology NOT model”, n = 48), tracer studies (“speleothem AND tracer”, n = 20), geophysics (“karst AND geophysics”, n = 76) and discharge modelling (“speleothem AND hydrology AND model”, n = 27). Methodological approaches and keywords were chosen through a review of the literature, and to capture a sufficiently large portion of the community in order to make analyses possible (respectively). Consequently, a broader search term for geophysics was used as “speleothem AND geophysics’ only returned one article. The databases are used to attribute sub-disciplines to the authors, and authors are assumed to have published in multiple disciplines if they are found in multiple databases. There was just one paper which appeared in two databases, and those authors were attributed to (at least) two disciplines. We also extracted a broader community set using “speleothem AND climate” (n = 860), and this was combined with the citations sourced to investigate the methodological approaches to form an overall SPCPC community database (duplicates between the two datasets were included but classified as part of the methodological subset). The Web of Science – Core Collection was chosen over other archives as it does not contain unpublished papers. Citations were exported in “.bib” file format. Citation data were analysed cumulatively at time-steps of 5 years between 1996 (the earliest entry for any of the search terms in the Web of Science- Core Collection) and 2017. Note that the final time step was 6 years. Bin size was a parsimonious choice to reflect change over time. The decision was made to present data in cumulative time-steps because discrete time windows do not adequately reflect the nature of the collaboration networks, in that they become more connected over time. The choice of bin size also impacts the analysis of discrete time windows. If authors were to collaborate extensively in the first time period, but not the second, it would appear as if they are not connected (or even present in the network) in the second time period, when in reality they may still be collaborating on unpublished work during this time. See Supplementary Figure 1 for an analysis of co-authorship in the sub-disciplines in discrete time windows.

Data analysis and network construction were undertaken in R using the `bibtext` and `igraph` packages (Csardi & Nepusz, 2006; Francois et al., 2017; R Core Team, 2017). Files were parsed to extract the unique author name, and names were considered to consist of one first initial and a surname. The term ‘unique
author’ refers to the unique authors present in the database. Efforts were made to ensure correct attribution of authorship to each parsed unique author. Instances of co-authorship between unique authors were recorded in an adjacency matrix, which was then converted into a graph of co-authorship. All network graphs were rendered in Gephi (Gephi, 2016), see Fig. 1 for an example of network components and how to interpret them.

Networks were evaluated using the average degree and the presence or absence and relative size of a giant component. The average degree is the mean number of co-authors that each unique author has, where higher average degree means that information propagates more readily through the network (Newman, 2001). The term ‘giant component’ refers to the largest subgraph (connected part of the network) (Holme et al., 2002). Here, we further restrict the definition to exclude subgraphs which are only comprised of the unique authors of one paper. Therefore, in Fig. 1, which shows two example networks and their key features, 1A does not include a giant component as the largest subgraph is the product of just one instance of collaboration (i.e. all of the authors who collaborated on one paper are connected). Figure 1B includes a giant component made up of unique authors collaborating in several different instances. Not all unique authors in the giant component are directly connected by co-authorship, but as members of the giant component they may still benefit from the easier sharing of ideas through the connected part of the network.

The full citation dataset and R scripts are in the Supplementary Material. Analysis was conducted on each: a) method database (see Section By discipline), b) the combined method database (see Section Combined discipline), and c) the overall SPCPC community database (see Section Whole community).

RESULTS AND DISCUSSION

By discipline

The CSNA identifies that the SPCPC methodological sub-disciplines have grown in both the number of unique authors and their level of connectivity, as shown by the increase in the average degree over time (Fig. 2), though some subtle but important differences are evident between the sub-disciplines. The stalactite discharge analysis sub-community is the second largest network, growing exponentially from 10 unique authors in 2001 to 213 in 2017. The discharge analysis network became more connected during this time, as the average degree increased from 4.8 to 7.47 between 2001 and 2017. The average degree of the discharge analysis network in 2017 was second only to that of the discharge modelling community. By 2017, 177/213 (83%) unique authors were part of the giant component, this is an increase from 58/249 (23%) in 2011, when the giant component was first observed.

The tracer study community is the youngest and smallest sub-community. The first citation found in the Web of Science Core Collection database was published in 2004, but the discipline has grown consistently from 13 authors in 2006 to 80 in 2017. Co-authorship has not been as pronounced within the tracer community, although it has increased over time. A small giant component had formed by 2011 (10/42 authors) but by 2017 this giant component comprised just 18 of the 80 authors (22%). However, these 18 are very well connected which reflects the relatively high average degree which increased from 4 in 2001 to 5.25 in 2017. Although members of the giant component were in the analysis in 2006, the giant component was not observed until 2011.

The discharge modelling community grew from 3 authors in 2001 to 107 in 2017. During this time co-authorship also increased - the average degree grew from 3.1 to 8.15 (the highest observed average degree of any of the disciplines). By 2011 a giant component was observed (12/30 authors), and by 2017 the giant component included 38/107 authors and a secondary subgraph had formed which included 29/107 authors. In both the giant component and the subgraph there are linking authors (two in each) who act as the only link between different groups of authors in the network (see also Fig. 1B). These linking authors clearly have a role as influencers, and are likely established members of the community.

The geophysics community is the largest and least well-connected community, although its size is an artefact of the broader search terms applied. Despite this, the average degree is consistently low relative to the other disciplines (4.17 in 2017). A giant component had formed by 2011, although it included just 9% of the community (12/137). By 2017 this had increased to 12% (27/232). Due to the relative lack of connectivity in the geophysics community there are no standout linking authors until 2017 when one member of the giant component had published with every other member.
Combined disciplines

Since 2001, the four sub-disciplines have become increasingly connected, as shown in Fig. 3 and by the increase in the average degree.

A giant component was present in 2001, which included 9 of 33 authors (27%). This remained stable through to 2006 (although the percentage of total authors in the giant component had fallen to 9%). By 2011 the giant component included 95 of 275 authors (34%), and by 2017 it included 269 of 563 authors (48%). The stalactite discharge analysis and discharge modelling disciplines are the most inter-disciplinary. In 2001, all authors from these two disciplines were part of either the giant component or a multi-disciplinary subgraph. In 2017 they continued to represent the largest proportion of the giant component, with 253 of the 268 authors in the giant component publishing in stalactite discharge analysis, discharge modelling, or both. There is a growing trend towards authors publishing in multiple sub-disciplines. In 2001, four authors had published in two sub-disciplines, this increased to five in 2006, 22 in 2011 and 46 in 2017. By 2011, one author had published in three sub-disciplines. This increased to 11 by 2017.

In 2017 geophysics dominated outside of the giant component (221 authors), followed by tracer studies (34 authors), then stalactite discharge analysis (19 authors), and modelling (12 authors). Although outside the giant component, inter-disciplinarity still occurred in subgraphs, with 33 authors publishing either across different disciplines or with co-authors from different disciplines. This includes six authors who themselves published across two different disciplines and an additional author who published across 3 different disciplines.

While authors publishing across different sub-disciplines are not necessarily linking authors as per our definition, it is likely that they may have been linking authors in the past and have played
Co-authorship analysis of the speleothem proxy-climate community

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**Fig. 3.** Combined co-authorship network, with unique authors colour-coded by the sub-discipline they published within. Stalactite discharge analysis is dark blue, tracer studies is orange, discharge modelling is light blue, and geophysics is red. Authors that have published within two sub-disciplines are yellow, and those that have published within three sub-disciplines are green.

Key roles in sharing information and methods, owing to their central positions in the giant component, and their experience in applying different methods. Since collaborating implies acquaintanceship and the communication of ideas, by publishing with co-authors from different disciplines, authors are exposed to different methods, approaches, and ideas (Huang et al., 2011; Newman, 2001, 2004).

**Whole community**

The rate of publication, and by inference, the broader SPCPC continues to grow. While the proportion of publications in the method-derived citation database which was used for the analyses in sections *By discipline* and *Combined disciplines* ("the subset") has not increased relative to the whole community (Fig. 4), co-authorship analysis shows that the authors in the subset are linked to the broader community, and that the broader community is itself highly connected (Fig. 5). Of 2433 unique authors in the broader community, 563 (23%) of them were found in the subset. A giant component included 2122 (87%) of the total unique authors, and authors from the subset made up 417/2122 (20%) of that giant component. Therefore, while co-authors in the subset database are well connected to one another (see section *Combined disciplines*), they are not as well-connected to the broader speleothem climate community. Note that the broad search terms used to define the broader community is likely to have included authors that have referred to the applications of speleothems in palaeo-climate science as a general comment. As such, the shortfall between the proportion of the subset in the whole community and in the giant component is not surprising.

**Fig. 4.** Citations data-mined from the web of science. The citations that make up the method-based subset are orange and the whole citation network is red.

**CONCLUSIONS**

Since 2001, the methodological sub-disciplines identified in this analysis have become more connected. This is most notable in the stalactite discharge analysis, tracer studies, and discharge modelling disciplines. Increasing levels of co-authorship has implications for the propagation of information through the community, and the growth of the community, as authors with high levels of co-authorship are statistically more likely to add new co-authors to the network (Barabási & Albert, 1999).

The methodological sub-disciplines have become more connected to one another over time. Again this
behaviour was most common in the discharge analysis, tracer studies and discharge modelling disciplines. There was also an increase in the number of authors publishing across multiple disciplines. The high level of cohesiveness and inter-disciplinarity means that the community has improved scope to tackle complex problems, and is able to quickly adopt and share new technologies and methodologies. The absence of geophysics from the giant component until after 2011 is surprising considering that the method is broadly applied in karst science, and that geophysicists were consistently well represented in the database. Its addition to the giant component of the network after 2011 is an indicator that this technology has begun to be adopted by the community. We may expect the use of geophysics to become more common due to the high levels of co-authorship in the broader SPCPC, and therefore easy pathways of knowledge sharing.

The broader SPCPC is highly connected, and the sub-disciplines are represented in the giant component. This means that, while many of the citations in the broader palaeo-climate community were not represented in the analysis of inter-disciplinarity, it is likely that they have access to the knowledge and expertise to adopt a diverse range of methods.

The speleothem palaeo-climate proxy community has become increasingly well-connected, and increasingly inter-disciplinary. While there remains a large part of the community that has not adopted any of the common methods to contextualise speleothem proxy climate records, the high degree of co-authorship between the members of the methodological sub-disciplines and the community at large indicates that it is likely that the broader community will a) adopt these approaches, and b) become more interdisciplinary over time, or c) become aware of these approaches through enhanced dissemination of ideas through a more integrated speleothem palaeo-climate proxy community. An interesting subject for future research is the speleothem palaeo-climate proxy community’s self-perception of collaborative behaviour between different sub-disciplines. This could use social science methods (such as interviews and surveys) to establish the methodological approaches of these sub-disciplines and where authors position themselves within or across them.

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Sulfur Cave (Romania), an extreme environment with microbial mats in a CO₂-H₂S/O₂ gas chemocline dominated by mycobacteria

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Abstract: Sulfur Cave (Puturosu Mountain, Romania) is an extreme environment, unique for displaying life in a gas chemocline. The lower part of the cave is filled with CO₂, CH₄, and H₂S of mofettic origin, while the upper part contains air that floats above the heavier volcanic gasses. S²⁻ and H₂SO₄ (from sulfur-oxidation) cover the cave wall at and below the CO₂-H₂S/O₂ gas/gas interface. On the cave wall, near the interface the pH is <1 and unusual microbial biofilms occur on the rock’s surface. We provide context information on the geology, mineralogy, chemistry and biology to better understand this unique environment. We have used X-ray diffraction, optical microscopy, scanning electron microscopy with EDAX capabilities, stable isotope analysis and 16S and 18S rDNA amplicon sequencing. The most common taxa in the microbial biofilms are Mycobacteria, Acidithiobacillus and Ferroplasmaceae. Liquid water in this system originates solely from condensation of water vapor onto the cave walls making inflow of organic carbon from outside unlikely. The most likely primary source of energy for this microbial community is sulfur oxidation with H₂S and S²⁻ as main reductants and atmospheric O₂ as the main oxidant. Ferric iron from the rock surface is another potential oxidant. In Sulfur Cave, gaseous CO₂ (from mofettic emission) maintains the stability of the gas chemocline. Sulfur Cave biofilms can help the search for extreme life in the subsurface, near volcanic systems on Earth and Mars. The Sulfur Cave example shows that a habitable environment can be established underground in gas chemoclines near CO₂-dominated gas discharge zones, where it can have a steady supply of water and energy.

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

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INTRODUCTION

Adopting a strategy to search for life on Mars or other extraterrestrial bodies is always fraught with uncertainties such as: (i) the best habitat to find life; (ii) the type of metabolism; and (iii) resemblance with life on Earth. Mars, in particular, is enigmatic in these regards, with a highly oxidized surface, a very thin (=17 mbars) atmosphere with little nitrogen (1.9%), very limited levels of oxygen (0.15%) and water vapor (mean 9.6 pr. precipitable microns), and 96% CO₂ (Mahaffy et al., 2013; Sindoni et al., 2011).
Due to profound differences in geological history, climate, atmospheric composition and electromagnetic shielding from cosmic radiation (among other things), there are no terrestrial environments that fully mimic the present conditions on Mars. However, a multitude of Mars-analog environments have been used for studying the effects of certain Mars-like conditions (i.e., temperature, aridness, radiation) on microbial life and biosignatures (Aerts et al., 2014; Amils et al., 2007; Navarro-González et al., 2003). Here we report on a terrestrial cave environment in Sulfur Cave (also known as “Peștera de la Turia”, Romania) that resembles several extreme conditions expected to exist, or to have existed, in the regolith’s subsurface near fumarole fields on Mars. Sulfur Cave is accessible for human exploration, but the target environment for this study is located in the deepest section of the cave and shielded from sunlight. Microbial communities colonize the cave walls at the level of a gas/gas interface between volcanic gasses of mofetic origin (and more abundant in the lower sections of the cave) and atmospheric air that floats above the heavier volcanic gases. Condensation of water vapor onto the cave wall is the only source of liquid water available for life.

The redox interface in Sulfur Cave may resemble an environmental niche for microbial life associated with it that could have been present on Mars in the distant past. Similar environments may even be present on Mars today in fumarole fields. The lower gas layer in Sulfur Cave shows similarities to the Martian atmosphere in terms of the relative abundance of CO₂, N₂, and O₂ (Althaus et al., 2000; Vaselli et al. 2002). An extinct near-surface fumarole field was found at the Gusev crater on Mars (Yen et al., 2008), and cave-like structures have been detected from orbit (Cushing et al., 2007). Learning what we can from similar terrestrial environments could offer valuable insights for future missions to Mars. Knowledge about the properties of the environment and about the structure of the bacterial community in Sulfur Cave may well contribute to better understanding of potential life forms in similar harsh environments on Earth and Mars.

History and geographic settings

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

Geology

The Ciomadul lava dome complex is composed of about 8-14 km³ of eruptive rocks (Szakács et al., 2015) and it is developed on the folded and thrusted Lower Cretaceous flysch units. Volcanism began at this site about 1 Ma, while the Ciomadul volcano has been built up in the past 200 ky. The youngest active stage (from 57 to 32 ky) of the volcanic activity was predominantly explosive and involved several events of lava dome collapses, accompanied by volcanic and sub-plinian eruptions (Harangi et al., 2015 a,b; Karátson et al., 2016). Most of the lava domes have preserved their original structure well, but some (e.g., Bálványos and Puturosu domes) exhibit signs of intensive erosion and alteration (Szakács et al., 2015). The volcanic products are predominantly potassium-rich dacites (Szakács et al., 1993; Vinkler et al., 2007). Petrogenetic as well as zircon dating studies indicate the existence of long-lasting (up to 350 ky), low-temperature (700-750°C) silicic crystal mush body, which was periodically remobilized by injection of hot basaltic magmas, rapidly triggering volcanic eruptions (Kis et al., 2014).

Hydrology, water chemistry and gas emissions

The aquifers of this area are marked by the dominant presence of CO₂ as free and dissolved gas (Althaus et al., 2000; Vaselli et al., 2002; Italiano et al., 2017). The chemical composition of the mineral springs found along the creeks and major tectonic
lines is highly variable, namely slightly acidic (pH 5-6): Na-K-Cl-HCO₃, Ca-Mg-HCO₃, to strongly acidic (pH ~1.6) Ca-SO₄ type of waters (Jánosi et al., 2011). The origin of the water is meteoric with a recharge area typical for mountain regions, as shown by their stable isotopic compositions (Förizs et al., 2010). CO₂-bubbling peat bogs can be found North-East (Buffogó Peat Bog, Hegyeli, 2008) and South of Puturosu Mt. (Zsombor-Valey, Jánosi et al., 2011). The youngest structures of Ciomadul volcanic area consist of the twin-craters hosting the St. Ana Lake and the Mohos Peat Bog. St. Ana Lake is the only existing volcanic lake within the East Carpathians, with a surface of 22 ha and the maximum depth up to 7 m (Magyari et al., 2006, 2009). Stable isotope data (δ¹³C, with a value of -5‰) suggests that magmatic degassing occurs at the bottom of the lake (Túri et al., 2016).

The term mofette is used to indicate gas emission sites on the Ciomadul Mt., but other terms such as fumarole and solfatara, although not suitable, have been used in the past to describe the diversity of gas emissions. The term mofette is generally reserved for low-temperature gas emissions mainly composed of dry CO₂, while the terms fumarole and solfatara are generally used to indicate high temperature and acidic fluid emissions that are directly related to recent volcanic activity (Martini, 1996).

In the Ciomadul area, emanations of CO₂ and H₂S in various proportions, are in the form of gas bubbling, mofettes, and naturally sparkling mineral water springs, often associated with rock alterations. The total output of CO₂ within the area of Ciomadul is 8.7 × 10⁶ kg y⁻¹ (Kis et al., 2017), consistent with other quiescent volcanoes worldwide. When emissions occur on a slope, the vegetation downhill from the vents is killed by toxic gases. The temperature of the gas when released from the rock fissures and vents is not higher than 10°C. Some CO₂-filled depressions such as the “Birds’ Cemetery” located close to the “Killer Cave” (Fig. 1), are suffocation traps for insects, birds and mammals. Native sulfur occurs in some fissures and cavities that convey the CO₂-rich gas to the atmosphere. Historically, the mofettes from Puturosu Mt. have been used for therapy and recreational purposes (Incze et al., 2016). In larger caverns, the gas level is indicated by the deposition of a layer of elemental sulfur on the walls. The chemical composition of the gas emissions in Sulfur Cave was first determined by L. Ilosvay as early as 1885. Modern gas geochemical data from a few sites (Bálványos, Sulfur Cave) were previously reported by Althaus et al. (2000), Vaselli et al. (2002) and Frunzeti (2013) (Table 1) with the CO₂ content of the gases ranging between 95.63 and 98.26%. Other gas components were also reported: N₂ (0.89 to 1.97%), CH₄ (0.65 to 2.35%), O₂ (0.02 to 0.04%), H₂S up to 0.012% and noble gases (Vaselli et al., 2002), including radon (Szabó & Szabó-Sellenyi, 1981).

The CAVES OF PUTUROSU MOUNTAIN

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

**Sulfur Cave**, also known as Peştera de la Turia, Peşteră Sulfuroasă / Puturoasă (in Romanian) and Bűdős-barlang (in Hungarian) is located at 1,044 m altitude (N46.119018; E25.949432). It is one of the most famous mofettes in Europe, with a daily gas outflow of up to 5.26 × 10⁶ kg/day (Kis et al., 2017). Ilosvay (1885) published a detailed description of Sulfur Cave. The main gallery is 14 m long, and about 7 m of it accessible to tourists (Fig. 2). The cave is characterized by the presence of a two-layer atmosphere. Continuous gas emissions from vents located on the cave floor fill the lower sections with a CO₂-rich gas (up to 98.26%, Vaselli et al., 2002, Table 1) denser than air (Fleischers, 1876). Sulfur deposits cover the cave floor and the cave walls in the lower sections of the cave, but they are absent from the upper part of the cave (Fig. 3a) which is exposed to atmospheric air. The zone where the two gas phases meet is a gas/gas redox interface (gas chemocline) that is well defined and relatively steady (within cm scale range) due to density differences between the two gas phases (Fig. 3b).
to Killer Cave. It is filled with CO$_2$ rich gasses, its entrance is relatively narrow, its depth appears to be approximately 7 m, and its lower sections are yet to be explored.

**MINERALOGY**

The most important minerals detected previously in Sulfur Cave are Alum-(K) (potassium alum; KAl(SO$_4$)$_2$·12H$_2$O, cubic) and sulfur (Brem, 1955; Nedopaca, 1982; Onac, 2003; Szakáll et al., 2006; Szakáll et al., 2010). Sulfur (S$_8$, predominantly orthorhombic) was reported in both Sulfur Cave and Alum Cave (Fridvaldszky, 1767; Orbán, 1869), but also in Killer Cave, Small Cave, and Vertical Cave. Sulfur is likely an oxidation product from the emitted H$_2$S present in the volcanic gasses. Other minerals described from this region are alunogen, celestine, cristobalite, gypsum, halotrichite, pickeringite and tamarugite.

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

**MATERIALS AND METHODS**

Mineralogy

Samples of mineral deposits from Sulfur Cave were analyzed by X-ray powder diffraction analysis using a Bruker D8 Advance powder diffractometer with Bragg-Brentano geometry. The diffractometer used a cobalt anode (CoKa$_1$ with $\lambda = 1.78897$ Å and CoKa$_2$ with $\lambda = 1.79285$ Å) and the K$\beta$ line was filtered with a 0.01-mm iron foil. The instrument was operated at 35 kV and 40 mA, and the diffracted X-rays were registered by a one-dimensional LynxEye detector.
The scanning 2θ angle range was between 5 and 64°, with a step size of 0.02° (2θ) and a measuring time of 0.5-2 s per step. The alignment of the diffractometer’s goniometer was verified using the NIST SRM1976a (corundum) standard. The DiffracEva software (Bruker Corporation) was used for mineral phase identification using the International Centre for Diffraction Data Powder Diffraction Files (ICDD PDF) database.

**XRD bulk analysis**

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

**Scanning Electron Microscopy**

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

**Stable isotope analyses**

Stable isotope analyses were performed at the Center for Stable Isotopes of the University of New Mexico. Carbon isotope ratios in gas samples were measured by headspace analysis using a Thermo Fisher Scientific Gasbench II coupled to a Delta Plus Isotope Ratio Mass Spectrometer. Calibration was performed using CO₂ resulting from the reaction of a carbonate standard (NBS 19) with phosphoric acid at 50°C. Nitrogen, carbon and sulfur isotope ratios were measured by continuous flow isotope ratio mass spectrometry using a Costech ECS 4010 Elemental Analyzer coupled to a Thermo Fisher Scientific Delta V Advantage mass spectrometer via a CONFLO IV interface. Sulfur isotope measurements were performed using the method of Fry et al. (2002). Isotope ratios are reported using the standard delta (δ) notation relative to V-AIR for nitrogen, the Vienna Pee Dee belemnite (V-PDB) for carbon and the Canyon Diablo troilite (CDT) for sulfur. Average analytical precision, based on routine analysis of a laboratory standard was better than 0.1‰ (1 σ) for both δ¹⁵N and δ¹³C and 0.2‰ for δ³⁴S.

**Microbial sampling and fungi cultures**

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

**DNA extraction and quantification**

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

**DNA sequencing and sequence processing**

The samples analyzed in this study were sequenced in parallel with over a hundred other unrelated samples resulting in a total of ±14 million reads using the Illumina sequencing platform. The four interface samples combined are composed of ~400,000 reads (varying from 32,000 to 194,000 reads). Pre-processing of the samples, was done separately in order to minimize the possibility of cross-contamination. *Mycobacterium* sequences were not detected in other unrelated samples, and thus are not an artifact. PCR reactions were performed in triplicate using Phusion Green Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Sweden). We targeted the V3-V4 region of the 16S rRNA gene, using the V3 forward primer S-D-Bact-0341-b-S-17, 5’-CTACTAGGGNGGCWGCAG-3’ (Herlemann et al., 2011), and the V4 reverse primer S-D-Bact-0785-a-A-21, 5’-GACTACHVGGGTATCTAATCC-3’ (Muyzer et al., 1993), giving rise to ~430 bp long dsDNA fragments. For fungal amplicons we have used the primers Euk528 (575–590) 5’-CGGTAATTCCAGCTCC-3’ and Euk690R (575–590) 5’-ATCCAAGAATTTCACCTCTGA-3’, giving rise to ~302 bp long dsDNA fragments. The primers were dual barcoded and were compatible with Illumina sequencing platforms as described previously (Caporaso et al., 2011). Performance of the PCR reaction was checked by running incorporated positive and negative controls from each triplicate plate on 1.5% (w/v) agarose gels. Triplicate PCR products were combined and each combined triplicate sample was purified using SPRI beads (Agencourt® AMPure® XP, Beckman Coulter, CA, USA). The DNA concentration in the purified samples was determined as described above. Samples were diluted to identical concentrations of 2 ng/μl prior to pooling the diluted PCR products together in equal volumes (10 μl) in one composite sample (including positive and negative controls). Samples were also taken from above and below the redox interface in Sulfur Cave, but did not reach sufficient product after amplification and thus were added undiluted to the pool. The number of reads of these samples was similar to those of the procedural blanks and negative controls, reflecting the low prokaryotic DNA content in these samples.

The composite samples were paired-end sequenced at the Vrije Universiteit Amsterdam Medical Center (Amsterdam, The Netherlands) on a MiSeq Desktop Sequencer with a 600-cycle MiSeq Reagent Kit v3 (Illumina) according to manufacturer’s instructions. High-throughput sequencing raw data were demultiplexed using bcl2fastq software version 1.8.4 (Illumina) and primers were trimmed using Cutadapt (Martin, 2011). Demultiplexed samples were further processed using a modified version of the Brazilian Microbiome Project 16S profiling analysis pipeline (Pyro et al., 2014). Paired-end reads were joined using PANDAseq (Masella et al., 2012) allowing for a minimum overlap of 10 nucleotides between the forward and reverse reads, a minimum sequence length of 285 and no mismatches in the primer region were allowed. PANDAseq addresses mismatches in overlapping regions by selecting the nucleotide with the best sequencer-assigned quality score. Because PANDAseq incorporates a base quality filter during read assembling, the threshold for consecutive high quality bases per read was set to zero. Metadata and demultiplexed samples were merged using add_qiime_labels.py (Caporaso et al., 2010) and sequence headers were changed using bnp-Qiime2UParse.pl (Pyro et al., 2014). UPARSE was used to dereplicate, filter chimeras, discard OTUs detected less than 2 times and OTU clustering at 97% similarity (Edgar, 2010, 2013). The OTU taxonomy was assigned using the UCLUST algorithm (Edgar, 2010) on QIIME (Caporaso et al., 2010) using SILVA compatible taxonomy mapping files (Silva database release 128) (Quast et al., 2013, Yilmaz et al., 2014) and aligned using align.seqs.py in QIIME (Caporaso et al., 2009). Taxonomy was manually curated and refined up to genus level based on 97% similarity of reference sequences. The reference tree was calculated using FastTree2 (Price et al., 2010). We generated a BIOM file using make.otu_table.py on QIIME (Caporaso et al., 2010). Prior to further analysis we produced an ANI file and a taxonomy table using BIOM scripts (McDonald et al., 2012). The OTUs detected in negative controls and procedural blanks were manually removed from the dataset.

**RESULTS**

**Mineralogy**

In Sulfur Cave, sulfur deposits are always present below the oxic/anoxic interface as a continuous layer covering the cave walls and the cave floors, while above the CO₂-H₂S-O₂ gas/gas interface the S² deposits are absent (Fig. 3a). There are also no sulfur deposits around the gas vents located on the cave floor most probably for lack of oxygen. In some areas of the cave, walls are covered by 5-20-cm-thick sulfur deposits (Fig. 4a, b), while in other area these deposits are thin (< 1 mm). Water films and water droplets found on the cave walls in S²-rich areas near the interface are very acidic (pH = 0.5 to 1).

The sulfur from Sulfur Cave was shown to be of various types. The most common type is an earthy aggregate of a powdery deposit with micron-sized yellow or pale yellow fine acicular crystals. Some crystals are tens of microns long (Fig. 4c, d) with a crystallite size of 860 Å (based on analysis using the program DifracEva). Twinning behavior ([001], 90°) of sulfur crystals is common in the sulfur deposit situated below the gas/gas redox interface from Sulfur Cave (Fig. 4e, f) and less frequent at the gas/gas interface where mostly orthorhombic crystals are...
Microbial mats in a CO$_2$-H$_2$S/O$_2$ gas chemocline in Sulfur Cave
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We used the WinXMorph program (Kaminsky, 2005; 2007) to model this twinning (Fig. 5). EDAX-SEM analysis confirmed that the thick yellow deposits from Sulfur Cave (Fig. 4) are dominated by sulfur. Also, X-ray diffraction analysis has confirmed that the thick wall deposits from Sulfur Cave (Fig. 6) are in fact sulfur crystals with a crystallinity index of 82%. Based on Rietveld analysis of the diffraction data using the FullProf Suite (Rodríguez-Carvajal, 1993; Roisnel and Rodriguez-Carvajal, 2000) and MAUD (Lutterotti et al., 2007) programs, the unit cell parameters for the sulfur crystals are: $a = 10.462$ Å; $b = 12.865$ Å; $c = 24.497$ Å; $α = 90°; β = 90°; and γ = 90°, almost identical to those published by Rettig and Trotter (1987). Besides sulfur, a very small amount of gypsum is present as transparent sub-millimeter-sized crystals on the surface of the sulfur deposit. The presence of this mineral was also confirmed by X-ray diffraction analysis.

Gas chemical composition and gas flux
The published gas analyses from Sulfur Cave are listed in Table 1. The CO$_2$ concentration is in the range of 96.7 to 98.2%, followed by other gases, here including N$_2$, CH$_4$, H$_2$, and H$_2$S. Noble gases (He, Ne, and Ar) also occur in low amounts. The differences in the gas composition are confined to a narrow range; they can be due to natural variations or to some extent, to the different sampling/analytical methods that have been used by the respective authors. Compared to the works of Frunzeti (2013), Vaselli et al. (2002), and Althaus et al. (2000) the data for carbon dioxide show similar values, while the other gases show small differences, which could be due to sampling strategy. Higher values were detected for N$_2$ and CH$_4$ by Frunzeti 2013, than the values reported by Althaus et al. (2000), and Vaselli et al. (2002).

Based on the measurements of Kis et al. 2017, the total output from Sulfur Cave is approximated to $1.92 \times 10^6$ kg y$^{-1}$ CO$_2$. The Sulfur Cave together with other high emission sites from the neighboring area show a total output derived from soil degassing and focused emissions of $8.70 \times 10^6$ kg y$^{-1}$ CO$_2$. The highest CO$_2$ gas fluxes at Ciomadul were found at the periphery of the youngest volcanic complex, at the intersection of the older lava domes, Puturosu and Bálványos (500-600 ky). The gas emissions are assumed to be controlled by tectonic features, like fractures or faults (Kis et al., 2017). Nevertheless, the locations of the strongest outgassing do not coincide with the youngest eruption centers of Ciomadul, but they are in a peripheral occurrence, like Puturosu Mt., where the caves are located. Compared to other locations through Europe, one may observe that the CO$_2$ emissions from Ciomadul complex are in the same order of magnitude as in the case of other European volcanic structures of similar age (Kis et al., 2017; Caracausi et al., 2015).
Stable isotopes

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

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

Microbial communities in Sulfur Cave

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

The 16S rDNA reads of four samples (collected in 2016 and 2017) from the gas/gas interface biofilm from Sulfur Cave were analyzed to genus level (97% similarity) and the relative abundances of the dominant phylotypes in each sample are summarized in Fig. 8 and Supplemental Table 1. For the four samples a total of 407,426 reads were obtained (after removal of contaminating sequences that were present in the negative controls and extraction blanks). The overall microbial diversity in the samples is low, with less than 50 genera detected and a total of 62 OTUs. Among them, only a few are abundant, which can be explained by the unusual and extreme conditions of the Sulfur Cave environment such as local anaerobiosis, high CO\textsubscript{2} levels, extremely low pH

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<tr>
<td>Wall-Biofilm 5</td>
<td>2.0</td>
<td>1.00</td>
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<td>-30.3</td>
<td>13.9</td>
</tr>
<tr>
<td>Wall-Biofilm 6</td>
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<td>-0.8</td>
<td>34.9</td>
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<td>10.6</td>
</tr>
<tr>
<td>Wall-Biofilm 7</td>
<td>3.3</td>
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<td>41.5</td>
<td>-31.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Wall-Biofilm 8</td>
<td>3.2</td>
<td>0.5</td>
<td>39.7</td>
<td>-31.5</td>
<td>12.3</td>
</tr>
</tbody>
</table>

Table 2. δ\textsuperscript{13}C values of the CO\textsubscript{2} gas from caves in the Ciomadul area.

Table 3. Nitrogen and carbon elemental and isotopic composition of eight replicates from the microbial biofilm at the redox gas/gas interface in Sulfur Cave.
and absence of sunlight. For fungi identification we have isolated colonies on organotrophic plates and then sequenced PCR-amplified DNA using fungal 18S rDNA primers. We have also repeated this sequencing on DNA extracted from the microbial community at the gas/gas interface. Images of fungal isolates and hyphae were taken by optical microscopy.

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

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

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

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

**DISCUSSIONS AND CONCLUSIONS**

**Mineralogy**

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

**Stable isotopes**

The relatively high $\delta^{13}C$ values for the CO$_2$ gas samples from Sulfur Cave (-2.8 and -2.5‰) are similar with the values measured in other caves from the Ciomadul area (Table 1; Vaselli et al., 2002; Althaus et al., 2000; Frunzeti, 2013). Vaselli et al. (2002) reported carbon isotope values of CO$_2$ gas derived from natural springs and dry volcanic mofettes from the inner part of the Eastern Carpathians, including the Ciomadul area, and showed that most of the $\delta^{13}C$ values lie within a relatively narrow interval ranging between -2.1 and -4.7‰. They suggest a deep crustal origin of the gases from a mixture of mantle derived CO$_2$ and CO$_2$ resulting from alteration and/or metamorphosis of marine carbonates. Our carbon isotope results are within the range reported by these authors, as well as other studies in similar geologic settings (see review in Fischer & Chiodini, 2015). Similar isotopic compositions were reported for other volcanic and non-volcanic regions worldwide (Tedesco et al., 2010; Barry et al., 2013; Ruzié et al., 2013, Oppenheimer et al., 2014; Rizzo et al., 2014; Mason et al., 2017 and references therein), giving a mean global volcanic carbon isotopic composition ranging between -3.8 and -4.6‰ (Mason et al., 2017). Considering the neighboring areas within the Carpathian Region, the Pannonian Basin, Cornides, (1993) reported an average $\delta^{13}C_{CO2}$ composition of -5‰, inferring “magmatic” origin, Palcsu et al. (2014) reported -3.3 to -2.1‰ from wells from the Pannonian Basin suggesting interaction between a magmatic and a crustal component, Bräuer et al. (2016) reported values of -3.5 to -7.5‰ for the westernmost Pannonian Basin, suggesting evidence for active lithospheric mantle degassing. Our data show slight $^{13}C$-enrichment comparable to the regional carbon isotopic compositions, suggesting a deep origin of the gases derived from a mixture of mantle CO$_2$ and CO$_2$ resulting from alteration and/or metamorphosis of marine carbonates.

Assuming a -30‰ fractionation between the inorganic carbon and the microbial mat (Sarbu et al., 1996), the stable isotope values for carbon and nitrogen in the microbial biofilms from the cave walls at the level of the redox interface (Table 2) are consistent with the CO$_2$-rich gases vented in Sulfur Cave serving as a main carbon source. The C/N ratios resemble those reported from biofilms consisting of chemoautotrophic microbes, found in other sulfidic cave environments (Engel et al., 2004).

Fig. 10. SEM images of microorganisms growing in Sulfur Cave at the gas/gas redox interface; a) Cells colonizing mineral surfaces coated with elemental sulfur; b) Cross section of a microbial mat from the rock walls of Sulfur Cave at the redox interface.
The $\delta^{34}$S values (-7.3 and -5.6‰) in the sulfur wall deposits are typical of reduced sulfur species with a possible volcanic origin. The sulfur isotopic composition of the H$_2$S gas on the Puturos Mt. has not been measured.

**Microbial communities**

Stable isotope values (Table 1) indicate that the organic carbon within the microbial biofilm from Sulfur Cave is of autotrophic origin. Apart from *Mycobacterium*, the other phylotypes we have found, are known acidophiles: *Acidithiobacillus* (Bacteria), Ferroplasmaceae (Archaea), and *Acidomyces* (Fungi). Several of them can grow chemolithoautotrophically as well (i.e., *Acidithiobacillus*, Ferroplasmaceae), (facultative) anaerobes (i.e., Ferroplasmaceae, *Acidithiobacillus*, *Metallibacterium*), and/or are capable of oxidizing sulfur compounds or iron in the presence of oxygen. In the absence of oxygen, alternative electron acceptors such as ferric iron (Fe$^{3+}$) or nitrate (NO$_3^-$) may be utilized by several community members (i.e., Ferroplasmaceae and *Acidithiobacillus*, respectively) (Baalsrud & Baalsrud, 1954; Golyshina & Timmis, 2005). Ferroplasmaceae, *Acidithiobacillus* and the fungus *Acidomyces* found in this system are known acidotolerants. Also, eukaryotic fungi can be found in this biofilm, most notably a fungus belonging to the genus *Acidomyces*, members from this genus can be isolated from extremely acidic soils and mine drainages and notably also from sulfur-containing soils. These fungi also show melanisation of their cell walls, which probably makes them more resistant to environmental stress.

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

Phylotypes belonging to the order of Rhizobiales and the family of Beijerinckiaceae were also found in the biofilm and could potentially be important in providing the community with a nitrogen source by N$_2$ fixation. Another interesting member of the Sulfur Cave microbial community was a species of the genus *Metallibacterium*. A recently described and widespread facultatively anaerobic, acid-tolerant, iron-reducing member of this genus (*M. scheffleri*) was shown to possess genes involved in sulfur oxidation and was capable to produce ammonium through protein (casein) consumption, thereby raising the pH in its proximate vicinity (Ziegler et al., 2013; Bartsch et al., 2017). Under acidic conditions ammonium is protonated to ammonia which can in turn serve as an easily accessible nitrogen source for other community members such as Mycobacteria.

The exact composition, function, metabolic properties and interactions between species of the Sulfur Cave microbial community remain to be elucidated. Presently, we speculate that Sulfur Cave contains an unusual community with a primary free energy transduction mode based on chemolitho-autotrophic growth. Free energy sources for the microbial community include sulfur deposits on the cave wall and the H$_2$S from the mofettic gas. Based on phylogenetic relatedness biomass formation in some of these species may occur via RuBisCO-based CO$_2$ fixation. Next to sulfur oxidation, iron oxidation may occur as well, by dedicated microorganisms such as Ferroplasmaceae, as well as oxidation of H$_2$S and S$^-$ with ferric iron, if iron becomes available from the weathering of bedrock minerals. Given the presence of residual methane in the Sulfur Cave (above the atmospheric background) the presence of methanotrophic activity is also possible. Yet, so far we have not identified any 16S sequences indicative of classical methanotrophs. *In situ* carbon and nitrogen fixation by species in the biofilm is suggested by the sequence data, as well as by the stable isotope data (Sarbu et al., 1996). The bedrock minerals from Sulfur Cave may provide the microbial community with other essential nutrients and metals such as phosphorus, magnesium, and manganese that are not available in the gas phase.

Sulfur oxidation has been observed in caves, such as Movile (Sarbu et al., 1996), Frassasi (Sarbu et al., 2000) and Ayyalon (Por et al., 2013) but in all of these cases the H$_2$S is dissolved in water and, as such, flows into these caves. Establishing a rich microbial community inhabiting a CO$_2$-H$_2$S:O$_2$ gas/gas interface in an aphotic environment and with CO$_2$ and H$_2$S brought in by mofettic emissions is, so far, unique to Sulfur Cave.

The discovery of abundant biofilms in Sulfur Cave is, to our knowledge, the first report of a non-
aquatic gas/gas interface (i.e., with energy from a redox gas chemoclione) in a volcanic zone, used by chemosynthetic microorganisms to fix carbon and nitrogen underground.

**Astrobiology**

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

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Antimicrobial activities of culturable microorganisms (actinomycetes and fungi) isolated from Chaabe Cave, Algeria

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Abstract: The discovery of new antibiotics and other bioactive microbial metabolites is a priority given the frequency of the emerging multi-drug resistant pathogenic microorganisms. Thus, scientists are searching for new antibiotics in microorganisms isolated from extreme habitats such as caves. In the present study, we aimed to perform the first analysis on culturable microorganisms in the Chaabe Cave (Algeria), and to test the antimicrobial activities of the isolates (Streptomyces spp. and Penicillium spp.). The potential for antimicrobial activity of 47 strains of actinomycetes and 23 strains of fungi were tested on Candida albicans, Staphylococcus aureus, Micrococcus luteus, Listeria monocytogenes, Bacillus subtilis, Escherichia coli, and Pseudomonas aeruginosa. The identification of the most active actinomycetes strains by 16S rRNA gene sequence analysis revealed that these isolates were exclusively affiliated to the genus Streptomyces. On the other hand, the fungi were determined by phylogenetic analysis based on the ITS region or on the β tubulin gene and were affiliated to the genera Readeriella, Cladosporium, Aspergillus, Penicillium, Beauveria, Alternaria, Rhizopus, and Rhizomucor. The strains showed strong inhibitory activity against pathogenic microorganisms and the diameters of the inhibition zones vary between 7.5 and 34 mm for Streptomyces strains, and between 6.5 and 19.50 mm for Penicillium strains. The data indicated that the majority (72.86%) of the 70 isolates were active against at least one of the tested microorganisms. The production of nonpolyenic antifungal substances by active Streptomyces isolates was investigated using several criteria (antibacterial activity, ergosterol inhibition, and UV-visible spectra) of active extracts. The results were promising and showed that the metabolites produced by the actinomycete strains do not have a UV-visible spectrum characteristic of a polyenic structure. The Chaabe Cave possesses a diversity of microorganisms that could lead to new antibiotics necessary in the fight against drug-resistant pathogens and warrant further study.

Keywords: antimicrobial activities, cave microorganisms, Streptomyces, Penicillium, Chaabe Cave

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INTRODUCTION

The World Health Organization stated that there is a serious lack of new antibiotics to fight the increasing risk of antimicrobial resistance, which represents a global health emergency (Kmietowicz, 2017). Antibiotics and other bioactive compounds have been isolated from microorganisms in different environments, mainly marine (Dharmaraj, 2010; Romano et al., 2017) and terrestrial (Charousová et al., 2017; Devi et al., 2017), although novel compounds from plants have recently been described (Romero et al., 2017, 2018).

In the last decades the search for novel bioactive compounds has included the exploration of little investigated environments of which caves are an
example. Karstic caves are widely distributed on all continents. Many caves have yet to be discovered or have barely been accessed, while other caves have received considerable attention due to tourist interest and have, therefore, been visited by a large number of people. This has contributed to their deterioration as can be seen with Lascaux Cave in France and Altamira Cave in Spain. (Bastian et al., 2010; Saiz-Jimenez et al., 2011).

Almost two decades ago, Groth et al. (1999) suggested that caves were a promising environment for finding novel actinomycetes able to produce bioactive compounds. From that time on, a number of papers reported the isolation of cave actinobacteria with production of bioactive compounds (Nakaew et al., 2009; Hodges et al., 2012; Cheeplham et al., 2013; Ghosh et al., 2016; Maciejewska et al., 2016). The most promising was the discovery of cervimycin A-D, a polyketide complex produced by a strain of Streptomyces tendae, isolated from Grotta dei Cervi, in Italy. Cervimycins showed significant activity against multi-drug-resistant staphylococci and vancomycin-resistant enterococci (Herold et al., 2005).

A number of caves should be considered oligotrophic in nature meaning that they have a very low organic carbon input. Given these circumstances, microorganisms compete for nutrients and their metabolic capabilities are enhanced over the bioactive substance production inhibiting the growth of other microorganisms (Cheeplham, 2013).

Algeria is a large country in Africa, with a remarkable abundance of flora and fauna. At present, there is little information on the microbial diversity of caves all over the world and, according to our knowledge, no publication has investigated the microbial diversity in cave ecosystems in either Algeria or Africa in general. In light of this, the main aim of the study was the identification of cultivable microorganisms (actinomycetes and fungi) with antimicrobial potential from Chaabe Cave (Tlemcen, Algeria). The isolates were identified by 16S rRNA gene analyses for actinomycetes and ITS region or β tubulin gene analyses for fungi and tested for their bioactive compounds.

**MATERIALS AND METHODS**

**Samples**

Samples were collected from Chaabe Cave in Tagema, near the city of Tlemcen (Algeria) (Western region: 34°53’N, 1°19’W, 1032 m altitude) (Fig. 1A; Carte du monde, 2017). The cavern access was characterized as difficult with a narrow entry and was not accessible to visitors. At present, there are no geological studies or physicochemical characteristics available about the cave. A total of four samples were collected aseptically and randomly from various sediments between February and March of 2016 (Fig. 1B). The samples (200-2,000 g) were taken from a depth of 0–20 cm below the surface with a large sterile spatula and placed in sterile polyethylene bags, closed tightly and stored at 4°C until used for further experiments.

**Measurement of pH**

An aliquot of the sediment was air-dried at room temperature for 7 days, crushed and passed through a 2 mm mesh sieve. Twenty grams of a dried sample were suspended in 50 mL of distilled water and mixed to ensure uniformity. After a few minutes of standing, the supernatant was used to determine the pH with a digital pH meter (HANNA Inst. pH 209).

**Total aerobic mesophilic microorganism counts**

Ten grams of each sample were added to 90 mL of 0.9% (w/v) solution of sodium chloride. After homogenization, this solution was diluted (10⁻¹ to 10⁻⁷) and 1 mL of the resulting solution was inoculated on Plate Count Agar (PCA). After incubation at 25°C, for 2 to 3 days, the colony forming units (CFU) were counted. Previous studies demonstrated that colonies from cave samples grow very slowly at cave temperature and that the diversity of the culturable genera was similar whether the bacteria were grown at cave temperature (13°C) or at laboratory temperature (28°C) (Groth et al., 2001; Laiz et al., 2003).

**Isolation of actinomycetes**

Under aseptic conditions, the sediment samples were air dried for 1 week prior isolation according to Jeffrey (2008) to decrease the population of Gram negative bacteria. The samples were then crushed in a sterile mortar. Isolation and enumeration of actinomycetes were performed by the dilution plate technique. Ten grams of sample was mixed with 90 mL of sterile water containing NaCl 9 g·L⁻¹. The mixture was shaken vigorously at room temperature. Portions (1 mL) of the suspensions (diluted 10⁻⁴) were transferred to 9 mL of saline solution and subsequently diluted until 10⁻⁶ and/or 10⁻⁹ after homogenization by vortexing. The inocula, consisting of 100 µL of these dilutions, were spread over the surface of a few isolation media: Chitin-B vitamin agar medium (Hayakawa & Nonomura, 1987), Olson’s medium (Olson, 1968), Bennett’s medium (Boudemagh et al., 2005) and Tryptic soy agar medium (Romanenko et al., 2008). The four media were supplemented with 50 µg·mL⁻¹ cycloheximide (actidione) and 30 µg·mL⁻¹ nystatin to inhibit the development of antagonist fungi and other eukaryotic microorganisms (Williams and Davies 1965; Ouhdouch et al., 2001; Badji et al., 2005). Ten µg·mL⁻¹ nalidixic acid were also added to plates in order to suppress Gram negative bacterial growth (Takizawa et al., 1993). All the inoculated plates were incubated in the dark at 28 ± 2°C for 2-4 weeks and examined daily for colony patterns and growth. Actinomycete colonies with different morphologies were selected and streaked onto fresh Bennett or Tryptic soy agar slants and incubated at 28 ± 2°C for 1 week. Pure cultures were stored at 4°C or as suspensions in culture medium-glycerol at -80°C.

**Phenotypic identification of actinomycetes**

Actinomycete strains were identified according to traditional morphological criteria, including the phenotypical aspect of the colony and growth characteristics on Bennett and starch-casein media.
morphology of aerial hyphae and spores. Microscopic morphologies were observed by light microscopy. The isolates were characterized using Gram’s staining, physiological and biochemical studies which included assessment of the isolates’ ability to utilize different carbon sources (glucose, lactose, saccharose, and citrate), formation of indole and H$_2$S, catalase and urease activities, hydrolysis of starch, casein, gelatine, production of melanin, nitrate reductase activity and action on skimmed milk (coagulation and peptonisation) (Shirling & Gottlieb, 1966; Marchal et al., 1991; Singleton, 1999).

Sequencing and molecular identification of actinomycetes

Bacterial DNA was extracted following the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR using the conserved primers 27F (5’-AGAGTTTGATCCTGGCTCAG) and 1522R (5’-AAGGAGGTGATCCAGCCGCA). PCR thermal conditions were as follows: 95°C for 60 s; 35 cycles of 95°C for 15 s, 55°C for 15 s, 72°C for 120 s; and a final extension cycle at 72°C for 10 min. Forward and reverse strands of the amplified DNA fragment were sequenced in an ABI 3700 sequencer (Applied Biosystems). Pairwise 16S rRNA gene sequence similarities for the most closely related strains were determined using the global alignment algorithm on the EzTaxon server (http://www.eztaxon.org) (Chun et al., 2007).

Isolation of fungi

Different culture media were employed to improve the isolation of fungi from the samples: Malt Extract Agar (MEA), Czapek Dextrose Agar with (CDAr) and without Rose Bengal (CDA), and Potato Dextrose Agar (PDA) with Rose Bengal or with 25% lactic acid (Atlas, 2010).

Samples (10 g) were suspended in 90 mL of 0.9% sterile saline solution and mixed thoroughly by shaking. Subsequently serial 10-fold dilutions were performed and 1 mL aliquots of $10^1$ to $10^9$ dilutions of each sample were plated onto the five media. All plates were incubated at 25°C for 1 week to reproduce the same natural conditions in the cave.

Fungal isolates were studied according to their phenotypic characteristics, such as color and texture of the surface colony, hyphal pigmentation, size and shape of conidia and conidiogenous cells, presence or absence of sclerotia, and growth rates. The number of colony forming units per g of dry weight (CFU/g dwt) was calculated for each sample. The isolated fungi were maintained as pure culture in PDA with 25% lactic acid and kept at 4°C until further study.

Sequencing and molecular identification of fungi

Fungal DNA was extracted from fresh mycelium grown on MEA. Extractions were performed using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer’s instructions. ITS and the β tubulin gene were amplified using primers sets ITS4/ITS5 (White et al., 1990) or Bt2A/Bt2B (Glass & Donaldson, 1995), respectively.

PCR amplifications were performed using a BioRad DNA Engine Peltier Thermal cycler with 30 cycles of 30 s at 94°C, 30 s at 55°C (for both primer set), 40 s at 72°C; 10 min at 72°C, in a 25 µL reaction mix, containing 12.5 µL genomic DNA (dilution: $10^{-2}$ after extraction), 5 µL PCR Direct Loading Buffer with MgCl$_2$ (Q-Biogen), 0.5 µL dNTPs (6.25 mM, dNTP Mix, Q-Biogen), 1 µL of each 10 µM primer (Eurogentec), 0.125 µL Taq DNA Polymerase (Q-Biogen, 5 units/µL), and 4.875 µL sterile water. PCR products were purified and sequenced by Genoscreen (Lille, France) in both directions to confirm the accuracy of each sequence. Sequences were assembled with CodonCode Aligner v. 3.7.1 (Codon Code Corporation), checked by visual inspection of the chromatograms and edited if necessary. Sequences were identified using the BLAST option at http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi.

In vitro screening of isolates for antimicrobial activity

Antimicrobial activity of selected strains was estimated by the agar cylinders method, against four Gram-positive bacteria: Staphylococcus aureus (ATCC 6538), Micrococcus luteus (CIP 53.45/ATCC 9341), Listeria monocytogenes (ATCC 19111) and Bacillus subtilis (ATCC 6633), three Gram-negative bacteria: Pseudomonas aeruginosa (ATCC 27853), Escherichia coli (CIP 53.126 / ATCC 8739), and Klebsiella pneumoniae (IBMC Strasbourg), and the yeast Candida albicans (CIP 444). All of these microorganisms were kindly provided by the National Museum of Natural
History (Paris, France) and the Laboratories of Natural Products and of Antibiotics (Tlemcen, Algeria).

Agar cylinders method

Inhibition zones were determined against selected pathogenic microorganisms using the disc diffusion method (Clinical and Laboratory Standards Institute, 2012), with necessary modifications. Bacterial suspensions were prepared in Mueller Hinton Broth (MHB) and fungal suspension in Sabouraud Dextrose Broth (SDB), and then incubated at 37°C for 24 h and at 30°C for 24-48 h, respectively. The tested microorganism suspension was adjusted to a similar optical density to that of McFarland 0.5 (10⁶ CFU/ml for bacteria in MHB and 10⁵ CFU/ml in SDB for C. albicans). The isolated actinomycete and Penicillium strains were grown on Bennett’s agar plates (Jones, 1949) for 14 days at 28°C and on Potato Dextrose Agar plates for 1 week at 25°C, respectively. Then a calibrated cylinder (6 mm in diameter) was cut out and placed on Mueller Hinton agar (Sabouraud Dextrose agar for C. albicans) plates inoculated with the adjusted suspension of an exponentially growing culture of test microorganisms using a sterile cotton swab. To compare the antimicrobial activities, standard antibiotic disks were used as positive controls, ampicillin (10 μg/disc; Biomaxima) and chloramphenicol (30 μg/disc; Sigma Aldrich) for bacteria, and nystatin (100 μg/disc; Sigma) and amphotericin B (100 μg/disc; Sigma) for yeast in order to control the sensitivity of the tested microorganism. Plates were first kept at 4°C for 2-4 h to allow the diffusion of any antimicrobial metabolites. Inhibition diameters were determined after 24 h for bacterial strains and 48 h for C. albicans. Plates were examined for evidence of antimicrobial activities, represented by a zone of inhibition of microbial growth around the cylinders (Lemriss et al., 2003; Melloulil et al, 2003; Kitouni et al., 2005).

Screening for actinomycete strains producing nonpolyenic antifungal metabolites

To select active actinomycete strains producing only nonpolyenic antifungal agents, the ergosterol inhibition and the production of active metabolites were investigated. Ergosterol inhibition was tested by the diffusion method (agar cylinders method) on Sabouraud’s agar medium with or without 50 mg·mL⁻¹ ergosterol, in the presence of C. albicans (Ouhdouch et al., 2001).

For the production and extraction of active metabolites, isolates showing antifungal activity were cultured in a 250 mL flask containing 25 mL of liquid Bennett’s medium and incubated at 37°C for 24 h. The culture was transferred into a 500 mL flask containing 225 mL of liquid Bennett’s medium. After 3 days of incubation at 37°C, either the whole culture was extracted twice with ethyl acetate (1:1, v/v), or the pellet and the supernatant (obtained after centrifugation at 4,500 rpm for 15 min) were extracted with methanol (1:5, v/v) and twice with hexane (1:1, v/v), respectively. The antifungal activity of the three extracts for each active strain was determined by the disc diffusion method (Lemriss et al., 2003). The UV-visible spectra of the active extracts were recorded in the 200-500 nm range with a spectrophotometer (JENWAY2750 UV.vis) (Hacène et al., 1994; Ouhdouch et al., 2001).

RESULTS AND DISCUSSION

Samples and isolation of actinomycetes

Actinomycete colonies were counted on different media plates after 14–28 days of incubation at 28°C. Actinomycetes were recognized by their morphological characteristics: tough leathery colony, branched vegetative mycelia, and, when present, aerial mycelia and spore formation. All bacterial strains were Gram-positive. Most of the colonies that grew on plates belonged to the genus Streptomyces since the colonies were slow growing, aerobic, glabrous or chalky, heaped, folded and with aerial and substrate mycelia of different colors (gray, white, red, yellow, green, beige, purple, brown, violet and orange) (Buchanan & Gibbons, 1974). Forty different media were used for the isolation of actinomycetes from cave: Chitin-B vitamin agar, Olson’s medium, Bennett’s agar, and Tryptic soy agar. In addition, the media MEA and CDA, used for the isolation of cave fungi, also allowed for the isolation of the actinomycetes.

Fifty seven actinomycete strains were isolated using different culture media from the four cave samples (Table 1). The number of isolates recovered from each sample varied widely and ranged from 2 to 14. Considering the used media, Olson’s medium yielded the greatest percentage of actinomycetes (29.79%), followed by Bennett’s agar (21.28%). However, the macroscopic morphological diversity noticed on Olson’s medium was substantially less than in the others. The higher morphological diversity was observed on both media TSA and Bennett’s Agar.

Identification of actinomycetes

The isolates were easily identified as actinomycetes by their morphology and strong adherence to the agar medium. Some isolates produced diffusible pigments on several agar media and melanin on peptone-yeast extract agar (ISP6) (Shirling & Göttlieb, 1966). The degradation of the substrates casein, starch and gelatin was variable according to each isolate. An isolate (strain A45) was affiliated to the genus Streptomyces using taxonomic features. For the identification of the remaining actinomycetes a molecular approach was used, resulting in the affiliation of all strains to the genus Streptomyces. In Table 2 the representative strains are shown. BLAST search revealed that the closest hits to each cave isolate were Streptomyces species displaying a similarity of between 98.76

Table 1. Number and percentage of actinomycetes isolated by culture medium.

<table>
<thead>
<tr>
<th>Medium</th>
<th>TSA</th>
<th>Bennett</th>
<th>Olson</th>
<th>Chitin</th>
<th>MEA</th>
<th>CDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of isolated actinomycetes</td>
<td>6</td>
<td>10</td>
<td>14</td>
<td>7</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Total %</td>
<td>12.76</td>
<td>21.28</td>
<td>29.79</td>
<td>14.89</td>
<td>17.02</td>
<td>4.25</td>
</tr>
</tbody>
</table>
and 99.93%. A few of these strains showed some morphological differences and were included in the taxonomic group of *S. griseus* and *S. fradiae*.

On the basis of the 16S rRNA gene sequences similarity, two strains (A36 and A27) were most closely related to several species belonging to the taxonomic group *S. griseus* which includes *S. griseus* subsp. *griseus*, *S. fulvissimus*, *S. anulatus*, *S. cinereorectus*, *S. microflavus*, *S. cyaneofuscatus*, *S. griseoplanus*, *S. setonii*, *S. fulvoreus*, *S. halstedii*, *S. griseolus*, *S. puniceus*, *S. griseorubiginosus*, *S. badius*, *S. parvus*, *S. sindenensis*, *S. pluricolorescens*, *S. rubiginosohelvolus*. Also strains A12, A2, A3, A5, A7, A8, A11, A16 and A47 were affiliated to the taxonomic group *S. fradiae*, which includes *S. celluloflavus* and *S. kasugaensis* among others.

Previous studies reported that the utilization of 16S rRNA gene sequences is not sufficient to discriminate between closely related species of the genus *Streptomyces* (Guo et al., 2008; Rong et al., 2009). To this end, a detailed study for species identification requires multilocus sequence analysis (MLSA): *atpD* (ATP synthase β-subunit), *gyrB* (DNA gyrase β-subunit), *recA* (recombinase A), *rpoB* (RNA polymerase β-subunit) and *trpB* (tryptophan synthase β-subunit) and DNA-DNA hybridization (Dominguez-Moñino et al., 2017), an approach that is out of our scope.

Cultivable members of the genus *Streptomyces* have been previously reported from different caves, however isolates belonging to other genera, have been found through both culture-dependent and independent approaches (Groth et al., 1999, 2001; Nakaew et al., 2009; Stomeo et al., 2009; Niyomvong et al., 2012). Taking into consideration the numerous studies on cave microbial diversity, it is worth noting that each cave seems to be unique and diverse in its microbial compositions, however, there is a trend of finding dominating actinobacterial communities in many caves (Cheeptham et al., 2013). The reason for the large recovery of *Streptomyces* in caves can lie in their spore dispersal, the ability to use a large variety of nutrient sources in synthetic rich media and their more rapid growth in comparison to other genera, recognized as rare Actinobacteria, which are isolated much less frequently (Subramani & Aalbersberg, 2013; Maciejewska et al., 2016).

The importance of cave actinomycetes as producers of bioactive substances was stressed in the past (Groth et al., 1999). Indeed, members of the genus *Streptomyces* are notable for their ability to produce a wide variety of pharmaceutically useful compounds as secondary metabolites (Huang et al., 2005). Many of these secondary metabolites are potent antibiotics, which has made streptomycetes the primary antibiotic-producing organisms exploited by the pharmaceutical industry (Jensen et al., 2007; Ramesh et al., 2009).

### Antimicrobial activity of actinomycetes

The antimicrobial potential of cave actinobacteria, and especially *Streptomyces*, has received the attention of many scientists and it is a topic of great interest (Herold et al., 2005; Hodges et al., 2012; Cheeptham, 2013; Rule & Cheeptham, 2013; Nimaichand et al., 2015).

The results of the initial screening of 47 strains for antibacterial and antifungal activity are summarized in Table 3. Overall, the tested actinomycetes showed antimicrobial activity against at least one of the tested pathogenic strains by agar cylinders method. The lowest activities were exhibited against Gram-negative bacteria. Indeed, 61.70% of the tested isolates were active against *S. aureus*, 57.45% against *M. luteus*, 36.17% against *B. subtilis*, 29.79% against *L. monocytogenes*, while 21.28% were active against *E. coli* and only 8.51% against *K. pneumoniae*. However, we observed an absence of antimicrobial activity against *P. aeruginosa*.

Maciejewska et al. (2016) studied the cultivable *Streptomyces* isolated from cave moonmilk deposits which showed a much stronger antibacterial activity against Gram-positive bacteria (94% of the phylotypes) than against Gram-negative bacteria (71% of the phylotypes). In addition, the data showed that the antifungal activity was exhibited in 14.89% of all isolates.

### Screening for antifungal polyenic and nonpolyenic metabolites

After primary screening for antimicrobial activity of the 47 isolates by agar cylinders method against pathogenic microbes, five strains of *Streptomyces* sp. (A5, A11, A16, A22, and A45) were selected for further analysis, since they showed significant antifungal activity against the yeast *C. albicans*. In addition, these strains exhibited good activity against tested Gram-positive bacteria compared to Gram-negative bacteria (Table 4). The inhibition zone diameter for *C. albicans* was more than 8.5 mm.

These five isolates appeared promising because of their activity against *S. aureus*, *M. luteus*, *B. subtilis*, and 99.58%.
### Table 3. Number (percentage) of active actinomycetes (n = 47) and Penicillium (n = 23) strains, according to diffusion method.

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Gram-positive bacteria</th>
<th>Gram-negative bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>C. albicans</em></td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actinomycetes</td>
<td>7 (14.89%)</td>
<td>29 (61.70%)</td>
</tr>
<tr>
<td>Penicillium sp.</td>
<td>10 (43.48%)</td>
<td>8 (34.78%)</td>
</tr>
</tbody>
</table>

**Standard antimicrobial compound**

<table>
<thead>
<tr>
<th></th>
<th>Ampicillin (10 µg/disc)</th>
<th>Chloramphenicol (30 µg/disc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30.00 ± 0.00  ( ^a,b )</td>
<td>26.00 ± 0.00  ( ^c )</td>
</tr>
<tr>
<td></td>
<td>(S)</td>
<td>(S)</td>
</tr>
<tr>
<td></td>
<td>40.20 ± 0.45</td>
<td>10.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>(S)</td>
<td>(I)</td>
</tr>
<tr>
<td></td>
<td>28.00 ± 0.00</td>
<td>22.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>(S)</td>
<td>(R)</td>
</tr>
<tr>
<td></td>
<td>33.67 ± 3.51</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>(S)</td>
<td>(R)</td>
</tr>
<tr>
<td></td>
<td>0 ± 0</td>
<td>(S)</td>
</tr>
<tr>
<td></td>
<td>0 ± 0</td>
<td>(R)</td>
</tr>
<tr>
<td></td>
<td>0 ± 0</td>
<td>(I)</td>
</tr>
<tr>
<td></td>
<td>0 ± 0</td>
<td>(R)</td>
</tr>
<tr>
<td></td>
<td>0 ± 0</td>
<td>(I)</td>
</tr>
</tbody>
</table>

\(^a\)Expressed as the size of the inhibition zones (mm) as an average of duplicates ± SD, including diameter of paper disk (6 mm).

\(^b\)Nystatin (100 µg/disc); \(^c\)Amphotericin B (100 µg/disc).

*S* Microorganism classified as *Susceptible* by CLSI criteria to the antimicrobial compound; *I* Intermediate; *R* Resistant.

### Table 4. Inhibitory effects of selected actinomycetes on indicator microorganisms.

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Gram-positive bacteria</th>
<th>Gram-negative bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>C. albicans</em></td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. (A5)</td>
<td>17 ± 1.41*</td>
<td>18 ± 0</td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. (A11)</td>
<td>13.5 ± 0.71</td>
<td>12.5 ± 0.71</td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. (A16)</td>
<td>8.5 ± 0.71</td>
<td>12 ± 0</td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. (A22)</td>
<td>8.5 ± 3.53</td>
<td>16.5 ± 0.71</td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. (A45)</td>
<td>14 ± 0</td>
<td>17 ± 0</td>
</tr>
</tbody>
</table>

\(^*\)Expressed as the size of the inhibition zones (mm) as an average of duplicates ± SD, including diameter of paper disk (6 mm).
and *E. coli* (cell membrane without sterols) but they showed a marked inhibition of antifungal activity by exogen ergosterol, which is considered to be a target of polyenic antifungal compounds (Bastide et al., 1986). A reduction of the inhibition zone in the presence of ergosterol is an indication of the presence of polyene metabolites. For nonpolyenic metabolites, no interaction occurs and the diameter of the inhibition zones remains constant (Hamilton-Miller, 1973). This allowed us to easily detect the absence or production of polyenic metabolites. The five *Streptomyces* strains showed an absence of inhibition zones (Table 5).

Several authors have used UV-visible spectroscopic analyses of the active extracts to distinguish between polyenic and nonpolyenic substances (Hacène et al., 1994; Ouhdouch et al., 2001; Lemriss et al., 2003). The spectra of polyenes are characterized by a series of bands between 260 and 405 nm (Hamilton-Miller, 1973). However the metabolites produced by the five strains of *Streptomyces* did not show an UV-visible spectrum characteristic of a polyenic structure.

To summarize, the five strains of *Streptomyces* sp. showed activity against *C. albicans* and bacteria, and the inhibition zones were markedly reduced after the addition of exogenous ergosterol. However, the extracts did not reveal polyene-type UV-visible spectra. These contradictory results have been explained by a coproduction of polyene and non-polyene products by the same strain (Lemriss et al., 2003).

### Table 5. Three step screening method for nonpolyenic antifungal agents.

<table>
<thead>
<tr>
<th>Antimicrobial activity</th>
<th>Ergosterol effect</th>
<th>UV-visible spectroscopic analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without</td>
<td>With</td>
</tr>
<tr>
<td><em>S. celluloflavus</em> (A5)</td>
<td>17 ± 1</td>
<td>0 ± 0</td>
</tr>
<tr>
<td><em>S. celluloflavus</em> (A11)</td>
<td>13.5 ± 0.5</td>
<td>0 ± 0</td>
</tr>
<tr>
<td><em>S. celluloflavus</em> (A16)</td>
<td>8.5 ± 0.5</td>
<td>0 ± 0</td>
</tr>
<tr>
<td><em>S. aureovericillatus</em> (A22)</td>
<td>10.5 ± 0.5</td>
<td>0 ± 0</td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. (A45)</td>
<td>14 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

*Activity measured by diameter of inhibition zones (in mm). S: Staphylococcus aureus ATCC 6538; M: Micrococcus luteus ATCC 9341; B: Bacillus subtilis ATCC 6633; E: Escherichia coli ATCC 8739.*

### Enumeration of viable counts and fungi

The enumeration of the aerobic mesophilic microorganisms was done in order to develop an idea about the cave microbiology and on the total microbial count of cave sediments where the strains were isolated.

Table 6 summarizes the microbial counts obtained and pH values from various samples in Chaabe Cave. The pH of the four samples ranged from 7.45 to 8.48.

In all samples, the count of total aerobic mesophilic microorganisms was significantly greater than the number of isolated fungi. CFU counts on PCA were the highest in sample 2 (6.8 × 10^5 CFU·g^-1) whereas samples 1, 3, and 4 with values of 2 × 10^5 CFU·g^-1, 2.78 × 10^5 CFU·g^-1, and 2.18 × 10^5 CFU·g^-1, respectively, were about three times lower. Fungal counts were higher in samples 1 and 2, when compared with the last two samples. Growth was better in the media CDAr and MEA as compared to PDA. Altogether 38 isolates of fungi were obtained from the four cave samples using different solid media. Most of the isolates were obtained from samples 1 and 2, those located less deep in the cave, which probably denotes a higher input from outside fungi.

### Mycobiota diversity

In total, 38 filamentous fungi isolates were picked from plates and identified. Species of *Penicillium*, *Cladosporium*, *Aspergillus* and *Alternaria* account for a high proportion of the strains examined, but representatives of other genera are also included.

Cultural analysis, macroscopic and microscopic characteristics of mycoflora on cave samples revealed the presence of eight genera indicating a diverse fungal community in our cave. The genera were (in the order of relative abundance) *Penicillium* spp. (60.53%; *n* = 23) as the most abundant, followed by *Cladosporium* spp. (10.53%; *n* = 4), *Alternaria* sp. and *Aspergillus* sp. (7.89%; *n* = 3), *Beauveria* sp. (5.26%; *n* = 2), *Rhizopus* sp., *Rhizomucor* sp., and *Readeriella* sp. (2.63%; *n* = 1) (Fig. 2). The identification at the species level was determined by sequence analysis of ITS rRNA or of partial β tubulin gene. Taking their similarities to sequences in GenBank, we identified 5 species: *Readeriella eucalypti*, *Cladosporium pseucladosporioides*, *Aspergillus wentii*, *Penicillium brevicompactum*, *Amphicorda felina* (= *Isaria felina* = *Beauveria felina*).

Man et al. (2015) studying the phylogenetic diversity of culturable fungi in the Heshang Cave (China) reported that the genus *Penicillium* was the most abundant and accounted for 40, 54, and 52% of cultivable fungi in the sediments, weathered rocks and bat guano, respectively. Some other abundant fungal genera were *Trichoderma*, *Paecilomyces*, and *Aspergillus*, but in weathered rocks the presence of bands between 260 and 405 nm (Hamilton-Miller, 1973). However the metabolites produced by the five strains of *Streptomyces* did not show an UV-visible spectrum characteristic of a polyenic structure.

To summarize, the five strains of *Streptomyces* sp. showed activity against *C. albicans* and bacteria, and the inhibition zones were markedly reduced after the addition of exogenous ergosterol. However, the extracts did not reveal polyene-type UV-visible spectra. These contradictory results have been explained by a coproduction of polyene and non-polyene products by the same strain (Lemriss et al., 2003).

### Table 6. The pH, total viable counts and fungi counts (cfu·g^-1) of different samples from Chaabe Cave.

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>PCA</th>
<th>CDA</th>
<th>CDAr</th>
<th>PDAr</th>
<th>PDAac</th>
<th>MEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>8.48 ± 0.04</td>
<td>2.10^3</td>
<td>4.10^3</td>
<td>5.10^1</td>
<td>5.10^1</td>
<td>3.10^1</td>
<td>10^1</td>
</tr>
<tr>
<td>Sample 2</td>
<td>8.35 ± 0.39</td>
<td>6.810^4</td>
<td>ND</td>
<td>5.210^1</td>
<td>1.110^1</td>
<td>1.4810^1</td>
<td>2.110^1</td>
</tr>
<tr>
<td>Sample 3</td>
<td>7.45 ± 0.05</td>
<td>2.7810^4</td>
<td>9.10^1</td>
<td>2.9510^2</td>
<td>4.510^1</td>
<td>1.510^1</td>
<td>6.10^2</td>
</tr>
<tr>
<td>Sample 4</td>
<td>7.54 ± 0.05</td>
<td>2.1810^3</td>
<td>ND</td>
<td>10^1</td>
<td>10^1</td>
<td>3.10^1</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Activity measured by diameter of inhibition zones (nm). S: Staphylococcus aureus ATCC 6538; M: Micrococcus luteus ATCC 9341; B: Bacillus subtilis ATCC 6633; E: Escherichia coli ATCC 8739.*
of Cladosporium and Beauveria felina were also significant. We also found the entomopathogenic fungus Amphicorda felina (= Beauveria feline) in cave floor surfaces collected by swabbing.

To the best of our knowledge, the present work is the first report on the isolation of the genus Readeriella from cave, a fungal genus associated to Eucaliptus leaves (Sánchez Márquez et al., 2011). For the other genera found in Chaabe Cave, Vanderwolf et al. (2013) stated that they are considered to be the most frequently reported with compare with other studies on cave mycology and that fungi are not distributed evenly throughout caves. The distribution of cave mycobiota is influenced by the susceptibility of the rock material to be colonized, their mineral composition, porosity, etc. and by environmental conditions (water availability, temperature, pH, and nutrient sources) (Gorbushina, 2007). Dickson and Kirk (1976) noted that more fungi and bacteria were found on the cave sediments than on the walls or ceiling, likely because organic carbon accumulates on the cave floor. In addition, bacteria are more uniformly distributed than fungi, which are often associated with invertebrates and organic matter (Jurado et al., 2008).

Antimicrobial activity of Penicillium spp.
A large number of fungal extracts and/or extracellular products have been found to have antimicrobial activity, mainly from species of the ubiquitous genus Penicillium (Rancic et al., 2006; Petit et al., 2009). A total of 23 strains of Penicillium were selected for screening of antimicrobial activity against pathogenic microorganisms.

The results of the initial screening of 23 strains for antibacterial and antifungal activity are summarized in Table 3. The Penicillium spp. tested showed antimicrobial activity against at least one of the tested microorganisms by agar cylinders method (except for P. aeruginosa which is the most resistant species) indicating that these fungi produce some type of antimicrobial substance(s) responsible for inhibiting the tested microorganisms. The active strains showed strong inhibitory activity opposed to the pathogenic microorganisms and the diameters of the inhibition zones vary between 7.5 and 26 mm. The results showed the best activity against C. albicans with a percentage inhibition of 43.38% (10/23).

Several surveys of the antibacterial substance production by fungi have been published recently, from which it appears that many of the Ascomycetes, Basidiomycetes and Fungi Imperfecti show marked antibacterial activity but few, if any, of the Phycomycetes. The present account deals with a number of Fungi Imperfecti examined mostly for antifungal activity; a preliminary examination for antibacterial activity has also been made in most cases.

CONCLUSION

This is the first report on microbial communities in a natural pristine cave ecosystem in Algeria to our knowledge, and sheds light on microbial assemblages that can be a remarkable source of antimicrobial molecules. This study was aimed at investigating the antagonistic activity of actinomycetes and fungi from Chaabe Cave, Algeria, against pathogenic microorganisms. The microbial assemblages associated with this Algerian cave were investigated by culture-dependent method together with the analysis of 16S rRNA gene sequences for actinomycetes and the fungal ITS or β tubulin gene sequences. A large number of isolated microorganisms exhibited activity against pathogenic bacteria and/or fungi according to their origin. The desired outcome of this publication is to provide preliminary data that indicate the cave is a useful potential source for the isolation of microorganisms producing biologically active products. A few strains of Streptomyces and Penicillium produced antibiotics and likely an array of other secondary metabolites, which merit a more detailed investigation.

ACKNOWLEDGEMENTS

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Drip water measurements from Carlsbad Cavern: implications towards paleoclimate records yielded from evaporative-zone stalagmites

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Abstract: Stalagmites can host numerous potential climate proxies (stable and radiogenic isotopes, trace elements, annual and non-annual banding, grayscale, growth hiatuses, mineral assemblage). Reproducibility and/or integration of proxy results between one or more stalagmites will become increasingly important, and ideally, climate records generated by multiple stalagmites from the same cave or cave room are expected to be near-identical. The reality is that stalagmites from the same cave room can yield differing results to some degree, especially in cave environments that are evaporative. Our drip water study in an evaporative shallow-depth cave environment in Carlsbad Cavern shows that adjacent drip sites produce differing drip rate behavior, but share some similarities. Drip water collected from four sites in this evaporative cave environment shows Ca, Mg, Sr, Ba, U, and Th elemental concentrations and $^{234}$U/$^{238}$U (expressed as $\delta^{234}$U) to vary seasonally, and all but U have higher values during the winter months when the instrumented cave site exhibited slower drip rates and was drier. Results from our Carlsbad Cavern drip sites indicate that increased relative humidity in the cave and decreased surface and cave atmospheric pressure combined with increased precipitation (rain and snow) are responsible for faster drip rates. Changes in atmospheric pressure play an essential role, although less directly during summer months. We therefore conclude that stalagmites within the same cave room may not record and produce identical proxy records in these more evaporative cave environments, but that differing records are simply recording the same climate signals expressed uniquely by the individual proxies, and that each stalagmite simply has differing sensitivities to the climate signals. Integrated, these proxy differences serve as important past climate indicators. Our drip sites respond to seasonal variations in climate more so than individual rain/snow events, and we favor the interpretation that seasonal changes reflect regional as well as local climate changes.

Keywords: cave, drip water, Carlsbad Cavern, climate, uranium isotopes

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INTRODUCTION

Stalagmites as paleoclimate indicators are advantageous in many ways over other indicators, producing some of the most robust and highly resolved records globally. Yet understanding the hydrological processes from the surface soil to the stalagmite lags behind the production of paleoclimate records, and this creates a need for more cave environment studies (Beddows et al., 2007). The numerous cave climate and drip studies that have been conducted leave researchers with countless questions related to stalagmite climate proxy reproducibility, correlation to precipitation and temperature, and effects due to ventilation, but with each study making valuable contributions towards understanding how cave climate translates into local and regional paleoclimate (Genty & Deflandre, 1998; Baldini et al., 2006; Fairchild et al., 2006; Verheyden et al., 2008; Poulin et al., 2015; Duan et al., 2016). Smart & Friedrich (1987), Baker et al. (1997), and Baldini et al. (2006) have produced widely accepted drip site classifications based on drip discharge and cave environment where drip rates of one drip per 1-1000 seconds are considered seepage flow and seasonal drip types, the classifications that our drip study falls under.

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Some studies of seepage flow and seasonal drip type dripping include multiple sites and show different dripping behaviors of these sites. Three drip sites from a cave in Belize show two sites with less drip rate variability having low drip rates (<35 drips/hr) and one site with high variability having drip rates >200 drips/hr (Ridley et al., 2015). Ten drip sites in Australian caves monitored by McDonald & Drysdale (2007) at different depths showed that increased bedrock overburden complicated the drip systems. A comprehensive study that includes 34 drip sites in 8 caves that covers a large region (southeastern China) has identified three types of drip sites based on δ^{18}O variability of drip water but with drip rate data included, and conclude that different hydrologic conditions associated with these three drip site types are important when considering interpretation of δ^{18}O variability in the stalagmites that grow under these sites (Duan et al., 2016). Six drip sites from Westcave Preserve in central Texas that include drip rate and elemental concentration data were intensively studied in an evaporative cave environment (Casteel & Banner, 2014) somewhat similar to our Carlsbad Cavern sites. Evaporative cave zones, however, are still largely avoided by paleoclimatologists because of problems related to kinetic fractionation of carbon and oxygen isotopes during calcite/aragonite crystallization (Lachniet, 2009, 2015), and consequently stalagmite samples from deep cave zones where relative humidity is perennially close to 100% are preferred. Nonetheless, stalagmites in somewhat evaporative zones are used as relative indicators of rainfall where kinetic fractionation can enhance the rainfall signal. Stalagmites from evaporative cave zones preserve added complexities, but also potentially hold additional benefits beyond relative rainfall indicators. Drip rate studies in these evaporative sites should yield beneficial information regarding the use of these cave zones in paleoclimate studies. Here we present results of a drip study from an instrumented site in Carlsbad Cavern, New Mexico USA that is evaporative and has yielded significant paleoclimate records covering the past two millennia of climate change (Polyak & Asmerom 2001; Rasmussen et al., 2006; Asmerom et al., 2007) and Asmerom et al. (2013) used samples from evaporative near-entrance cave zones with the premise that these samples will better preserve a more direct link to surface climate changes. This near-entrance cave zone is where most annually banded stalagmites grow. Our instrumented sites in Carlsbad Cavern experience relatively large swings in temperature, relative humidity, atmospheric pressure, and drip rate during the winter months related to cold winter air sinking into the cave. The monitoring period for this study (May 2002 to September 2004) overlapped with the 2002 and 2004 El Niño events and a short-lived shift from negative to positive Pacific Decadal Oscillation (PDO), both of which provide the southwestern United States with greater precipitation (Rasmussen, 2006). Surface precipitation during the monitoring period was near

![Fig. 1. Study area in Carlsbad Cavern. Drip site area is below the main entrance where rock overburden is thinnest.](Image)
normal during 2002, very dry during 2003 CE, and above normal during 2004. Four drip sites, WBC1, WBC2, WBC3b, and WBC4, were instrumented (Figs. 1 and 2).

The Guadalupe Mountains range in elevation from 2667 m at Guadalupe Peak on the southwestern end of the mountains to 1340 m at Carlsbad Cavern on the northeastern end. The area is semi-arid, forested at higher elevations and covered with desert vegetation (cactus and desert shrubs) at Carlsbad Cavern. As much as 50% of the 380 mm of annual precipitation comes from the North American Monsoon summer rains from July to September (Leavitt et al., 2011). The El Niño phase of the El Niño Southern Oscillation (ENSO) brings extra winter and spring precipitation to the study area every 2 to 8 years, and particularly when El Niño is in phase with the Pacific Decadal Oscillation (PDO; Wang et al., 2014).

METHODS

We used accelerometers to count drips, where all individual drips are counted as drips per hour. An accelerometer was attached to a Plexiglas paddle that was tilted so that each drip that strikes the paddle produces an event that is recorded by an Omega NOMAD OM-CP-PULSE101 datalogger (Fig. 2). The sensitivity of the accelerometer was adjusted so that aftershocks were not recorded. The water from these drips flows down the paddle and into a one-liter bottle for collection. The small size of the bottle neck significantly dampens evaporative effects relative to open bottles. Drip rates were sufficiently slow that the bottles did not over-fill at the end of a one-month period, except during the summer humid months in some cases. An Omega NOMAD OM-CP-PRTEMP101 temperature, relative humidity, and atmospheric pressure datalogger was placed near the drip counters. The two dataloggers, the one for drip counts and the other for climatology, were independently recording data at hourly resolution. Each drip counter was powered with a 6V motorcycle battery that lasted one month. Dataloggers were downloaded and batteries exchanged each month.

Mg, Sr, Ba, and Ca elemental concentrations were determined by inductively coupled mass spectrometry (ICPMS) on a Fison VG PQ-2 (Rasmussen, 2006) and Thermo X-series II ICPMS. U and Th concentrations, \(^{234}\)U, and \(^{230}\)Th/\(^{232}\)Th were measured on a Thermo Neptune multicollector (MC) ICPMS. Samples run on our MC-ICPMS were prepared and analyzed using our standard methods described in Polyak et al. (2012) and Asmerom et al. (2013). The drip study was not designed for stable isotope measurements.

RESULTS

Of our four monitored drip sites, site WBC1 fed stalagmite BC8, and site WBC4 fed stalagmite BC7. Both of these stalagmites were collected. The other two drip sites, WBC2 and WBC3b were set up where stalagmites likely existed but were detached when the area was damaged by early visitors to the cave decades before our study. This area is decorated with many stalagmites, stalactites, columns, and flowstone, but located in the path of bat guano miners from 1906 to 1924 and the first tourists that rode the guano bucket down the Bat Cave entrance to bypass the vertical drop at the main entrance from 1924 until 1926 when the stairs were built (Nymeyer & Halliday, 1991). As a result, much damage was done to these galleries, although Asmerom et al. (2013) dated the top of stalagmite BC2 to be \(\sim1970\) CE, indicating that stalagmite BC2 was broken after the stairs going into the main entrance were installed. All of the drip sites and the temperature-humidity-pressure datalogger are located within a 50-meter perimeter along the south wall of the passage (Fig. 1).

Monitored cave site climatology and drip rates

Drip water monitoring sites included WBC1, WBC2, WBC3b, and WBC4 (Fig. 1). Climatology of the monitored cave site (Carlsbad Cavern) versus surface climate monitored at the city of Carlsbad municipal airport 26 km east of the cave show positive correlations as expected. The temperature time-series in the cave versus that on the surface has a high correlation (\(R = 0.84\); Rasmussen, 2006). The cave relative humidity time-series versus surface also exhibits a positive correlation, although weaker (\(R = 0.28\); Rasmussen, 2006). Atmospheric pressure time-series of the surface and cave site are almost identical (\(R = 0.99\); Rasmussen, 2006), with the cave pressure slightly higher, a similar scenario to that measured by McCann (2013) in a Guam Cave. The
monitored cave site in Carlsbad Cavern experiences increased temperature and relative humidity during the warmer, more humid monsoonal summer months. Sites WBC1 and WBC3b had ceilings too high to observe the origin of dripping. Sites WBC2 and WBC4 had no obvious soda straws or stalactites that could be observed over the drip sites, partly because of damage done to the site by early mining or tourism. The two longer-record drip sites are compared to daily precipitation that is based on the average of three nearby climatological stations, Queen, Carlsbad, and Carlsbad Caverns obtained from the Western Regional Climate Center (2013). All drip sites produce a seepage flow type drip, with the exception of drip site WBC2, which can be categorized as a seasonal drip type because this site quits dripping in the winter months (Fig. 3).

Fig. 3. Drip type classification from maximum measured drip rate (liters/second) versus coefficient of variation (standard deviation/mean *100). See Baldini et al. (2006). Three of the four drip sites fall into the seepage flow type drip category. Drip site WBC2, however, quit dripping during the winter months, and when that period of non-dripping is included with the period of dripping, the coefficient of variation increases and the results plot in the seasonal drip category. For the period of dripping, drip site WBC2 plots in the seepage flow classification. Drip site WBC4 is similar to WBC2, where missing data (available only by manual drip rate measurements) would produce greater coefficient of variation. Drip sites WBC2 and WBC4 have assumed drip volumes of 0.1 ml.

**Drip site WBC2** stopped dripping during both winters of the monitoring period (Fig. 5). The drip rate at this site was higher by a factor of 3 to 10 over site WBC1 and varied from 0 to 280 drips per hour (Fig. 4). This site is obviously far more sensitive to seasonal climatological changes. One could assume from this drip data that a stalagmite under this drip site would exhibit more growth rate variability in response to climatic swings. The drip rate at this site peaked long before the cave temperature and humidity peaked, and before site WBC1 drip rate peaked. Two years of manually measured drip data and one year of instrumented data were collected.

![Drip rate record for site WBC1 compared to cave relative humidity, cave temperature, and daily surface precipitation records for the same period. Surface precipitation (rainfall) data are from Western Regional Climate Center (2013). Yellow circles and blue dashed curve represent manually measured drip rate data.](image)

**Fig. 4.** Drip rate record for site WBC1 compared to cave relative humidity, cave temperature, and daily surface precipitation records for the same period. Surface precipitation (rainfall) data are from Western Regional Climate Center (2013). Yellow circles and blue dashed curve represent manually measured drip rate data.

The tip of this stalagmite visually indicates that the top is modern calcite, and there was some calcite deposited on the paddle of the drip rate counter. Two years of manually measured and instrumented drip data were collected at this site.

**Drip site WBC1** dripped continuously over the two-year monitoring period. The drip rate changed seasonally by ~10 drips per hour, with warmer, more humid summer months coincident with higher drip rates (Fig. 4). While this drip site dripped continuous over the two-year monitoring period, our preliminary examination of stalagmite BC8 below this drip site exhibited discontinuous growth with hiatuses illustrating that the current drip period was likely not continuous in much of the late Holocene. The tip of this stalagmite visually indicates that the top is modern calcite, and there was some calcite deposited on the paddle of the drip rate counter. Two years of manually measured and instrumented drip data were collected at this site.

![Drip rate record for site WBC2 stopped dripping during both winters of the monitoring period. The drip rate at this site was higher by a factor of 3 to 10 over site WBC1 and varied from 0 to 280 drips per hour. Two years of manually measured drip data and one year of instrumented data were collected.](image)

**Fig. 5.** Temperature, relative humidity, and site WBC2 drip rate show that seasonal variations correlate well with drip rate. The WBC2 drip site stopped dripping in both winters that were monitored. Note that the drip rate is exceptionally higher for site WBC2. Yellow circles and blue dashed curve represent manually measured drip rate data.
Drip site WBC3b dripped continuously over the monitoring period, and behaved similar to site WBC1 (Fig. 6). Both sites exhibit a similar drip rate between 5 and 25 drips per hour. Almost two years of drip data were collected at this site, but there are gaps due to instrument problems.

Drip site WBC4 dripped continuously over the monitoring period. The drip rate varied between 25 and 70 drips per hour (Fig. 7). This drip site fed stalagmite BC7, which has a fast growth rate. Stalagmite BC7 was a broken stub with recently precipitated calcite growth on the broken surface. The stalagmite was likely broken during guano mining operations, or by the earliest tourists within the last 110 years, and a U-series date on this new growth is 30 ± 100 yrs BP. The recent calcite deposit exhibits faint annual banding from which the age of this overgrowth on the stub was determined to be 64 ± 10 yrs BP with a growth rate of 147 µm/year (Rasmussen, 2006). The stub represents a segment of Late Holocene growth between 2900 and 1900 years ago that has a faster growth rate of ~300 µm/year. About 1½ years of manually measured drip rate data and less than a year of instrumented data were collected,

Elemental concentrations and U-series values

Ca, Mg, Sr, Ba, and U concentrations of two years of monthly drip water samples show higher elemental concentrations (Fig. 8) and higher δ²³⁴U (Fig. 9) during slower drip rates in the winter months. Drier cave climate at these drip sites during winter months is consistent with high concentrations of elements in the drip water due to evaporation or prior CaCO₃ precipitation. Slower drip rates during winter is also consistent with lower infiltration rates that drive high δ²³⁴U values of drip water.

At site WBC1, δ²³⁴U of the top two years of stalagmite BC8 calcite has a value between the higher winter values and lower summer values of the corresponding drip water, showing that at site WBC1 deposition of calcite is likely occurring during all seasons, but predominantly in the spring and summer seasons. U concentration is negatively correlated with δ²³⁴U in WBC1 drip water (Fig. 9). Stalagmite BC11, collected from a different drip site after the drip study, has similar δ²³⁴U values. The degree of δ²³⁴U changes is a response to hydrological variation (Hellstrom & McCulloch, 2000) suggesting that sites with similar δ²³⁴U have comparable plumbing. These changes could occur from seasonal cessation of dripping, where winter season drip rates were more important than summer season, or they could result simply from drier/wetter annual periods (Polyak et al., 2012; Cross et al., 2015). Th isotope values for WBC1 drip waters are also included in Fig. 9, and give insight into initial ²³⁰Th/²³²Th atomic ratios used to correct U-series dates.

Drip site WBC2, only 5 meters from WBC1, did not drip during the winter months over the monitoring period, showing that stalagmite growth could be sensitive to differences in seasonal dripping periods. This would suggest, for instance, that two stalagmites from the same cave room could produce significantly different geochemical proxy records, even though these records might uniquely record the same climate signal. Elemental, δ²³⁴U, and probably stable isotope values should be different in a sample that grew from summer-only dripping, compared to a sample that grew from summer and winter dripping, and would be higher for elemental values during drier climate, where oscillations primarily follow decadal drivers such as PDO (Rasmussen et al., 2006; Asmerom et al., 2013). Seasonal values of drip water are complicated by infiltration times and evaporative conditions in the caves.
Fig. 8. Comparison of Mg, Ca, Sr, Ba, U concentrations, and δ²³⁴U for drip water of site WBC1 showing higher values during winter months when drip rates are lower. All y-axes are increasing in value upward except for U concentration.

A comparison of Ca concentration and Mg/Ca, Sr/Ca, and Ba/Ca shows that these ratios increase as Ca concentrations decrease in the WBC1 drip water (Fig. 10). Johnson et al. (2006) suggest that strong covariance of Mg/Ca, Sr/Ca, Ba/Ca, and U/Ca in stalagmites is linked to both increased CO₂ degassing combined with calcite precipitation on the stalagmite surface, and/or prior calcite precipitation before drip waters impact stalagmites. While evaporation could play a role, prior CaCO₃ precipitation is the likely cause of decreased Ca concentrations and disproportionate increases in Mg/Ca, Sr/Ca, and Ba/Ca in our WBC1 drip water. The monthly anomaly that indicates prior CaCO₃ took place was a winter month in 2003 CE, during a particularly dry year. It suggests, in agreement with Johnson et al. (2006), that dry climate leads to increased prior CaCO₃ precipitation. Oster et al. (2012) suggest that a strong positive correlation between Mg/Ca and Sr/Ca in drip water is an indication of prior CaCO₃ precipitation and this pattern should also be preserved in stalagmite CaCO₃ (Oster et al., 2012).

Initial ²³⁰Th/²³²Th

The ²³⁰Th/²³²Th atomic ratios and corresponding ²³²Th concentrations (ppt) were measured for 20 monthly water samples collected at site WBC1. Stalagmite studies commonly assume an initial ²³⁰Th/²³²Th atomic ratio value (time = 0) to be 4.4 ppm (0.0000044) used to correct uranium-series dates (Dorale et al., 2004). However, in our study area, the initial ²³⁰Th/²³²Th atomic ratios were measured to be higher (4.4 to 100 ppm) and varied with ²³²Th concentration, where the ²³⁰Th/²³²Th atomic ratio decreases non-linearly as ²³²Th concentrations increase (Fig. 9B; Polyak & Asmerom, 2009). This was modeled as the high value end member Th source being the bedrock and the low value end member Th source being the soil. Recently, closed and open stable isotope systems have been modeled similarly, with the closed system (percolating water isolated from soil CO₂ reservoir) causing higher δ¹³C values in speleothem CaCO₃ due to greater input from bedrock carbon, and an open system causing lower δ¹³C values in speleothem CaCO₃ due to greater input from soil carbon (McDermott, 2004). Greater initial ²³⁰Th/²³²Th atomic ratio values would seemingly be predicted in samples that have higher than expected δ¹³C values (i.e., closed system samples). Our drip waters yield values that are consistent with our study of stalagmite CaCO₃ from this region, where ²³⁰Th/²³²Th atomic ratio decreases non-linearly as ²³²Th concentrations increase (Fig. 9B).

Volume of individual drips

We determined drip volume at two sites based on number of drips counted versus monthly volume collected. The volume is therefore a monthly average.
At site WBC1 and WBC3b, drip volumes were measured to be 0.087 ± 0.005 ml/drip (n = 5), and 0.057 ± 0.008 ml/drip (n = 6), respectively, which is less than the 0.17 cm$^3$ (ml)/drip reported by Baker et al. (1997) as typical, and the 0.114 ml/drip reported by Genty & Deflandre (1998). This difference in volume between WBC1 and WBC3b is presumably because of the shape of the surface where the drip originates (Tate, 1864). This was quantitatively determined by Collister & Mattey (2008) to be $m = 0.0255r + 0.00981$ for stalactites, where, $m =$ mass in grams (1 ml of water = ~1 gram) and $r =$ radii of the drip tube/surface. Their formula would suggest that drip sites WBC1 and WBC3b were fed by stalactites or sharp projections having diameters of 6.0 and 3.7 mm, respectively, for which sizes are consistent with soda straw stalactites. Site WBC1 and WBC3b recorded similar drip rates, but our results suggest that site WBC1 having a larger drip volume delivers ~50% more volume over time to the stalagmite.

Evaporation at the drip source (i.e., tip of soda straw), during the distance that the drip falls, at impact (including some loss of volume due to aerosol loss from the splash), and in the collection bottle could affect drip volume results that are measured using monthly bottle volumes. The standard errors on the mean drip volumes are low and show that the volume does not change linearly with volume of drip water in the collection bottles or with season, which would be the case if evaporation critically changed water volume in the bottles. While the effects from evaporation are somewhat significant in open pans even at 80 to 90 % RH (McClean, 1971; Hill, 1987), we measured an 11 times less evaporation rate in the narrow-neck bottles than in open pans/bottles in the lab. Other factors mentioned above should change volumes at each site similarly. Therefore, we interpret that final results showing such big differences between drip volume at these two sites are meaningful.

Cave and surface temperature

There is a positive correlation between cave temperature and drip rate at all four drip sites, with all sites showing higher drip rates in the summer months as illustrated using site WBC1 drip versus surface and cave climatological data (Fig. 11). This would seemingly suggest a direct link between higher temperatures and increased effective precipitation, in some ways contrary to a previous interpretation that faster stalagmite growth correlates to wetter cooler climate (Polyak & Asmerom, 2001). However, we suggest that the drip rate and seasonal surface temperature positive correlation is biased because seasonal increases in precipitation are linked to the summer monsoon, the period of maximum seasonal precipitation largely coincident with the period of maximum seasonal temperature. Seasonal correlation between temperature and drip rate is likely independent of long-term correlations between the two. In Figure 11, drip rate

![Graph](image)
increases through summer, and continues to increase after surface temperatures begin cooling, and starts decreasing at the end of the summer monsoon in synchronization with surface and cave relative humidity, and cave temperature.

**Relative humidity at the drip sites**

There is a positive correlation between cave relative humidity and drip rate, with both having higher values in the summer months (Figs. 4-7, 11). This would suggest a link between increased effective precipitation and drip rates. Several studies show a very good correlation between drip rates and individual surface precipitation events (Oster et al., 2012; Khazmutdinova & Nof, 2013; Poulain et al., 2015). However, like other drip rate studies (Onac et al., 2008; Casteel & Banner, 2014), we have no obvious direct link between occurrences of precipitation events and drip rate (Rasmussen, 2006), but this could be partly due to the drip water collection period being within a lengthy period of drought that started in the early 1990s, although year 2002 CE yielded near average precipitation and 2004CE yielded ~40% above average precipitation. Like the correlation with temperature, the correlation with surface and cave relative humidity is seasonal and caused by the summer monsoonal months.

**Atmospheric pressure at the drip sites**

Overall correlation between cave atmospheric pressure and drip rate in the two longest drip records, WBC1 and WBC3b, are $R^2 = -0.0064$ and $R^2 = -0.017$, respectively (Rasmussen, 2006), but during the winter and spring months there is a significant negative correlation showing that drip rate responds to changes in cave pressure. Drip site WBC4 had good correlation between drip rate and cave pressure in both spring and summer, and exhibits an hourly untuned correlation of $R^2 = 0.57$ and $R^2 = 0.44$, respectively, for a one to two month period (Fig. 12). Temperature and relative humidity in the cave did not respond the same, although individual storms can significantly lower the cave temperature at the drip sites. Genty & Delflandre (1998) saw this same relationship for drip sites having <200 drips per hour. Low pressure systems (storms) during the winter and spring months result in increased drip rates during those events at our monitoring site. Other sites have recorded similar results (Baker & Burnsdon, 2003; Fernández-Cortés et al., 2007; McCann, 2013; Tremaine & Froelich, 2013). Spring and summer of 2003 show less variability (Fig. 11) than fall and winter.

Our results show that low pressure systems associated with storm events during the winter months cause measurable drip rate increases followed by decreases. This is followed by high pressure and corresponding decreasing drip rates. This negative correlation between drip rate and atmospheric pressure shows that drip rate increases during short-term decreases in pressure due to individual winter and spring storm systems. This correlation is not so clearly observed during the summer months, but we interpret from these results that summer monsoons having more storm events will have an overall lower seasonal atmospheric pressure, and therefore we interpret an indirect or less obvious tie of the drip rate to seasonal changes in atmospheric pressure rather than individual rainfall events.

**Spectral signals**

Analyses using Redfit (Schulz & Mudelsee, 2002) of fall and winter data show that drip rate and atmospheric pressure share the same diurnal periodicities of 24, 12, and ~3 hours, and both show an ~7-day periodicity (Fig. 13). Analyses of cave humidity and temperature data do not show the diurnal periodicity, but also show the ~7-day signal. The 7-day periodicity could be related to increased visitation to the cave.
on weekends. However, significant changes in both atmospheric pressure and temperature strongly indicate that these changes in the cave are driven by winter storms that push cold air into Carlsbad Cavern’s large entrance. Significant drops in daily low temperature accompanied by changes in atmospheric pressure associated with the storm events on the surface match these excursions measured in the cave (Fig. 12).

**DISCUSSION**

Our drip study results show hourly to seasonal variability. The Carlsbad Cavern drip sites are similar to the seasonal and medium variability drip sites reported by Duan et al., 2016. In their study which focused primarily on oxygen stable isotope values of 34 drip sites from eight caves, only 12% were defined as seasonal type drip sites and 6% were defined as medium-variability drip sites, while 82% of the drip sites were described as static and did not show significant variability over a three-year period. Most of their cave sites also experienced distinctly greater rainfall events seasonally, for which drip sites seemed to have responded. Our sites are relatively close to Carlsbad Cavern’s big entrance and some variability in our drip data was expected due to cold winter air sinking into the cave in winter and warm moist monsoonal air circulating through the cave during the summer. Hourly to weakly variability was likely caused by individual storm events, and are interpreted to be mainly caused by atmospheric pressure changes and not individual storm event precipitation amount (Rasmussen, 2006). The seasonal variability is not directly related to individual storm or rainfall events, but rather related to seasonal changes driven by relative humidity, and temperature. These seasonal changes translate to annual banding in stalagmites of the study area and are tied to regional climate change more so than local individual climate events.

While temperature correlates best with drip rate (positive correlation) in our two-year study ($R^2 = 0.76$, $\rho = 0.00001$ for site WBC1; Rasmussen, 2006), we suggest that it is a seasonal relationship, and that temperature itself is not directly causing increased drip rates, because over the long-term and annually, the correlation between temperature and drip rate is likely negative. For instance, greater spring and summer rainfall is correlated to cooler spring and summers (Fig. 14) in the historical period, not warmer spring and summers (Western Regional Climate Center, 2013). Personal observation shows that in general wetter spring and summers result in wetter caves. Temperature alone is not driving variations in drip rate, but rather that the relative humidity is positively correlated to drip rate, and that higher summer temperatures occurring during every monsoon season indirectly drive drip rates. For example, higher relative humidity in the cave and on the surface occurs during the summer months when monsoonal flow and temperatures are higher. So we interpret that it is mostly a seasonal coincidence that temperatures are higher during periods of higher drip rate.

Dry hot summers equate to higher evaporation and less moisture available to enter the joints that serve as conduits for ground water infiltrating from the surface into the cave. Our monitoring period did not experience any abnormally wet winter precipitation, and drip rates slowed significantly or stopped during winter months, so our drip rate results would suggest that winter may equate to less or no calcite growth simply because of moisture deficit in the cave. Therefore, while drip rates correlate best with cave and surface temperatures seasonally, long-term correlations are related to spring and summer periods where cooler spring-summer periods equate to overall greater seasonal precipitation, and therefore equate to higher drip rates. This is indirectly consistent with wetter years equaling thick annual bands suggested by Polyak & Asmerom (2001).

The warmer monsoonal season may cause higher drip rates in other ways. We introduce an additional interpretation that invokes monsoonal moisture and ‘infiltration system relaxation’ during the summer months that cause the infiltration routes to open up

![Fig. 13. Spectral analyses show the (A) periodicity of drip variability and (B) cave atmospheric pressure of drip site WBC4, with dashed lines representing 95% confidence.](image)

![Fig. 14. Carlsbad Caverns spring-summer temperature versus precipitation for 72 of the 77 years from 1935 to 2013 shows a significant negative correlation. Cooler spring-summers represent wetter spring-summers. Data are from the Western Regional Climate Center (2013).](image)
or ‘relax’ enough to allow greater amounts of water to infiltrate through the bedrock overburden. In this hypothetical interpretation, higher relative humidity and higher effective rainfall would further enhance variations in drip rate. This relaxation is likely related to thermal expansion of the bedrock surface (Yan et al., 2009), increased organic activity within joints (i.e., root wedging; Maeght et al., 2013), physical variations of joint-filling materials by wetting/drying cycles (Dahan et al., 2000), and decreased surface tension and viscosity of water during the warmer months (Shukla, 2013), all more effective during the monsoonal months. In this way, higher drip rates during the summer months are directly related to infiltration system relaxation and increased relative humidity, both of which we expect to be enhanced by greater precipitation and more storm events.

Our area is moisture-limited, meaning that during very dry periods, the caves dry up, and as a consequence drip rates decrease or stop. So intuitively, higher precipitation means greater supply of water into the infiltration system, and therefore faster drip rates. However, we did not find a direct link between drip rate and precipitation event amount. But instead, the more indirect link is likely robust. During summer months, drip rates increase, not due directly to local temperature, but to increased relative humidity and relaxation of the infiltration systems related to the monsoonal period. Atmospheric pressure likely plays a long-term role and is related to climatic oscillations such as Pacific Decadal Oscillation and El Niño Southern Oscillation. Wetter seasonal cave climate should be caused by a greater number of storms and not solely correlated to precipitation amount from those storms. Thus we argue that spring-summer periods with more storms and moisture are cooler than spring-summer periods with less storms and moisture.

CONCLUSION

Our drip and stalagmite sites in Carlsbad Cavern are relatively close to the cave’s large entrance, and yielded climate data that shows this area of Bat Cave Passage near the Main Corridor is an evaporative cave zone. Two drip sites exhibited drip rates between 10 and 30 drips per hour, and one drip site measured drips between 30 and 70 drips per hour. A fourth drip site had the highest drip rate (up to 280 drips per hour), but quit dripping in the winter months. At these sites, relative humidity varies seasonally from ~90% in the summer to ~80% in the winter, and temperature changes from ~13°C in the summer and ~11°C in the winter. Atmospheric pressure changes match those at the surface, and during winter months, there is a high correlation between pressure and drip rate changes. Elemental concentrations and δ18O in the drip water change seasonally with higher concentrations and lower δ18O values during winter months. Because our study area is moisture limited compared to more humid regions, more atmospheric moisture means faster drip rates. Our interpretation is that the higher temperature associated with the monsoonal period causes higher drip rates due to infiltration system relaxation and increased seasonal humidity that is associated with spring and summer storms. We predict that annual dripping will decrease during years with drier, hotter spring and summers. Therefore, cooler, wetter spring and summer periods represent greater effective moisture and increased drip rates in these caves. Exceptionally wet winters should also translate to faster drip rates as well, but this did not occur during our study period. A direct link between precipitation and drip rate is not necessary, but may play a small role as well.

ACKNOWLEDGMENTS

Our engineer, the late Bob Macy designed and built our drip monitors. Installation, drip water collection and collection of stalagmites BC2 and BC7 were permitted through Carlsbad Caverns National Park. We thank D. Pate, S. Allison, T. Bemis and P. Burger for coordination and field support during this study. We also thank Z. Rasmussen and P. Provencio for field assistance. This material is based upon work supported under a National Science Foundation Graduate Research Fellowship (J.B.T.R.), and by NSF grant ATM-0214333 (V.P. and Y.A.). We are grateful to three anonymous reviewers who helped improve this paper.

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Bacterial diversity associated with mineral substrates and hot springs from caves and tunnels of the Naica Underground System (Chihuahua, Mexico)

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Abstract: The Naica Underground System (NUS) in Northern Mexico comprises a lead, zinc, and silver producing mine and displays the largest gypsum crystals ever found in natural caves. The caves are now closed to the public and mining activities have been suspended for an undefined period since October 2015. Besides its geological, economical, and tourist importance, the bacterial diversity in the NUS has not been fully explored yet. This study surveyed for bacteria present on different mineral substrates (gypsum crystals, iron oxide crusts) and hot spring samples collected before the NUS was inaccessible, using culture-dependent and culture–independent (PCR-DGGE) methods. This study is the first reporting the isolation of microorganisms from Naica. Cluster analysis of DGGE fingerprints revealed slight differences between communities from caves and tunnels and according to their mineral substrate type while communities from solid substrates and water samples appeared to be more distant. Both approaches, culture-dependent and independent, revealed the presence of bacteria from the Firmicutes, Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria in gypsum crystals, iron oxide crusts, and hot springs, respectively. Deinococcus-Thermus and Actinobacteria were only detected by DGGE in hot spring samples. According to 16S rRNA sequencing, heterotrophic bacteria isolated under aerobic conditions were affiliated with Bacillus, Brevibacillus, Paenibacillus, Schlegelella, Cupriavidus, Pseudoxanthomonas, and Lysobacter. Most of the isolates and sequences retrieved by DGGE were related to organisms previously detected in other extreme subsurface environments. Some of the isolates were able to precipitate calcium carbonate and precipitate Fe(III) in solid media but their possible participation in biomineralization processes in situ has still to be investigated. Microbial communities found in the NUS are likely autochthonous with some allochthonous components due to human intervention. Their role in geobiological processes requires further investigation.

Keywords: gypsum crystals, iron oxide crusts, hot springs, 16S rRNA, bacterial isolates, Naica

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INTRODUCTION

Deep subsurface environments (natural caves, tunnels, shafts, fractures, aquifers, mine systems) are integral ecosystems of the lithosphere, with exchanges of gases, matter, and life at different rates and magnitudes with surface environments and the atmosphere (Parkes et al., 2005; Barton & Northup, 2007; Parkes et al., 2011; Kallmeyer & Wagner, 2014). These environments bear important resources (water, ores, energy) and have attracted the attention of microorganisms in the last decades because they represent largely unknown pools of microbes with interesting biological activities and potential...
applications in mineral processing, exploration and bioremediation, among others (Colwell & D’Hondt, 2013; Tomova et al., 2013).

Culture-dependent and -independent surveys of deep mines and caves worldwide have revealed the presence of complex and phylogenetically diverse prokaryotic microbial communities, comprising microbes with a variety of metabolic capabilities, such as sulfate-reducers, chemoheterotrophs, hydrogen-oxidizers, thermophiles, and heavy-metal resistant organisms (Moser et al., 2003; Onstott et al., 2003; Barton & Northup, 2007; Zhang et al., 2007a; Rastogi et al., 2009; Engel, 2010; Epure et al., 2014). The microbiota of Mexican subterranean environments has, however, been poorly explored.

In the last century, the Naica Underground System (NUS) located in the State of Chihuahua (Mexico) has been an important source of ores, mainly lead and zinc. Besides its industrial and economic relevance, it is also exceptional for its caves of giant selenite (gypsum, CaSO₄·2H₂O) crystals. Some of these can reach >10 m in length and >1 m in width, being thus the world’s largest gypsum crystals. Other conspicuous and abundant chemical precipitates are also found on the inner walls of the caves. The most common are extensive iron oxide crusts, often mixed with other minerals, such as celestite (SrSO₄) and calcite (CaCO₃). Man-made tunnels provide direct access to the host rock, which is mainly composed of limestone and other minor minerals, such as sulfides, sulfates, as well as iron and manganese oxides (Forti et al., 2009; Forti & Sanna, 2010). The presence of putative biogenic structures in iron oxide crusts suggests that microbes may play a role in the formation or modification of mineral deposits within the NUS (Forti & Sanna, 2010). Ragon et al. (2013) have explored the microbial diversity of hydrothermal waters of the NUS using culture-independent methods. These authors detected a low bacterial and archaeal diversity in these waters and suggested that some of the retrieved sequences corresponded to thermophilic chemolithoautotrophs adapted to energy-limited environments. However, to date, there are no reports concerning the diversity of microorganisms present on solid mineral substrates of the NUS. Furthermore, no isolated microorganisms have been reported from this system, these are particularly valuable for experimentation on their geobiological capabilities.

Therefore, herein, we report on a survey of the bacterial diversity associated with conspicuous solid substrates (gypsum crystals and iron oxide crusts) and hot springs in natural caves and man-made tunnels of the NUS using culture-dependent and -independent methods. This is the first report on culturable bacteria from the NUS, some of them presenting biomineralization activities and, thus, a potential role in minerals transformation.

MATERIALS AND METHODS

Site location and description

Naica’s entrance to the mine opens at 1,385 m a.s.l. and is located on the NE flank of the Sierra de Naica (27.852470°-105.496469°) in Chihuahua, Mexico. Caves and tunnels occur from -120 to -800 m. The underground system formed from dissolution of the enclosing Mesozoic limestone of the Aurora Formation (Marín Herrera et al., 2006; Briceno-Prieto, 2011). The overall geology and hydrology of the NUS is described elsewhere (García-Ruiz et al., 2007; Forti & Sanna 2010; Garofalo et al., 2010; Badino et al., 2011; Briceno-Prieto, 2011).

Sampling

Samplings were carried out in two different events and, for logistical and access permissions constraints within the mine, it was not possible to sample the same sites. So, in 2009, samples were collected from three natural caves at -290 m (Cueva de los Cristales, Ojo de la Reina, and Cueva del Tiburón) while in 2011 samples were collected from four mine tunnels with low or no mining activity at -290, -430, -780, and -790 m (Fig. 1-2, Table 1). Three types of substrates were sampled: a) gypsum crystals growing on the walls, b) iron oxide crusts covering calcareous walls; and c) hot springs (sulfated and calcic type waters) percolating in caves and outflowing hot springs in tunnels. One additional sulfide (galena sphalerite-pyrite) sample was collected from a tunnel wall.

Rock and mineral substrates were sampled by scraping the surface (up to 1 cm deep) with sterile spatulas and chisels, and placing the fragments directly into sterile, 50 ml centrifuge tubes. Hot spring water was collected into sterile plastic containers thoroughly rinsed with the same water before sampling. Nitrile gloves were used at all times, and tools were cleaned and flamed with alcohol before and after each sampling. Samples for cultures were stored at 45°C during transport and until use in the laboratory; samples for DNA extraction were stored at -80°C until use. The mineralogy and chemical composition of the major and minor elements were determined by X-ray diffraction and X-ray fluorescence for each sample type (data not shown).

Sample codes used herein are based on the substrate type (gyp = gypsum; ox = iron oxides; w = water; sulf = sulfide) and collection site (CC = Cueva de los Cristales; OR = Ojo de la Reina; CT = Cueva del Tiburón; Tun = tunnel); numbers indicate depth below ground in meters. Gypsum crystals (gyp) and iron oxide crusts (ox) were collected from the CC, OR and CT caves (gypCC, oxCC, gypOR, oxOR, gypCT, and oxCT, respectively) from outside the CT (gypTun290) and CC caves (oxCCTun290, ox+gypTun290) and outside the Cueva de las Velas (oxTun290) at -290 m (Fig. 2A-D). Additional iron oxide crusts and sulfides (ox+gypTun430 and sulfTun430) were collected at -430 m, and one gypsum crystals sample from a fracture with flowing hot water at -790 m (gypTun790) (Table 1 and Figs. 1A and 2F).

Environmental DNA isolation and PCR-DGGE analysis

Environmental DNA was isolated from 1 g of solid samples or 50 ml of hot spring water, using a commercial isolation kit (UltraClean Soil DNA, 0.625 mg/ml) soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). Extracted DNA was amplified using primers specific for total prokaryota (Eske et al., 2011) and DGGE analyses were performed as described (Ulibarri et al., 2011).
Fig. 1. Latest version (2011) of the NUS and location of sampled sites. A) Cross section of the NUS oriented N80°E, facing NW, showing the mineshafts and tunnels (yellow lines) and the approximate depth of the phreatic level (blue line). The dashed line is an inferred depth. Three sampling locations: -430 m, -780 m and -790 m, are indicated. The box with 'B' corresponds to level -290 m and is shown in the right pane. B) Plan view the -290 m level indicating sample locations. Numbers between brackets indicate the year of collection. See Table 1 for sample codes. Maps were modified from the originals by Ing. Roberto Carlos Reyes-Ramirez and José Manuel Estrada-Conchos, Minera Maple S.A. de C.V.
MoBio Laboratories Inc., Carlsbad CA), following the manufacturer’s instructions. DNA quality and concentration were determined by electrophoresis in agarose gels and by spectrophotometry (ND-2000 Nanodrop spectrophotometer, Thermo Scientific, Wilmington DE). The V3 hypervariable region of the bacterial 16S rRNA was amplified by PCR using the GC-338F (5’- CGC CCG CCG GCG GCG GCG GCG GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG -3’) and Univ529R (5’-ACC GCG GCK GCT GGC-3’) primers (Muyzer et al., 1993). The PCR products from five replicate reactions were pooled, purified and concentrated into a final volume of 30 µl using a DNA Clean & Concentrator kit (Zymo Research Co., Irvine CA). The purified DNA (~150 ng) was used for DGGE analysis in a Dcode System apparatus (Bio-Rad Co, Hercules CA). The experimental conditions for DGGE were as follows: 8% (w/v) polyacrylamide gels with a 40-60% urea-formamide denaturing gradient, electrophoresis in 1X Tris-acetate EDTA (TAE) buffer at 60°C with a voltage of 55 V during 16 h and silver staining following the Bio-Rad silver stain protocol [http://www.bio-rad.com/LifeScience/pdf/Bulletin_9057.pdf].

Fig. 2. General aspect of the sampled mineral surfaces and hot spring: A) giant Selenite (gypsum) crystals in Cueva de los Cristales [CC]; B) Close up of cave walls showing selenite crystals (gypCT) and well consolidated iron oxide crusts (oxCT) from CT; C) Semi-soft iron oxide deposit from a tunnel (oxTun290); D) Soft and hard iron oxide crusts with gypsum and carbonates from a tunnel (oxCCTun290); E) Outflowing (arrow) hot spring from a tunnel (wTun430); F) Sulfide mineral deposit (galena-sphalerite-pyrite) from a tunnel (sulfTun430). See Table 1 for sample codes.

Clustering analysis of DGGE fingerprints and sequencing of DGGE bands

Digitized images of DGGE gels were used to quantify the number of bands per lane and their horizontal position with respect to neighboring bands using the ImageJ software (Rasband, 2012). Presence-absence matrices were then built from the DGGE banding patterns and used to calculate their hierarchical clustering using the Pearson correlation coefficient for similarity and complete linkage with the XLSTAT plugin v 2009.3.02 software for Microsoft Excel [www.xlstat.com]. Selected bands were excised with sterile scalpels and soaked into 50 µl of autoclaved MilliQ water overnight at 4°C for DNA elution. The eluted DNA was reamplified and purified as described above and sent out for sequencing. Retrieved sequences were compared with those available at the GenBank and Greengenes databases using the BLAST algorithm.

Isolation and identification of bacteria

Bacteria were isolated in the laboratory from 1 g of crushed solid samples or 1 ml of aqueous samples, dissolved into 9 ml of buffered saline phosphate (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) adjusted to pH 7.4. The suspensions were incubated for 48 h at 45°C. Serial dilutions were spread onto solid media by the extension method using sterile
Table 1. Type of samples collected at the NUS.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Sample code*</th>
<th>Collection year</th>
<th>Depth (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gypsum crystal</td>
<td>gypCC</td>
<td>2009</td>
<td>-290</td>
</tr>
<tr>
<td>Gypsum crystal</td>
<td>gypOR</td>
<td>2009</td>
<td>-290</td>
</tr>
<tr>
<td>Gypsum crystal</td>
<td>gypCT</td>
<td>2009</td>
<td>-290</td>
</tr>
<tr>
<td>Gypsum crystal</td>
<td>gypTun290</td>
<td>2011</td>
<td>-290</td>
</tr>
<tr>
<td>Gypsum crystal</td>
<td>gypTun790</td>
<td>2011</td>
<td>-790</td>
</tr>
<tr>
<td>Oxide crusts</td>
<td>oxCC</td>
<td>2009</td>
<td>-290</td>
</tr>
<tr>
<td>Oxide crusts</td>
<td>oxOR</td>
<td>2009</td>
<td>-290</td>
</tr>
<tr>
<td>Oxide crusts</td>
<td>oxCT</td>
<td>2009</td>
<td>-290</td>
</tr>
<tr>
<td>Oxide crusts+carbonates</td>
<td>oxCCTun290</td>
<td>2011</td>
<td>-290</td>
</tr>
<tr>
<td>Oxide crusts+gypsum</td>
<td>ox+gypTun290</td>
<td>2011</td>
<td>-290</td>
</tr>
<tr>
<td>Oxide crusts+gypsum</td>
<td>ox+gypTun430</td>
<td>2011</td>
<td>-430</td>
</tr>
<tr>
<td>Metal sulphides</td>
<td>sulfTun430</td>
<td>2011</td>
<td>-430</td>
</tr>
<tr>
<td>Hot spring water*1</td>
<td>wTun430</td>
<td>2011</td>
<td>-430</td>
</tr>
<tr>
<td>Hot spring water*2</td>
<td>wTun780</td>
<td>2011</td>
<td>-780</td>
</tr>
<tr>
<td>Percolated water</td>
<td>wCC</td>
<td>2009</td>
<td>-290</td>
</tr>
<tr>
<td>Percolated water</td>
<td>wOR</td>
<td>2009</td>
<td>-290</td>
</tr>
</tbody>
</table>

*Codes are based on the substrate type/collection site: substrate type (See Materials and Methods).

In situ measurements of sampled hot spring water at tunnels: *1) T (ºC) = 51.9, pH = 6.81, EC (mS) = 2.97, TDS (µg/l) = 1.48. *2) T (ºC) = 52.0, pH = 7.29, EC (mS) = 3.08, TDS (µg/l) = 1.54.

RESULTS

Substrates characteristics

The sampled gypsum crystals were eudrad, colorless, with tabular habit (Fig. 2A-B). Only the gypTun790 sample was mixed with carbonates. Iron oxide crusts were often mixed with carbonate and sulfate fragments from the walls of caves and tunnels (Fig. 2C-D). Iron crusts from the CC and CT caves were thicker (up to about 1 cm) compared to those from OR (which only consisted of a thin iron patina on the host rock). It is assumed that the iron oxide crusts found at -290 m were deposited before the giant gypsum crystals formed (Forti et al., 2009; Briceño-Prieto, 2011). Environmental parameters of the NUS, including temperature and relative humidity, have already been thoroughly studied (Badino, 2009; Badino et al., 2011). According to these authors, the NUS is characterized by a high relative humidity (>80%) and temperatures above 44°C. Although the mining activities inside the NUS have provoked a decrease in air temperature due to ventilation, this system is therefore still considered a thermophilic environment. The temperature of sampled hot spring water sources in the tunnels at the -430 m and -780 m levels were around 52°C (Table 1) and their pH was 6.81 and 7.29, respectively. Chemical characterization of the water has been previously reported showing that it is saturated with calcium sulfate and carbonates (Garcia-Ruiz et al., 2007; Briceño-Prieto, 2011).
Analysis of DGGE banding patterns

Differences in DGGE band positions (Figs. 3A and 4A), resulted in distance matrices that separated samples collected in caves and tunnels as shown by the obtained dendrograms (Fig. 3B and 4B). The community profiles presented low likeness (<50%) among clusters in their main branches, indicating heterogeneity between sampled substrates (gypsum crystals, iron oxide crusts, and hot springs). The grouping of hot spring samples was more cohesive regardless of their source (caves or tunnels). Interestingly, cluster analysis of the different mineral substrates and hot spring water from caves (Fig. 3B) segregated samples from the CT cave, which is a smaller hidden cave, without steel doors and more exposed to the effects of detonations and mining activities than the CC or OR caves (Fig. 1B). Likewise, the differences observed in the banding pattern of samples taken in CC and adjacent tunnels (Fig. 4B), could be due to periodic disturbances by tourism or mining activities. In addition, DGGE fingerprints displayed at least eight common band positions in all the samples despite their dissimilar substrate source and location (Fig. 3A and 4A), suggesting that they represented ubiquitous bacteria. The number of bands per sample ranged from 6 to 28 with several similar horizontal band positions across lanes. The gypTun790 sample, located under an outflowing hot spring, had the highest number of bands of all solid samples. This sample was possibly ‘enriched’ with bacteria from the water. In general the number of bands was higher for hot spring water samples and intermediate for iron oxide crusts, despite these being very porous (allowing for biomass accumulation) and of probable biogenic origin (Forti et al., 2009; Forti & Sanna, 2010).

Sequence analysis of DGGE bands

A total of 43 sequences were obtained from representative bands excised from the DGGE gels. The obtained sequences were related to 12 bacterial genera belonging to 6 bacterial Phyla: Actinobacteria (Propionibacterium sp.), Deinococcus-Thermus (Meiothermus sp.), Firmicutes (Bacillus sp. and Staphylococcus sp.), Alphaproteobacteria (Sphingomonas sp. and Hyphomicrobium sp.), Betaproteobacteria (Delftia sp. and Comamonas sp.), and Gammaproteobacteria (Acinetobacter sp., Halomonas sp., Pseudomonas sp., and Hydrocarboniphaga sp.) (Table 2). These OTUs were present in both sampling campaigns except Delftia (Betaproteobacteria), which was only retrieved from iron oxide crusts inside caves CC and CT (collected in 2011). Common bands for all types of samples (e.g. bands B-14b, A-12, B-9, B-20, B-29, B-26, B-23c, B-24, B-25b, B-27, B-55a,b,c, B-56, B-57) (Figs. 3A-B and Table 2) corresponded to Comamonas, Acinetobacter, Hydrocarboniphaga and Bacillus, indicating a continuous presence of these bacterial genera in the NUS despite samples being collected two years apart and in different locations inside the NUS. The sequences retrieved from calcium sulfate-rich substrates (either gypsum crystals or hot springs) mainly corresponded to Sphingomonas and Meiothermus. Less common sequences corresponded to Hyphomicrobiurn in the gypTun790 sample, Delftia in iron oxide crusts from caves, Staphylococcus in the gypCC sample, Propionibacterium in some gypsum crystals and hot spring water samples, and some uncultured bacteria (Table 2). The bands corresponding to Meiothermus, Pseudomonas and Halomonas were only present in hot spring water samples (wTun430 and wTun780) and in a gypsum sample under a hot spring water seepage (gypTun790).

Isolation and identification of bacteria

Several heterotrophic bacterial isolates were obtained in pure culture from gypsum crystals, iron oxide crusts
Fig. 4. DGGE fingerprint and corresponding cluster analysis from Cueva de los Cristales and mine tunnels. A) DGGE fingerprint of samples from Cueva de los Cristales and mine tunnels. Std corresponds to the 100 bp molecular weight marker. Excised bands are indicated to the left of each band and with arrows; B) Dendrogram obtained from the cluster analysis of DGGE fingerprint. Green lines show clusters of solid substrates (gypsum crystals and iron oxides crusts) from caves. Red lines show clusters of solid and hot springs water samples. Blue lines show clusters of hot springs water samples (see Methods for details). See Table 1 for sample codes.

(or gypsum with iron oxides), and hot spring water samples (Table 3). Based on their 16S rRNA sequences, the isolates grouped within the phyla Firmicutes, Betaproteobacteria and Gammaproteobacteria (Table 3). The Firmicutes included Bacillus subtilis, Bacillus licheniformis, Bacillus oceanosediminis and Paenibacillus sp. Firmicutes were isolated from all sample types. The Betaproteobacteria included Schlegelella aquatica and Cupriavidus sp. Schlegelella isolates were retrieved from gypsum and hot spring water samples but not from iron oxide crusts while Cupriavidus isolates were only obtained from iron oxide crusts. The Gammaproteobacteria included Pseudoxanthomonas taiwanensis and Lysobacter thermophilus obtained from hot spring water and gypsum crystals, respectively.

Table 2. Phylogenetic affiliations of DNA sequences retrieved in DGGE bands.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Phyla</th>
<th>Identity* (%)</th>
<th>Environmental source of blasted match</th>
<th>Accession Number*</th>
<th>Band*</th>
</tr>
</thead>
<tbody>
<tr>
<td>wCC</td>
<td>Actinobacteria</td>
<td>97</td>
<td>Clone. Alkaline hot spring sediment</td>
<td>HG797141</td>
<td>A-25a</td>
</tr>
<tr>
<td>gypTun790</td>
<td>Propionibacterium acnes</td>
<td>100</td>
<td>Strain. Ditch sludge</td>
<td>HG797149</td>
<td>B-14a</td>
</tr>
<tr>
<td>wTun430</td>
<td>Propionibacterium acnes</td>
<td>100</td>
<td>Strain. Ditch sludge</td>
<td>HG797165</td>
<td>B-50b</td>
</tr>
<tr>
<td>wCC</td>
<td>Deinococcus-Therms</td>
<td>98</td>
<td>Strain. Hot spring water</td>
<td>HG797138</td>
<td>A-19</td>
</tr>
<tr>
<td>wTun430</td>
<td>Meiothermus timidus (AJ871170)</td>
<td>99</td>
<td>Hot spring water</td>
<td>HG797163-64</td>
<td>B-49/B-50a</td>
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<tr>
<td>wCC</td>
<td>Meiothermus sp. (FN666194)</td>
<td>99</td>
<td>Uncultured. Geothermal springs</td>
<td>HG797139</td>
<td>A-20</td>
</tr>
<tr>
<td>gypCC</td>
<td>Staphylococcus sp. (X86633)</td>
<td>99</td>
<td>Strain. Deep subsurface environment</td>
<td>HG797130</td>
<td>A-1</td>
</tr>
<tr>
<td>oxOR</td>
<td>Bacillus sp. (FJ889612)</td>
<td>100</td>
<td>Strain. Psychro tolerant from sea</td>
<td>HG797145</td>
<td>A-31</td>
</tr>
<tr>
<td>ox+gypTun290</td>
<td>Bacillus sp. (KC809940)</td>
<td>100</td>
<td>Strain. Heavy metal-contaminated soil</td>
<td>HG797153-55</td>
<td>B-23c/B-24/B-25a</td>
</tr>
<tr>
<td>ox+gypTun290</td>
<td>Bacillus sp. (KC809940)</td>
<td>99</td>
<td>Strain. Heavy metal-contaminated soil</td>
<td>HG797156,58</td>
<td>B-25/B-27</td>
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<tr>
<td>wTun430</td>
<td>Bacillus sp. (KF561879)</td>
<td>99</td>
<td>Strain. Fresh water sediment Mn(ll)ox.</td>
<td>HG797161</td>
<td>B-43</td>
</tr>
<tr>
<td>wTun780</td>
<td>Bacillus sp. (KF576647)</td>
<td>100</td>
<td>Strain. Marine from xenobiotic sediment</td>
<td>HG797169</td>
<td>B-55b</td>
</tr>
<tr>
<td>wTun780</td>
<td>Bacillus pseudofirmus (HG513117)</td>
<td>100</td>
<td>Strain. Extremophile from soda lake</td>
<td>HG797168.</td>
<td>B-55a/B-56</td>
</tr>
</tbody>
</table>
Calcium carbonate and iron(III) precipitation by bacterial isolates

Calcium carbonate-precipitating isolates formed visible crystals or flakes within or around colonies after a week of incubation at 45°C in solid medium supplemented with CaCl₂. All the Bacillus isolates could precipitate copious amounts of calcium carbonate as well as the Pseudoxanthomonas, Lysobacter, and Cupriavidus isolates, although to a much lesser extent in the case of Schlegelella and Cupriavidus. Brevibacillus and Paenibacillus could not precipitate calcium carbonate under the tested conditions. Some heterotrophic bacteria may precipitate poorly crystalline Fe(III)-oxides via the utilization of carbon from Fe (III) organic complexes (in this case from ferric ammonium citrate) (Tuhela et al., 1993). All the Bacillus, Brevibacillus, and Cupriavidus strains could produce Fe(III) precipitates evidenced by a red-brown color in solid medium amended with ferric ammonium citrate (Fig. 5). Paenibacillus, Pseudoxanthomonas and Schlegelella isolates did not produce Fe(III) precipitates under these conditions.

Lysobacter did not grow in the medium amended with ferric ammonium citrate.

**DISCUSSION**

Natural caves and other subsurface environments are considered oligotrophic and in these systems, energy sources and nutrients for microbial growth normally enter as gases and condensates, soil-derived aromatic and polyaromatic compounds, groundwater, and metal ions dissolved from rocks and minerals (Mulec, 2008). This energy and nutrient input can dramatically change due to human impact via polluted underground or percolating waters, infiltrations from impacted soils or air contaminated during tourist visitation and mining activities, all of which can carry allochthonous organic and inorganic matter and new microbial populations, altering indigenous microbial communities (Onstott et al., 2003, Mulec, 2008, Chelius et al., 2009, Rastogi et al., 2009, Adetutu et al., 2012, Griffin et al., 2014). The active exploitation of the Naica Mine initiated
in 1900 and groundwater pumping started in the 1970s (Marin Herrera et al., 2006) exposing rocks and minerals below 150 m. After the giant crystals were discovered at the -290 m level in year 2000, human presence became more frequent inside the caves and adjacent tunnels, followed by nearly four years (2006-2009) of intense documentary filming activities (Badino, 2009). These activities at different depths probably promoted contamination from the surface to the subsurface and across natural caves and artificial tunnels, influencing microbial diversity and distribution. The existence of human-associated bacteria in caves has been noted before (Campbell et al., 2011) and could therefore be expected in the NUS, especially in locations with continuous human visits or mining activities in touristic caves and tunnels. The occasional presence of crickets and bats may also be of importance. Thus, in contrast to Ragon et al. (2013) who suggested that only indigenous microbes thrived in a deep subsurface aquifer of the NUS, the bacterial communities analyzed here seemed to be

mixed communities of both native and allochthonous organisms.

The clustering analysis of DGGE profiles showed a segregation of bacterial communities from caves and tunnels, and a correlation according to their substrate, suggesting that the bacterial community composition was similar between substrates of the same type (gypsum crystals, iron oxide crusts or hot springs), despite such samples being collected at different times, depths and locations. Similar banding patterns for samples collected two years apart suggest little or no variation in the community composition regardless of their substrate, even with continuous and ongoing mining activities and tourist visitations. This seems consistent with a relatively steady environment expected for underground systems (Barton & Northup, 2007). Combined environmental (temperature, water chemistry) and geochemical (types of minerals) factors may influence the development and overall distribution of microbial communities in artificial tunnels and natural caves in the NUS. The main

### Table 3. Summary of bacterial isolates retrieved from the NUS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phyla</th>
<th>Closest matcha</th>
<th>Identity (%)</th>
<th>Environmental source of blasted matchb</th>
<th>Accession Numberc</th>
<th>Max growth</th>
<th>Strain code</th>
<th>Precipitation tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>gypCC</td>
<td>Firmicutes</td>
<td>Bacillus subtilis (NR_025198)</td>
<td>100</td>
<td>Strain. Lead and zinc mine</td>
<td>MG282294</td>
<td>55</td>
<td>Cc.1.1a</td>
<td>✓</td>
</tr>
<tr>
<td>oxCC</td>
<td>Firmicutes</td>
<td>Bacillus subtilis (NR_109621)</td>
<td>100</td>
<td>Strain. Soil with petroleum</td>
<td>MG282296</td>
<td>55</td>
<td>Cc.1.15</td>
<td>✓</td>
</tr>
<tr>
<td>wCC</td>
<td>Firmicutes</td>
<td>Bacillus subtilis (KM8322599)</td>
<td>100</td>
<td>Strain. Soil</td>
<td>MG282293</td>
<td>55</td>
<td>Ca.1.7b</td>
<td>✓</td>
</tr>
<tr>
<td>oxCC</td>
<td>Firmicutes</td>
<td>Schlegelella licheniformis (HQ143568)</td>
<td>99</td>
<td>Strain. Soil</td>
<td>MG282295</td>
<td>58</td>
<td>Cc.1.5</td>
<td>✓</td>
</tr>
<tr>
<td>wCC</td>
<td>Firmicutes</td>
<td>Schlegelella licheniformis (KM893455)</td>
<td>99</td>
<td>Strain. Contaminated soil</td>
<td>MG282292</td>
<td>58</td>
<td>Ca.1.1</td>
<td>✓</td>
</tr>
<tr>
<td>ox+gypTun430</td>
<td>Firmicutes</td>
<td>Bacillus oceaniensis (KX007772)</td>
<td>99</td>
<td>Strain. Soil from gold mine</td>
<td>MG282302</td>
<td>55</td>
<td>NO.4.4</td>
<td>✓</td>
</tr>
<tr>
<td>ox+gypTun430</td>
<td>Firmicutes</td>
<td>Brevibacillus limnophilus (NR_024822)</td>
<td>99</td>
<td>Strain. Thermophilic</td>
<td>MG282301</td>
<td>45</td>
<td>NO.4.2</td>
<td>✓</td>
</tr>
<tr>
<td>ox+gypTun430</td>
<td>Firmicutes</td>
<td>Paenibacillus sp. (CP013653)</td>
<td>99</td>
<td>Strain. Soil-Thermophile</td>
<td>MG282304</td>
<td>58</td>
<td>Oy.4.3</td>
<td>-</td>
</tr>
<tr>
<td>gypTun790</td>
<td>Firmicutes</td>
<td>Schlegelella aquatica (FR774570)</td>
<td>100</td>
<td>Strain. Paper mill</td>
<td>MG282291</td>
<td>50</td>
<td>Cc.1.1</td>
<td>✓</td>
</tr>
<tr>
<td>gypTun790</td>
<td>Firmicutes</td>
<td>Schlegelella aquatica (FR774570)</td>
<td>100</td>
<td>Strain. Paper mill</td>
<td>MG282298</td>
<td>50</td>
<td>NC.7.1</td>
<td>✓</td>
</tr>
<tr>
<td>wTun430</td>
<td>Firmicutes</td>
<td>Schlegelella aquatica (FR774570)</td>
<td>99</td>
<td>Strain. Paper mill</td>
<td>MG282286</td>
<td>50</td>
<td>A.4.1</td>
<td>✓</td>
</tr>
<tr>
<td>wTun430</td>
<td>Firmicutes</td>
<td>Schlegelella sp. (FR774567)</td>
<td>99</td>
<td>Strain. Paper mill</td>
<td>MG282287</td>
<td>50</td>
<td>A.4.4</td>
<td>✓</td>
</tr>
<tr>
<td>wTun430</td>
<td>Firmicutes</td>
<td>Schlegelella sp. (FR774567)</td>
<td>99</td>
<td>Strain. Paper mill</td>
<td>MG282290</td>
<td>50</td>
<td>A.4.9</td>
<td>✓</td>
</tr>
<tr>
<td>wTun430</td>
<td>Firmicutes</td>
<td>Schlegelella sp. (FR774567)</td>
<td>100</td>
<td>Strain. Paper mill</td>
<td>MG282297</td>
<td>58</td>
<td>NA.4.3</td>
<td>✓</td>
</tr>
<tr>
<td>oxTun290</td>
<td>Firmicutes</td>
<td>Cupriavidus taiwanensis (EU915711)</td>
<td>95</td>
<td>Strain. Without source information</td>
<td>MG282303</td>
<td>50</td>
<td>OV.2.1</td>
<td>✓</td>
</tr>
<tr>
<td>oxTun290</td>
<td>Firmicutes</td>
<td>Cupriavidus taiwanensis (EU915711)</td>
<td>95</td>
<td>Strain. Without source information</td>
<td>MG282300</td>
<td>50</td>
<td>NOV.2.5</td>
<td>✓</td>
</tr>
<tr>
<td>wTun430</td>
<td>Firmicutes</td>
<td>Pseudoxanthomonas taiwanensis (NR_025198)</td>
<td>100</td>
<td>Strain. Thermophilic from hot springs</td>
<td>MG282288</td>
<td>55</td>
<td>A.4.5a</td>
<td>✓</td>
</tr>
<tr>
<td>wTun430</td>
<td>Firmicutes</td>
<td>Pseudoxanthomonas taiwanensis (NR_025198)</td>
<td>99</td>
<td>Strain. Thermophilic from hot springs</td>
<td>MG282289</td>
<td>55</td>
<td>A.4.5m</td>
<td>✓</td>
</tr>
<tr>
<td>gypTun790</td>
<td>Firmicutes</td>
<td>Lyssobacter thermophilus (NR_109621)</td>
<td>99</td>
<td>Strain. Geothermal soil</td>
<td>MG28222</td>
<td>55</td>
<td>NC.7.4a</td>
<td>✓</td>
</tr>
</tbody>
</table>

a Accession numbers of the closest match in GenBank
b Accession numbers of the isolates in GenBank
c Growth in liquid nutrient media; NG = No Growth

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disadvantages of DGGE are that it sometimes detects only the top 1% population (Muyzer et al., 1993); intra-specific and intra-isolate microheterogeneity in ribosomal sequences can produce different bands for a same bacterial species (Nakatsu et al., 2000); different DNA sequences of different bacterial species can migrate at the same position (Muyzer et al., 2004). However, DGGE fingerprints are advantageous when multiple samples are processed and are suitable for comparing patterns across different sites, allowing an exploratory approach and a quick “view” of microbial communities composition (Fry et al., 2006; Cleary et al., 2012). Although only a small proportion of bacterial taxa can be successfully retrieved by PCR-DGGE techniques, the diversity recovered here provides valuable information about the bacteria present on different substrates, depths, locations and environmental conditions at the NUS.

Concerning the Firmicutes, Bacillus species were detected by DGGE and isolated from all types of samples (gypsum crystals, iron oxide crusts, and water samples) at different sampling sites in the NUS. Nevertheless, Bacillus spp. were not detected by Ragon et al. (2013) although these authors detected 2 OTUs close to Paenibacillus, which was isolated in the present study from an iron oxide crust sample together with Brevibacillus sp. Under laboratory conditions the Bacillus isolates precipitated Fe(III) and copious amounts of calcium minerals. Bacillus spp. are known for their ability to precipitate calcium-containing minerals (calcite, aragonite and gypsum) both in natural environments and under laboratory conditions (Barabesi et al., 2007; Baskar et al., 2009; Achal et al., 2010; López-Moreno et al., 2014). It has been suggested that Bacillus may induce calcite precipitation in speleothems and gypsum in cave walls (Baskar et al., 2009). Additionally, both Paenibacillus and Brevibacillus have been previously reported from Fe(III)-reducing microbial communities in Uranium(VI) contaminated sediments (North et al., 2004). Particularly, Paenibacillus sp. has the ability to reduce soluble Fe(III) complexes (Ahmed et al., 2012) and may play a similar role in the NUS. Another Firmicutes, Staphylococcus sp., was detected by DGGE in the gypCC sample from the CC cave (the most visited cave). Staphylococcus has also been previously reported in caves and mines (Zhang et al., 2007a; Griffin et al., 2014). Bacillus and Staphylococcus have long been considered as human indicator bacteria in caves together with Escherichia coli (Lavoie & Northup, 2006). Staphylococcus belongs to the list of genera of human origin according to Human Microbiome Project Consortium and has been repeatedly associated to human contamination in cave environments (Griffin et al., 2014; Leuko et al., 2017). Thus, the fact that several of these Firmicutes were detected may reflect a degree of human disturbance. However, concerning Bacillus, this genus has also been considered indigenous in other studies (Spilde et al., 2005; Mulec, 2008; Baskar et al., 2009; Adetutu et al., 2012). For instance, halotolerant B. licheniformis isolates have been obtained from subsurface environments (Yakimov et al., 1995) including thermotolerant varieties. Bacillus sp. exhibiting high tolerance to Pb toxicity has been detected in mine tailings (Zhang et al., 2007b; Govarthanan et al., 2013) and has also been associated with biomineralization processes (bioaccumulation and solubilization) of metallic ions such as copper, lead, cadmium and zinc (Zastrow & Straube, 1991; da Costa & Duta, 2001). The presence of Bacillus sp. and their potential role in biogeochemical processes in the NUS needs to be further investigated.

Here, Hyphomicrobium sp. and Sphingomonas sp. (Alphaproteobacteria) were found by PCR-DGGE in gypsum crystals ([gyp Tun290] and gypCC samples, respectively) as well as in hot spring water at -430 m for Sphingomonas. Hyphomicrobium has been previously detected in ferromanganese deposits in cave crusts and stromatolites, where it is thought to influence iron/manganese redox processes (Northup et al., 2003; Engel, 2010; Lozano & Rossi, 2012). It has also been found as a major component of bacterial communities in moonmilk deposits, a cave and mine deposit constituted by hydrated carbonate crystals (Portillo & Gonzalez, 2011)]. Sphingomonas has also been found in water pools and sediments of caves and mines (Moser et al., 2003; Campbell et al., 2011; Adetutu et al., 2012). Although Ragon et al. (2013) did not detect Hyphomicrobium, they found several OTUs related to Paracoccus and Sphingomonas, this last one consistent with our results. As Hyphomicrobium, Paracoccus is a metabolically versatile bacterium capable of methylotrophy, chemolithotrophy on sulfur and denitrification, all of which are relevant metabolic processes in cave ecosystems (Kumaresan et al., 2015).

The genus Delftia sp. (Betaproteobacteria), detected by DGGE in iron oxide crusts in the CC and CT caves, was also reported by Ragon et al. (2013) in hot spring water. A Delftia strain capable of zinc and lead biosorption was isolated from mine tailings, which is a
related environment (Bautista-Hernández et al., 2012) and this genus seems to be common in natural caves (Barton et al., 2007). Coincidentally, *Cupriavidus* isolates, previously *Ralstonia* (*Betaproteobacteria*), capable to produce Fe(III) precipitates in solid medium at 45°C were obtained from iron oxide crusts at the -290 m site. *Ralstonia* has been isolated from highly human impacted rock surfaces in a limestone cave (Iknner et al., 2007). Both *Delftia* and *Cupriavidus* are well known for their resistance to heavy metals and their ability to produce siderophores (Diels et al., 2009; Ubalde et al., 2012). *Delftia* was also reported to play a role in the formation of gold nuggets together with *Cupriavidus metallidurans* (Reith et al., 2010). Another *Betaproteobacteria, Comamonas* sp., was detected here by DGGE in a gypsum crystal sample located under a hot spring outflow. *Comamonas* have been found in speleosols (cave soils) and associated to iron, manganese, lead, cadmium and other metallic deposits (Spilde et al., 2005; Barton et al., 2007; Zhang et al., 2007b). Finally, within the *Betaproteobacteria*, two isolates of *Schlegelella* were obtained from gypsum crystals and hot spring waters that can precipitate calcium minerals under laboratory conditions at 45°C. The *Schlegelella* genus comprises two moderately thermophilic species (optimum growth at 45-50°C): *S. thermodepolymerans* (Elbanna et al., 2003) and *S. aquatica* (Chou et al., 2006) recovered from activated sludge under thermophilic conditions and a hot spring, respectively. To our knowledge, *Schlegelella* has not been reported from caves before and Ragon et al. (2013) did not retrieve this genus in their 16S clone library study although the characteristics of this microorganism are consistent with a hot spring environment. In their study, Ragon et al. (2013) report that *Bacteria* were dominated by *Betaproteobacteria*, which is somehow consistent with the present DGGE and culturing results.

Several *Gammaproteobacteria* were detected by PCR-DGGE, as for example, *Hydrocarboniphaga* sp. in an iron oxide-gypsum sample and *Acinetobacter* sp. in iron oxide crusts and gypsum in caves and tunnels. These genera have been previously found in subsurface environments (Jimenez, 1990; Webster et al., 2006; Barton et al., 2007; Zhang et al., 2007b; Jones et al., 2008; Adetutu et al., 2012; Rurszynák et al., 2012) being *Acinetobacter* the most frequently reported, generally in non-disturbed areas of caves, *i.e.*, inaccessible to tourists (Adetutu et al., 2012; Rurszynák et al., 2012; Griffin et al., 2014) although some authors also report it as a potential pathogen possibly translocated into caves by human and animal visitors (Tomova et al., 2013). *Halomonas desiderata* (detected in gypsum crystals by DGGE) is a moderately halophilic bacterium with ability to precipitate magnesium and calcium carbonates (Rivadeneyra et al., 1998), both widespread minerals in the NUS. *Pseudomonas* sp. (also detected by DGGE in gypsum crystals and hot springs) is a cosmopolitan, aerobic metal tolerant bacterium that has also been found in caves and mines (Jimenez, 1990; Cho et al., 2003; Onstott et al., 2003; Spilde et al., 2005; Zhang et al., 2007a; Adetutu et al., 2012; Rurszynák et al., 2012) and associated to the formation of moonmilk (Portillo & Gonzalez, 2011). Some studies have reported the presence of *Pseudomonas* in sites impacted by human visitation (Iknner et al., 2007; Griffin et al., 2014) while others detected it in pristine caves (Rurszynák et al., 2012) and in association to manganese oxide deposits deep into caves (Leuko et al., 2017). Several *Gammaproteobacteria*, were isolated here, namely *Pseudoaxanthomonas* and *Lysobacter* species, from hot spring samples and from a gypsum crystal under hot spring flow, respectively. *Pseudoaxanthomonas taiwanensis* and *Lysobacter thermophilus* are thermophilic species already isolated from hot springs (Chen et al., 2002) and geothermal soils (Wei et al., 2012) which coincide with some of the environmental parameters found in the NUS. *Lysobacter* has been isolated from cave environments both from high and low human impact sites (Iknner et al., 2007; Herzog Velijonka et al., 2014). Both the *Pseudoaxanthomonas* and *Lysobacter* isolates could precipitate calcium minerals and grow at 45-55°C under laboratory conditions. These features make the presence of these two genera consistent with the environmental conditions at the NUS, with interesting biogeochemical potential in this system. Contrastively, Ragon et al. (2013) did not detect any *Gammaproteobacteria* in their study.

The genus *Meiothermus* (*Deinococcus-Thermus*) was only detected in water samples (wCC and wTun430). It is a moderately thermophile (50-65°C) heterotroph, generally found in neutral and alkaline hot springs. It has been associated to the formation of speleothems in caves, geothermal mines (Spear et al., 2007), and geothermal steam vents (Benson et al., 2011). Finally, members of the *Actinobacteria* (detected in gypsum crystals and hot springs in caves and tunnels) have also been found in caves and mines (Zhang et al., 2007a; Rastogi et al., 2009), and may have a role in carbonate precipitation (Cuezva et al., 2012). The genus *Propionibacterium* sp. has been found associated with biospeleogenesis at a sulfide rich cave hot spring (Barton & Luizier, 2005) and moonmilk deposits (Portillo & Gonzalez, 2011). *Propionibacterium* is a major inhabitant of the human adult skin that belongs to the list of genera of human origin in caves (Griffin et al., 2014; Leuko et al., 2017). Again, in contrast, members of the *Deinococcus-Thermus* and *Actinobacteria*, as, those detected here, were not reported by Ragon et al. (2013).

**CONCLUSIONS**

PCR-DGGE techniques and culturing showed that bacteria are conspicuous throughout the NUS and revealed the presence of bacteria affiliated to the *Actinobacteria, Deinococcus-Thermus, Firmicutes, Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria* in mineral substrates and hot springs water samples in caves and tunnels at different depths and locations of the NUS. Bacteria within the *Deinococcus-Thermus* and *Actinobacteria* lineages were only detected by DGGE in hot springs water samples.

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Most of these results are somehow consistent with those obtained by Ragon et al. (2013) considering different sampling events, substrates and depths within the NUS and the different methodology used (16S clone library). Ragon et al. (2013) focused on the microbial diversity of saline hydrothermal waters at -700–760 m and detected bacteria belonging to the Candidate Phyla OP3, Firmicutes, Alpha, and Betaproteobacteria. They did not find Gammaproteobacteria and concluded that most of the detected lineages appeared to be autochthonous. The microbial ecosystem of the NUS is probably not so isolated and microbial communities in Naica may contain both indigenous and allochthonous organisms, considering that natural caves and human-made tunnels have been impacted by human activities. Most of the detected bacteria (isolates or DGGE bands) were related to organisms previously found in subsurface environments, hydrothermal springs, and heavy metal-contaminated mine tails. The ability of the obtained isolates to precipitate calcium and Fe(III) minerals may indicate a possible contribution in biomineralization processes inside the NUS. However, the influence of the detected bacteria in the formation and alteration of minerals and groundwater chemistry in the NUS is not known and requires further studies.

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Extremely high diversity of sulfate minerals in caves of the Irazú Volcano (Costa Rica) related to crater lake and fumarolic activity

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Abstract: The caves of the Irazú volcano (Costa Rica), became accessible after the partial collapse of the NW sector of the Irazú volcano in 1994, offering the opportunity to investigate active minerogenic processes in volcanic cave environments. We performed a detailed mineralogical and geochemical study of speleothems in the caves Cueva los Minerales and Cueva Los Mucolitos, both located in the northwest foothills of the main crater. Mineralogical analyses included X-ray diffraction (XRD) and Raman spectroscopy, while geochemical characterization used Energy Dispersive X-ray spectroscopy (EDX) coupled to Scanning Electron Microscopy (SEM). In addition, measurements of environmental parameters in the caves, cave drip water and compilation of geochemical analyses of the Irazú volcanic lake (~150 m above the cave level) and fumarole analyses were conducted between 1991 and 2014. We identified forty-eight different mineral phases, mostly rare hydrated sulfates of the alunite, halotrichite, copiapite, kieserite and rozenite groups, thirteen of which are described here as cave minerals for the first time. This includes the first occurrence in cave environments of aplowite, bieberite, boyelite, dietrichite, ferricopiapite, ferrinatrite, lausenite, lishizhenite, magnesiocopiapite, marinelite, pentahydrite, szomolnokite, and wupatkiite. The presence of other new cave minerals such as tolbachite, mercallite, rhomboclase, cyanochroite, and retgersite, is likely but could not be confirmed by various mineralogical techniques. Uplifting of sulfurous gases, water seepage from the Irazú volcanic lake and hydrothermal interactions with the volcanic host rock are responsible for such extreme mineralogical diversity. These findings make the caves of the Irazú volcano a world-type- reference locality for investigations on the formation and assemblage of sulfate minerals and the biogeochemical cycle of sulfur, with potential implications for Astrobiology and Planetary science.

Keywords: hydrated sulfates, sulfate speleothems, volcanic caves, crater lake, cave minerogenesis

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INTRODUCTION

Although still poorly studied compared to karstic carbonate environments, lava tubes and volcanic caves have drawn the interest of many speleological and mineralogical investigations over the past 20 years (Forti, 1994, 2005, Forti et al., 2003, 2004; Daza & Bustillo, 2014; Miller et al., 2014, among others). Increasing attention is being paid to minerogenesis and weathering processes in terrestrial magmatic materials, and in particular to subterranean volcanic environments, as analogues of mineral-forming mechanisms on Mars and other planets (e.g., Morse et al., 2011; Miller et al., 2014; Lalla et al., 2015, 2016). Indeed, volcanic caves host over 40% of the total number of cave minerals reported to date, whereas...
10% of the mineral species found in caves worldwide are limited to subterranean volcanic environments (Hill & Forti, 1997; Forti, 2005; Onac & Forti, 2011a).

Silicate minerals (e.g., opal and clay minerals) and carbonate speleothems (predominantly made of calcite and/or aragonite), sometimes derived from biological processes, are relatively common in volcanic caves (Daza & Bustillo, 2014; Miller et al., 2014). However, sulfates are the main group in terms of mineralogical diversity, representing over 70% of the different minerals reported from volcanic caves (Hill & Forti, 1997; Forti, 2005). The five main processes leading to sulfate speleothem precipitation in volcanic caves include: (1) deposition from aerosols, (2) evaporation of $\text{SO}_4^{2-}$-rich waters, (3) alteration and weathering (e.g., oxidation of reduced sulfur species), (4) biomediated mineral precipitation, and (5) bat guano mineralization.

Sulfur-rich caves commonly present sulfate speleothems (secondary mineral deposits formed in caves) such as stalactites, stalagmites, and crusts. Less common are geysermites, vent-shaped speleothems with a central hole that continues in the cave floor and are formed by rising thermal fluids (vapor) (Hill & Forti, 1997 and references therein); rims and blisters, formed in fractures with fumarole vapors influence (Onac & Forti, 2011b). In some of these sulfur-rich caves [e.g., Cueva de Villa Luz in Mexico, Hose et al. (2000); Frasassi cave in Italy, Jones et al. (2016)] white mucous-like soda straw biofilms hanging from cave walls and known as “snottites” (Hose & Pisarowicz, 1999) have been reported.

We conducted a detailed mineralogical and geospeleological study of Cueva Los Minerales (CMI) and Cueva Los Mucolitos (CMU), in the Irazú Volcano, Costa Rica (Fig. 1A). This area is characterized by its intense volcanic influence, with relevant $\text{CO}_2$ emissions (Galindo et al., 2004; Epiard et al., 2017) and evidence of passive fumarole activity and hydrothermalism (Alvarado et al., 2006). Here, we discuss factors related to minerogenesis, the formation of the caves themselves and their linkage with the hydrothermal system. In addition, we provide measurements of environmental parameters in the cave, hydrochemical analyses of cave infiltration waters, those present in the Irazú volcanic lake and in fumaroles (from 1991 to 2014). Abundant sulfur gases and dissolved sulfur species in the cave water are proposed as being responsible for the precipitation of an unusual diversity of sulfate minerals, many of which are reported here as cave minerals for the first time. The exact ages of the investigated caves are unknown, although access from the surface to these caves exist since December 8th, 1994, when a large landslide on the volcano flank occurred. Thus, minerogenetic processes in the caves of the Irazú Volcano can be considered as a recent and ongoing example of sulfate mineral precipitation in the volcanic subterranean environment.

**STUDY SITES**

The Costa Rica Volcanic Front is developed parallel to the Middle American Trench that separates the Coco and Caribbean plates. The convergence rate
between the subducting Coco Plate and the Caribbean Plate in Costa Rica is close to ~90 mm/yr (DeMets, 2001) (Fig. 1B). Upper plate deformation is affected by differences in subducting plate morphology, age, and dip, resulting in different deformation styles in northern and southern Costa Rica (Protti & Güendel, 1995). The Central Costa Rica Deformed Belt is the onshore expression of the transition of these two deformation styles (Marshall et al., 2000; Montero et al., 2013). It is characterized by shallow seismicity (<15 km) and a broad array of conjugate northwest-striking dextral faults and northeast-striking sinistral faults (Montero et al., 2013).

Irazú Volcano is located on an area of structural weakness dominated by the Atirro-Río Sucio Fault System. It consists of a 150 km long northwest-trending zone of dextral strike-slip faults with strong morphotectonic expression (Montero et al., 2013), it presents active seismicity, and it is composed of different active faults (Montero & Alvarado, 1995; Montero, 2001; Montero et al., 2013).

The two main craters of the Irazú Volcano, the highest volcano in Costa Rica (altitude 3,432 m a.s.l.), are located in the 35 by 10 km wide pull-apart basin comprised between traces of the Eastern and Western Río Sucio Faults (Montero et al., 2013). The Irazú Volcano evolved from Early Pleistocene to Holocene in age. The Irazú summit hosts the Main Crater “Crater Activo” (active 1962-1965). The older crater “Diego de la Haya” is located to the east of the summit. The remaining structure of a composite oldest crater called “Playa Hermosa” together with a prehistoric scoria cone and tuff rings can also still be recognized. Evidence of a debris avalanche can be seen to the northwest of the main crater (Alvarado et al., 2013). An intermittent volcanic lake has been present on “Crater Activo” after the last eruptive period (1965). This lake lasted until March 2013 when it disappeared, but it started forming again in 2017. Subsequent activity took place during the last century, the most recent eruptive phase being in 1963-1965. Geological units in the surroundings of Irazú Volcano are Pleistocene to Holocene in age.

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Survey and sampling

Since 2011, four surveys for sampling and measuring environmental variables in the caves have been carried out. The caves were surveyed using a laser-distance meter, compass, and clinometer. Cave temperature, humidity (wet bulb/dry bulb), and light intensity were measured with a portable thermohygrometer (Kestrel 4500, ± 1°C), humidity probe (Kestrel 4500, ± 3%), and photometer equipment (CEM DT-8820 4-in-1 digital Multifunction Environment Meter). Thermal images were obtained using a thermal camera FLIRSC660. In CMI and CMU the CO₂ and H₂S concentrations at the floor level and in the cave atmosphere were measured with a West System WS0820 flux meter. Mineral and water sampling in CMI and CMU were conducted in 2011, 2013, and 2014. Representative mineral samples were collected based on their visual appearances (e.g., color, texture) and location in the caves. Different types of speleothems (e.g., stalactites, geysermites, crusts on the walls) were collected, preferably broken specimens to minimize impact on the cave. Water samples for trace element analyses were taken in amber glass 50 ml bottles. Samples for analyses of heavy metals were acidified with HNO₃ to a pH < 2 to prevent precipitation during storage.

Hydrogeochemical and fumarole gas analyses

Analyses were carried out following certified procedures (INTE-ISO/IEC17025:2005) at Agrotec Analytical Laboratory (San Jose, Costa Rica). Trace element analyses were measured by inductively coupled plasma optical emission spectrometry (Thermo Trace Jarrel-ash 51-i) (Eaton et al., 1998, Standard Method 3120b). Calibration was conducted using synthetic standards with a relative standard deviation (RSD) of 2% or better.

In this paper, we used cation and anion analyses of 25 water samples collected by the Instituto Costarricense de Electricidad (ICE) from June 1991 to February 1993 and 17 samples collected by the Observatorio Vulcanológico y Sismológico de Costa Rica (OVSICORI) since March 1999 to December 2010 (Supplementary Table 1). Water samples were collected in two 500 ml bottles. Cation samples were preserved with 5 N HNO₃ (one ml per 100 ml). The water samples were analysed for cations by Atomic Absorption Spectrophotometer and for anions by titration methods (bicarbonate), spectrophotometric methods and ion chromatography.
Errors in the determination of the different ions are lower than 10% in all cases.

The compiled eight fumarole gas analyses for 1991, 2003, 2006m and 2007 (Table 1), were sampled by ICE and OVSICORI using bottles under vacuum and partially filled with 4N NaOH (Giggenbach, 1975). The caustic solution was used for the analyses of F and Cl by ion chromatography and SO$_2$ as SO$_2$\textsuperscript{2-} after oxidation with H$_2$O$_2$ by ion chromatography.

**Table 1. Historical fumarole analyses in Irazú Volcano.**

<table>
<thead>
<tr>
<th>Date</th>
<th>pH</th>
<th>Temp (°C)</th>
<th>Conductivity (µS/cm)</th>
<th>Salinity (g/L)</th>
<th>F (mg/l)</th>
<th>Cl (mg/l)</th>
<th>SO$_2$ (mg/l)</th>
</tr>
</thead>
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<tr>
<td>7/17/1991</td>
<td>2.35</td>
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<td></td>
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<td>1.08</td>
<td>1.06</td>
<td>503</td>
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<tr>
<td>5/27/2003</td>
<td>88</td>
<td></td>
<td></td>
<td></td>
<td>0.048</td>
<td>2.673</td>
<td>42.116</td>
</tr>
<tr>
<td>6/12/2003</td>
<td>88</td>
<td></td>
<td></td>
<td></td>
<td>0.39</td>
<td>8.535</td>
<td>496.798</td>
</tr>
<tr>
<td>10/13/2006</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n.a.</td>
<td>1.791</td>
<td>n.a.</td>
</tr>
<tr>
<td>12/13/2006</td>
<td>2.88</td>
<td>87</td>
<td>648</td>
<td>0.1</td>
<td>2.235</td>
<td>2.173</td>
<td>99.224</td>
</tr>
<tr>
<td>2/4/2007</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.863</td>
<td>86.589</td>
<td>57.761</td>
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<tr>
<td>5/5/2007</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.525</td>
<td>15.214</td>
<td>190.662</td>
</tr>
<tr>
<td>5/15/2007</td>
<td>2.41</td>
<td>83</td>
<td>1854</td>
<td>0.8</td>
<td>5.525</td>
<td>86.589</td>
<td>503</td>
</tr>
<tr>
<td><strong>Maximum</strong></td>
<td>2.88</td>
<td>88</td>
<td>1854</td>
<td>0.8</td>
<td>5.525</td>
<td>86.589</td>
<td>503</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>2.55</td>
<td>86.5</td>
<td>1251</td>
<td>0.45</td>
<td>2.023</td>
<td>16.862</td>
<td>231.594</td>
</tr>
<tr>
<td><strong>Minimum</strong></td>
<td>2.35</td>
<td>83</td>
<td>648</td>
<td>0.1</td>
<td>0.048</td>
<td>1.06</td>
<td>42.116</td>
</tr>
</tbody>
</table>

The spectra were compared with the RRUFF Raman mineralogical database (http://rruff.info/) for most of the analyses using CrystalSleuth software (Laetsch & Downs, 2006). Spectra that did not match any in the database were not included in the list and might be related to mineral mixtures or unknown minerals. The comparison spectrum, percentage of match, Raman peaks, integration time and respective number of accumulation are reported in Supplementary Table 2 for micro-Raman analyses and Supplementary Table 3 for FT-Raman.

**Mineralogical analyses Raman spectroscopy**

Micro-Raman and FT-Raman analyses were carried out at the Unidad Asociada UVA-CSIC of Centro de Astrobiología, University of Valladolid (Spain). Micro-Raman was useful to analyze particular parts of the samples at microscale, based on different colors and textures. A total of 134 micro-Raman spectra were performed in different mineral facies. The excitation source was a Laser Research Electro-Optics (REO) working at 632.8 nm. The KOSI HoloSpec f/1.8i spectrometer from Kaiser Optical covered a spectral range of 150–3800 cm\textsuperscript{-1} and a spectral resolution of 5 cm\textsuperscript{-1}, while the CCD (charge coupled device) employed was a D420A-OE-130 model from Andor. The Raman head used was KOSI MKII, HFPH-FC-S-632.8 model from Kaiser Optical Systems coupled by optical fiber to a Nikon Eclipse E600 microscope, which in turn was attached to a JVC TK-C1381EG videocamera for visual analysis and precise control of the measured spots. Two objectives were used, 50x and 100x allowing microanalyses of 37 and 15 μm diameter spots, respectively. The laser power on the sample was maintained around 2 mW to ensure no thermal damage occurred to the samples. Typical integration time for spectral acquisition was 10 s and 10 accumulations were done. The sample was manually scanned, while the height of focus was varied in order to optimize the intensity of the spectra signals.

In addition, 53 powdered bulk samples were analyzed using a FT-Raman Bruker instrument (model RFS100/S). The coherent laser was a Compass 1064-500 model (Nd:YAG, 1064 nm, 500 mW). The coherent laser was a Compass 1064-500 model (Nd:YAG, 1064 nm, 500 mW). The laser power on the sample was approximately 1 μm. The limit of detection of this technique enables major elements such as Fe, Mn, O, Si, Al, Ca, Pb, Zn, and Ba to be analyzed.

**RESULTS**

**Elemental composition and microphotographies**

Energy Dispersive X-ray spectroscopy (EDX) coupled to Scanning Electron Microscopy (SEM) analyses were performed at the Servicios Centrales de Investigación of the University of Almeria (Spain). SEM microphotographs of 26 samples were taken using a HITACHI S-3500 instrument in variable-pressure mode. The elemental chemistry was determined by EDX microprobe at different points with different typology over several grains of the samples, using an Oxford INCA 7210 diffractometer, with a Cu anode (CuKα, λ= 0.154 nm) and graphite monochromator. A Ni filter and Al sample holders were utilized. Tension and current produced by the generator were 40 kV and 30 mA respectively, for all analyses. The analysis used the 20 scanning method, using 0.400 seconds per step and within the angular limits of 5 to 70°.

**X-ray diffraction**

X-ray diffraction analyses were carried out at the Unidad Asociada UVA-CSIC of Centro de Astrobiología, University of Valladolid (Spain). A total of 53 powdered samples were analyzed (Supplementary Table 4) using a Philips PW1710 diffractometer, with a Cu anode (CuKα, λ= 0.154 nm) and graphite monochromator. A Ni filter and Al sample holders were utilized. Tension and current produced by the generator were 40 kV and 30 mA respectively, for all analyses. The analysis used the 20 scanning method, using 0.400 seconds per step and within the angular limits of 5 to 70°.

**Geological observations**

The host rock of the caves consists of interbedded pyroclastic rocks, with centimetric to decimetric...
stratification and poorly selected volcanic breccia with angular volcanic clasts associated with pyroclastic density current deposits. Pyroclastic rocks are light grey, white to yellowish color, and in some areas present evidence of silicification. These rocks are normally poorly to moderately compacted or welded and weathering is intense due to harsh volcanic conditions (e.g., high temperature and volcanic gases). The dip direction (318/26°) is well defined by the strata where the caves are located, and this plane served as rupture surface of the 1994 debris avalanche. Pyroclastic rocks close to the CMU entrance, displays synthetic and antithetic Ridel shears with sub-horizontal striation and dextral motion. This fault corresponds to the Central Río Sucio Fault (Montero et al., 2013) and extends to CMI and Crater Principal (Fig. 1A and 2). To the south of the main entrance of CMI, there is a sector with evidence of runoff watermarks and mud deposits.

**Hydrogeochemistry**

Evidence for multiple aquifers has been reported in Irazú Summit (Alvarado, 1993). An ephemeral upper water level (~3,170 m a.s.l.) is associated with the geological contact between recent volcanoclastic deposits and hydrothermally altered rocks. The ephemeral main crater volcanic lake is located between ~3,080 and <3,050 m a.s.l. We provide geochemical water analyses from 1991 to 2010 for the lake waters (Supplementary Table 1). This volcanic lake has undergone important changes in physico-chemical conditions over time. For example, pH values range from very acid (pH 2.85 in November 1991) to alkaline conditions (pH 8.43 in March 2005) and temperatures registered a maximum of 93°C (July 1993), although the “normal range” is between 14 and 30° C (Fig. 3A). The Irazú lake analyses show relatively high content in sulfates ($\text{SO}_4^{2-}$), with values over 2,500 ppm in 1991-1999 and 2,219 ppm on average for the entire period of this study. Chlorine increases with the sulfates and presents maximum values of 1,020 ppm (in April 1992) and average value of 420 ppm (Fig. 3B). A single infiltration water analysis, which likely comes from volcanic lake seepage waters, was performed in 2013 for selected elements (Fig. 3C). The existence of a deeper aquifer is suggested (Alvarado et al., 2006) associated with phreatomagmatic eruptions and the hydrothermal system in the area. This deeper aquifer could be the source of sulfate-rich water and also one brownish-yellow drain (possibly acidic) close to the base of the cliff (at ~2,825 m a.s.l.) could be associated with the upwelling of these deeper waters.

**Chemical fumarole gas composition**

Fumaroles are located ~300 m northeast of the cave area (Fig. 1) and ranged in temperature from 83 to 88°C and pH from 2.35 to 2.88. Table 1 displays the chemical composition of main fumarole components...
Environmental parameters

CMI presents a wide entrance ~50 m (Fig. 4A), with a main chamber developing 27 m from the cave entrance to the end of the cave and approximately 40 m wide, whereas Sala de los Cristales Verdes is 10 m wide and presents green stalactites (Fig. 4B). Due to the cave morphology (Fig. 5A), the cave is exposed to sunlight (>1.32 lux during the day), because of the very large cave entrance. Temperature in the cave ranges from 9 to 16 °C in the main chamber. Thermal map (Fig. 5B) was drawn showing the highest temperatures (28-30°C) close to the north wall fissures (where important sulfate crusts are located) and some unmapped areas close to Sala de los Cristales Verdes show temperatures over 40°C. The southern part of the main chamber presents the highest elevation and acts as a hot air trap, with temperatures between 30-32°C. Lower mineral diversity was found in this section of the cave. Based on 2013 field trip measurements, the relative humidity in CMI varies from 74.2% (close to entrance) to 92.4% (inner area of the main chamber). CO₂ concentrations up to 950 ppm were measured; H₂S was 29 ppm and SO₄ and H₂S were below detection limits.

CMU is smaller in volume, the entrance is small (~50 cm) and is developed in a collapsed area, 38.2 m long and 14.6 m deep. This cave showed temperatures ranging between 16 and 18 °C and relative humidity of 96.8 % in 2013. Unlike CMI, this cave is totally dark.
Sulfate minerals in caves of the Irazú volcano (Costa Rica)

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Fig. 5. A) Cave map of CMI, showing the morphology and mineral distribution; B) Thermal mapping of the cave.

Sulfates
Sulfates were the most common mineral group in the caves of the Irazú Volcano (91.5% of the total diversity). Most of the identified sulfates correspond to hydrated acid and sulfates (33). Also, sulfates belonging to hydrated sulfates containing hydroxyl or halogen (4), anhydrous acid and sulfates (3), anhydrous sulfates containing hydroxyl or halogen (3) have been detected (Fig. 6).

Anhydrous acid and sulfates
Minerals of this group are present only in CMI. Anglesite has been found forming part of crusts on the walls, floor, and roof of the cave. Under binocular microscope, anglesite shows transparent to green or turquoise green crystals, botryoidal masses or needles and was detected by micro-Raman. Anhydrite has been identified by XRD in two different samples from crusts and stalactites, in association with native sulfur (Fig. 7A and B). The presence of mercallite is possible, although it relies on a single Raman spectrum.

Anhydrous sulfates containing hydroxyl or halogen
Jarosite has been found only in CMU, whereas natroalunite and natrojarosite are present in both caves. These minerals are predominantly yellow in color, and occur on the walls of the caves. Jarosite in CMU was associated with gypsum in an area of acid dripping (pH < 2). Identification by Raman spectroscopy has been confirmed by XRD for all the samples.

Hydrated acid and sulfates
This group comprises 28 minerals considered as “confirmed” and 5 minerals which presence is “possible” (Table 2). All of these minerals were found in CMI, except for gypsum that is also present in...
Table 2. List of the minerals identified in CMI and CMU. The numbers correspond to how many times the mineral species were detected using different techniques.

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Chemical formula</th>
<th>New cave Mineral</th>
<th>Cave</th>
<th>Cave location</th>
<th>Color</th>
<th>Habit</th>
<th>Crust crystal</th>
<th>Stallactites</th>
<th>Geysermite</th>
<th>2015 XRD (#)</th>
<th>2011 XRD (#)</th>
<th>micro-Raman (#)</th>
<th>FT Raman (#)</th>
<th>Total</th>
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<tr>
<td><strong>NATIVE ELEMENTS (1)</strong></td>
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<td></td>
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<tr>
<td>Sulfur</td>
<td>S₈</td>
<td>C</td>
<td>CMI</td>
<td>W, R, F</td>
<td>Y, O</td>
<td>MA</td>
<td>X</td>
<td>X</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>11</td>
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<tr>
<td>Tolbachite</td>
<td>CuCl₂</td>
<td>X</td>
<td>P</td>
<td>CMI</td>
<td>R</td>
<td>LG</td>
<td>X</td>
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<td>CMU</td>
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<tr>
<td>Kaolinite</td>
<td>Al₂Si₂O₅(OH)₄</td>
<td>C</td>
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<tr>
<td>Marinellite</td>
<td>Na₄Ca₂Al₂Si₃O₉(SO₄)₅Cl·6H₂O</td>
<td>X</td>
<td>C</td>
<td>CMI</td>
<td>W, F</td>
<td>Y, O</td>
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<td>W, R, F</td>
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<td>CMI</td>
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<td>BR, GR, R</td>
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<td>W, R, F</td>
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<td>CMI</td>
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CMU too. Hydrated acid sulfates are sub-classified according to Dana’s classification (Mindat, 2018).

Hydrated acid and sulfates with the general formula \((\text{A})_2\text{(XO}_4\text{)}_y\cdot\text{x(H}_2\text{O})\) are represented by alunogen, coquimbite (Fig. 8), and lausenite in crusts on the walls, roof, and floor of the cave. Coquimbite was also found in geysermites. Alunogen and coquimbite have been identified by Raman techniques and XRD, lausenite only by FT and micro-Raman. The presence of rhomboclase is suggested by micro-Raman (Fig. 9) and the presence of Fe in EDX analyses.

Hydrated acid and sulfates belonging to the group with general formula \((\text{A}^+\text{B})_2\text{(XO}_4\text{)}_z\cdot\text{x(H}_2\text{O})\) are represented by the possible presence of blödite (identified by XRD) and cyanochroite (identified by micro-Raman, Fig. 9), whereas ferrinatrite and metavoltine, have been found on walls and roof of the cave.

Some hydrated acid and sulfates with formula \(\text{A}\cdot\text{B}\cdot\text{(XO}_4\text{)}_z\cdot\text{x(H}_2\text{O})\) are difficult to differentiate by Raman because of the similarity in the spectra (e.g., apjohnite, dietrichite, halotrichite, pickeringite), with peaks usually at 994 and 975 cm\(^{-1}\), and because they commonly appear in mineral mixtures. However, the combination of XRD-Raman and EDX has made identification possible. Some samples also show a minor peak at 1,025 cm\(^{-1}\) (CR-1C-A and MIM-9A) possible associated with the presence of coquimbite. Minerals from this group, have been found forming part of crusts on the walls and geysermites (Fig. 10A), in some cases associated with ferricopiapite (Fig. 10B), and generally showing acicular habit (Fig. 10C, D). Kalinite, mendozite, and tamarugite, have been detected in several samples.

Hydrated acid and sulfates with formula \(\text{A}\cdot\text{XO}_4\cdot\text{x(H}_2\text{O})\) are especially common in these caves. Thirteen species have been found as crust on the walls. Melanterite and rozenite are also part of geysermites, szomolnokite is part of stalactites (Fig. 11A, B, and C). Gypsum is a very abundant mineral in CMI, covering the north walls of the cave and often associated with some microbial mats. Other new cave minerals from this sub-group are pentahydrite and szomolnokite, both identified by Raman and XRD techniques. Chalcanthite is another possible mineral but was identified only from one micro-Raman analysis; however, it matches 98% with the reference Raman spectrum (982, 609, and 455 cm\(^{-1}\)).

Retgersite is the only nickel sulfate identified by micro-Raman (984, 609 cm\(^{-1}\)) (Fig. 9) that belongs to...
the sub-group with the formula $A \cdot XO_4 \cdot x(H_2O)$. Finally, voltaite displays black pyramidal shaped crystals under the SEM (Fig. 8D) and is the only member of Hydrated Acid and Sulfates with miscellaneous formula. Generally, it is present in the cave as crusts and crystals on the walls, floor, and roof. The presence of voltaite has been confirmed by XRD and micro-Raman techniques.

Hydrated sulfates containing hydroxyl or halogen

This group comprises minerals of the copiapite group, represented in CMI by aluminocopiapite, copiapite, ferricopiapite, and magnesiocopiapite. These minerals are common forming part of geysermites and normally show yellow colors, and sometimes a tabular habit (Fig. 10B). Ferricopiapite and magnesiocopiapite are new cave minerals confirmed by XRD.

Other minerals (native elements, halides, oxides, and silicates)

Native sulfur was detected in 11 samples from CMI by FT-Raman, micro-Raman, and XRD. Native sulfur deposits in this cave appear close to volcanic vents as crusts made of crystals <5 cm large, in high temperature areas, acid conditions (pH ~2) and sometimes forming stalactites in association with anhydrite (Fig. 7).

The possible presence of the halide tolbachite is suggested by XRD as accessory mineral associated with römerite, gypsum, melanterite, rozenite, and szomolnokite, detected in the same stalactite. This mineral was forming part of a light green stalactite (>8 cm length) in CMI. Tolbachite was not reported from cave environments yet.

The only oxide detected, goethite, was found in CMU as dark orange and reddish masses filling cracks in the walls. It was identified by XRD in only one sample. The only phyllosilicate identified is kaolinite, found in a single muddy yellow sample from CMU. It was associated with natrojarosite and minor gypsum in snottite-like speleothems hanging from the roof of the cave, probably related to extremophile microorganism activity (pH 1–2). Finally, marinellite was identified by micro-Raman spectrometry in two different samples, but it was not confirmed or detected by XRD.

Mineral distribution and speleothems

Mineral distribution in CMI is represented in Fig. 5, however due to the high diversity and possible spatiotemporal variable distribution of minerals (e.g., different temperature and relative humidity affecting the hydration state of some hydrous minerals), this study did not aimed at quantitatively estimating this spatial diversity. Other techniques, including photogrammetry or LIDAR scanning may be necessary.

Most of the reported sulfate minerals are very soluble, therefore, the only reason why they exist in the tropical weather of Costa Rica (rainy season from May to November, with 2,387 mm/yr) is because of the particular cave climate conditions. Most minerals in CMI occur as crusts on the wall and roof and are concentrated on the northern side and also covering walls and roof of the Sala de los Cristales Verdes (where most of the stalactites are located). Other speleothems such as stalactites and stalagnates are associated with $SO_4^{2-}$ and Fe-rich infiltration waters (Fig. 11 and 4B), or Ca-rich solutions as a particular anhydrite-sulfur stalactite (Fig. 7). Geysermites are associated with $SO_4^{2-}$ rising thermal waters, and present high-mineral diversity with more than 11 mineral species in a single sample. Most of the identified mineral species present yellow, white, and translucent color followed by green. The most common mineral habits are acicular followed by massive. Most of the crust minerals are centimeters in size, while stalactites and geysermites are normally decimeters.

Elemental analyses and microphotography

The results of 93 EDX analyses on samples from both caves allowed the detection of 17 chemical ions and their relative abundance (Supplementary Table 5). Presence of iron is related with abundant iron sulfate mineral phases (e.g., coquimbite, copiapite, ferricopiapite, rozenite). Other metals such as aluminum have been detected in several samples associated with sulfates containing Al (e.g., marinellite, alunogen, dietrichite, halotrichite,
Fig. 9. Micro-Raman spectra of the new cave minerals reported only by this technique.

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The presence of Si can be associated with the silicates (e.g., kaolinite or marinellite) and diatom skeletons found in the sample MU-170514-4 next to snottites (at pH ~2). Alkaline elements, such as calcium correspond to gypsum but can also be related to other minerals such as marinellite, anhydrite, and copiapite. The other alkaline cations detected by EDX (Na, Mg, K) are quite common in many samples. V, Br, and Sr were also reported but no mineral containing those elements has been identified in this work. This suggests that there could be more minerals present in the samples. SEM photographs of the samples show complex associations between these minerals and are useful to distinguish the different habits (Fig. 7, 8, 10, and 11).

**Sulfur in the caves**

Sulfur is common in sulfuric acid caves [e.g., Cueva Villa de la Luz (Mexico; Hose et al., 2000); Carlsbad and Lechuguilla caves (New Mexico; Hill, 1995); Lower Kane Cave ( Wyoming; Egemeier, 1981); Diana Cave (Romania; Onac et al., 2009); Santa Cesarea Terme (Italy; D’Angeli et al., 2017)] as wall crusts and stalactites, in places associated with gypsum (Hill & Forti, 1997). On Irazú Volcano, elemental sulfur has been reported in fumaroles (Stoiber & Rose, 1974) and as sulfur flow in the main crater in 1982 (McClelland et al., 1989). In the Irazú caves, elemental sulfur is present in some volcanic vents on the eastern wall of the cave (pH 1-2) and crystals can be up to 6 cm in length. Furthermore, elemental sulfur was found in a ~10 cm along active faults in the Irazú caves area may have caused the formation of the underground voids.

This pseudokarstic mechanism is evidenced by the presence of a cylindrical hole ~1 m in diameter in the roof of the cave, similar to holes formed in dissolution caves. Linked to this, in Llano Grande mine quarry (6.5 km SW of the Crater Active), there are channels ~1 to 2 m wide in similar pyroclastic hydrothermally altered rocks, completely filled with native sulfur. Likewise, these presumed proto-channels were found during a drilling campaign, when a borehole intersected a possible hypogenic cave, ~1 to ~5 m depth. Therefore, a combination of tectonic, weathering, high temperature degasification, leaching, and erosion could be involved in the genesis of the caves on Irazú Volcano.

**DISCUSSION**

**Speleogenesis**

The caves of the Irazú Volcano have a complex origin, which differs considerably from the speleogenetic mechanisms associated with normal carbonic and sulfuric acid caves worldwide (Hose et al., 2000b; Klimchouk, 2009; Onac et al., 2009). Ulloa et al. (2013) analyzed different possible mechanisms for the origin of CMI (i.e., landslide, phreatic explosion, or water runoff). These authors concluded that the landslide model with a stepped fault plane generated these caves. However, preliminary results of Baldoni et al. (pers. comm.) estimated a rock removal rate of ~3,000 m³/yr in the Irazú-Turrialba system by chemical leaching from the springs. Also, based on an infrared survey in 1964, thermal anomalies were also identified (Gawarecki et al., 1980), which coincide with the Río Sucio fault traces and some of them were located close (~250 m NW) to the actual position of the caves (covered by the material that slipped in 1994). Therefore, the possibility exists that dissolution weathering and underground removal of volcanic pyroclastic rocks by very acid waters
long stalactite (Fig. 7A). Similar sulfur speleothems have been reported in strongly acid environments as a result of H2S oxidation (Forti & Mocchiutti, 2004; D'Angeli et al., 2017). Sulfur is present in all the EDX analyses (Supplementary Table 5), ranging from 5.2 to 70.8 wt%. The abundant presence of sulfur in these minerals, is related to the typically sulfur-rich volcanic environments. Sulfur is present in a variety of valence states; the most reduced form as hydrogen sulfide (H2S) in volcanic gases is -2, elemental sulfur (valence 0) and the most oxidized form as sulfate (SO4^2-) is +6. Some of the changes in valence are attributed to the biogeochemical reactions, in which microorganisms frequently play a main role as catalysts, taking advantage of the associated electron fluxes to satisfy their metabolic needs (Morse et al., 1987).

Minerogenetic mechanisms in the Irazú caves

The transport of ions and chemical compounds in the caves and the consequent mineral precipitation can occur from mineralized solutions and this may happen both in subaerial or subaqueous settings. Each of the transport mechanisms can trigger different minerogenetic processes. Mineralized solutions plays a very important role in the transportation of chemical elements and compounds into the caves. Water geochemistry analyses indicate considerable concentrations of sulfates in the main crater lake (Supplementary Table 1), and infiltration water analyses indicated the presence of Se, Fe, Al, Ca, Mg, Na, Mn, K, Zn, Ni, and Co ions (Fig. 3C). Selenium is the only element that is not reflected in the elemental composition of the reported minerals. Enrichment in selenium (993.1 mg/l) might be associated with the input of geothermal waters (Floor & Román-Ross, 2012). Iron concentration is 440.2 ppm in the infiltration waters and is present in the elemental composition of 20 minerals, followed by calcium present in 14 different minerals. Stalactites and stalagmites (usually green to blue in color) are associated with dissolution-precipitation processes of Fe-sulfate-rich infiltration waters. They are found in “Sala de los Cristales Verdes”, formed by iron hydrated sulfates including melanterite, szomolnokite, römerite, rozenite, and volatile with presence of gypsum (probably in lower quantities) and possible tolbachite. In the case of iron sulfate minerals as szomolnokite-rozenite-melanterite-römerite, they differ from each other by their content in water molecules, with hydration and dehydration processes possibly causing their transformation from one species to another. The growth of the blue-green stalactite (Fig. 4B) is relatively fast: based on field observations (growth and collapse of green-stalactites) they can grow in the order of ~30 cm/a. Similar melanterite stalactites and stalagmites have been reported in caves in California, Nevada, and Tennessee (USA), Sicily (Italy), and mine caves (Hill & Forti, 1997).

At least 7 different geysermites have been identified in CMI distributed in the main chamber and ranging from ~15 to ~40 cm height and ~8 to ~15 cm width (Fig. 5 and 10). Field observations revealed no thermal anomalies in these speleothems suggesting that the process that formed them, is not currently active. The minerogological composition of the analyzed geysermites consists of hydrated acid sulfates (apjohnite, coquimbite, dietrichite, halotrichite, kalinite, pickeringite, and wupatkiiite) and hydrated sulfates containing hydroxyl or halogen (aluminocopiapite, copiapite, ferricopiapite, magnesicopiapite). The genesis of these geysermites is related to upwelling sulfate-rich waters of the Irazú hydrothermal system. The cooling of the rising thermo-mineral fluids induced supersaturation and mineral precipitation (Onac & Forti, 2011b) and may explain the high mineralogical diversity of such speleothems. The presence of geysermites suggests the existence of lower thermal aquifers which can be responsible of acid drainage in the Rio Sucio River basin (Arce-Rodríguez et al., 2017) and some thermal springs in the surroundings.

Aerosols (solid or colloidal particles, 10^-7 to 10^-2 cm) and hydrosols (liquid droplets) are another way to transport chemical compounds, which is a feasible mechanism where there is a mixture of air at different temperatures in caves (Cigna & Hill, 1997). Speleothems associated with aerosols are related to thermal caves (preferable associated with fumarole vapors), where temperature and humidity gradients are present (Onac & Forti, 2011b). Speleothems associated with aerosols as transport-media of chemical compounds is strongly suggested for CMI because: (1) presence of minerals close to hydrothermal vents, higher density and radial distributions around them; (2) elevated concentrations of SO4^2- in fumarolic analyses (Table 1) and presence of H2S in the cave atmosphere (29 ppm) being evidence of sulfur-compounds in the air; (3) some minerals present morphologies similar to “rims and blisters” (according to the description of Onac & Forti, 2011b), which are associated with speleothems developed by aerosols and vapors. CMU did not present considerable thermal anomalies and shows less mineral diversity, which could be suggesting that aerosols are probably one of the most

Fig. 11. Example of szomolnokite stalagmite ~15 cm long (Photography taken by Víctor Carvajal in 2014) in CMI (A); SEM image of similar szomolnokite samples (B and C), in association with römerite (C).
active mechanisms in the higher mineral diversity of CMI.

The presence of elemental sulfur crystals in CMI could be associated with cooling down of fumarole gases (sublimation at temperatures >100°C), which is suggested as minerogenetic mechanism (Forti, 2005; Onac & Forti, 2011b). The atmosphere of CMI is rather complex, and shows significant variations in temperature (9 to 30°C) and relative humidity (74.2 to 92.4%). These variations can influence precipitation of aerosol minerals and also rule the hydration-dehydration processes for those mineral species that only differ in their water content (e.g., aplowite-bieberite, epsomite-hexahydrite and melanterite-rozenite-szomolnokite series).

Finally, another possible mineral-forming mechanism in the cave is microbial activity. This mechanism is suggested based on field observations where gypsum-stalactite are intrinsically related to snottites in some parts of the cave.

Relative abundance of elements and minerals

CMI may be the cave (or one of the caves) in the world with the greatest mineralogical diversity. Because of the particular conditions in an active volcano environment (SO$_4^{2-}$-rich waters and influence of volcanic gases), 48 minerals species have been identified, 41 are confirmed and 7 mineral species are suggested. According to Onac & Forti (2011a) in the past 20 years, on average, three new cave minerals per year have been discovered. This study adds 18 minerals to the list Onac & Forti (2011a), including 12 confirmed sulfates (aplowite, bieberite, boyleite, dietrichite, ferrinatrite, ferricopiapite, lausenite, lishizhenite, pentahydrite, magnesiocopiapite, szomolnokite, and wupatkiite), one confirmed silicate (marinellite), four other possible sulfates (cyanochroite, merrallite, retgersite, and rhomboclase, Fig. 9), and also the possible presence of an halide (tolbachite). Five of these new cave minerals (aplowite, ferrinatrite, magnesiocopiapite, wupatkiite, and boyleite) in CMI were previously reported by Ulloa et al. (2013) and apjohnite is not in the cave minerals list of Onac & Forti (2011a), but was reported by Onac et al. (2009). However, it must be considered that the number of minerals present in these caves is still underestimated because it was not possible to associate some of the Raman spectra with any specific mineral species of the Raman database, as well as the presence of more elements (e.g., Br, Sr, V) identified by EDX analyses (Supplementary Table 5) which are not reflected in the composition of the minerals listed in Table 2.

Apart from selenium, the majority of elements in infiltration waters (Fig. 3C) corresponds to the most abundant elements present in the identified sulfates (Fig. 6D). Fe, Al, Ca, Na, and Mg are the most abundant cation species in sulfates and also the major elements in infiltration waters. Thus, the abundance of accessory elements in sulfate minerals is proportional to the abundance of elements in the infiltration waters.

Gypsum is probably the most abundant mineral in the cave. The north walls of CMI are totally covered with gypsum crystals, mainly along the external edge of the cave, and this mineral is also present in CMU. Gypsum tends to be idiomorphic and some of the crystals presents an interesting association with microbial mats.

Summit volcanic crater lake and hydrothermal system

The last eruptive activity of the main Irazú crater stopped after 1965 and in July of the same year the volcanic lake was formed in the main crater. The last eruptive activity of the main Irazú crater stopped after 1965 and in July of the same year the volcanic lake was formed in the main crater. This lake has an ephemeral behavior, being repeatedly dry (years 1977, 1979, 1982-83, 1987, 1990, and 2013-17) and precipitation has not been the main factor in its change in volume. Fumaroles and sub-aquatic fumaroles have also been reported at different time-intervals, during 1973, 1977-1978, 1982, 1985-1986, 1991-2000, and 2002-2008 (Ramírez et al., 2013 and references therein). The lake of the main crater showed important changes in pH, temperature, chemical composition (Fig. 3A and B; Supplementary Table 1) and water level since 1991, making this lake a complex geochemical system. An acidification trend in the lake was measured in the years 1991-1993 and was associated with enrichment in SO$_4^{2-}$ and Cl$^-$. After 2000, lower concentrations of SO$_4^{2-}$ and Cl$^-$ were detected and an alkaline trend predominated. The average temperature of the lake is 29.5°C, although it showed temperatures over 50°C in 1991 and 2003.

These variations in water levels and volcanic gas emissions are strongly associated with the active faulting and secondary permeability in the volcanic conduit. Based on historical reports it is suggested that rising of hot fluids and volcanic gases from the hydrothermal system has changed over time, sometimes reaching the volcanic crater-lake level. Hydraulic communication between the main crater lake, and the caves and hydrothermal system is suggested. The main supporting signs are: the presence of diatom skeletons in a sample in a dripping zone at CMU, which may come from lake; the enrichment in sulfates in historical water analyses of the main crater lake associated with infiltration waters in the caves and presence of sulfate minerals in the cave. The hydraulic connection lake-caves coinciding with the model proposed by Ramírez et al. (2013) is associated with high-permeability pathways within the faults. According to this hypothesis, variations of regional tectonic forces, seismic activity, clays on cracks, and raining may be factors controlling the ephemeral behavior of Irazú Volcanic Lake in the Crater Principal.

For periods for which water geochemical analyses are available (Supplementary Table 1) in Irazú Volcanic Lake, evidence of sub-aquatic fumarole activity is reported for years 1991, 1992, and 2003 to 2008 (Ramírez et al., 2013). These ephemeral volcanic emissions are connected with different pulses of the hydrothermal system activation and probably directly linked with variations in the geochemical lake system. According to this model, cave inputs of sulfate-rich
waters can present two origins, one related with volcanic lake infiltration, which was presently active during field trips. A second sulfate-rich water source is suggested, related with the rising of geothermal fluids through faults, which can provide permeable paths. Infiltration sulfate-rich solutions are responsible for speleothem precipitation, such as stalactites and stalagmites, whereas geothermal rising sulfate-rich waters probably caused the formation of geysermites.

Fumaroles and diffuse gas at Irazú System

Sulfur and gypsum minerals have been reported as fumarole minerals in the volcano (Stoiber & Rose, 1974). Fumarole locations have changed over the last decades, but actually at least two still active fumaroles are present in the northwest part of Irazú Volcano (Fig. 1A). In the area affected by the 1994 landslide another fumarole was present, maybe associated with the actual location of the caves. The only published data from Irazú fumaroles shows concentrations of CO$_2$ from 98.0% to 99.8%, SO$_2$ from 0.106% to 0.007% and H$_2$S from 0.015% to 0% in 1981 and 1982, respectively (Global Volcanism Program, 1983) and CO$_2$ 98.9% and H$_2$S 0.8% in the year 1993 (Global Volcanism Program, 1993). The compiled historical analyses of Irazú fumaroles (Table 1) shows an important content of SO$_2^2$ ranging from 42 to 503 mg/l, being SO$_2^2$ the most abundant compound, making aerosols mineralogenesis a potential mechanism.

Irazú Volcano also presents an important diffuse CO$_2$ flux: Galindo et al. (2004) established a CO$_2$ base flux at ~20 gm$^{-2}$ d$^{-1}$ and maximum values of 316 g m$^{-2}$d$^{-1}$ for sites studied in 2001, while Epiard et al. (2017) reported maximum values of 2.71 x 10$^{4}$ gm$^{-2}$d$^{-1}$ for the northern flank and 2.17 x 10$^{4}$ gm$^{-2}$d$^{-1}$ north of the main crater in 2015. Those diffuse CO$_2$ flux anomalies are located in areas close to active fault traces, being the structural control very important for the escape of gases and associated rock alteration, but probably with less relevance for the formation of speleothems in the caves of the Irazú Volcano.

Implications for planetary exploration

Caves are considered extreme environments for life because they are resource-limited due to the absence of light and implicitly of photosynthesis; however, chemolithoautotrophic organisms can metabolize inorganic elements such as iron, sulfur, and manganese in caves (Northup et al., 2011, 2012; Riquelme & Northup, 2013). Thus, terrestrial subterranean environments and secondary cave minerals (speleothems) have drawn attention as potential analogues of life niches for planetary exploration, especially Mars (Boston et al., 2001; Bost et al., 2013; Gázquez et al., 2014). During the past decades important efforts have been done by the scientific community to understand primary composition of Mars and alteration products that are associated with paleo-environmental conditions of the planet. Chemical analyses suggest that the Martian crust is basaltic, where mineralogy is dominated by primary silicates (e.g., olivine, clinopyroxene, orthopyroxene, plagioclase, alkali feldspars) and secondary minerals groups (e.g., oxides, clay minerals, hydrated silicates, carbonates, sulfates, and chlorides). These secondary minerals are associated with alteration related to presence of liquid water in some stage of the Martian evolution (Mustard et al., 2008; Ehlmann & Edwards, 2014). The discovery on the Martian surface of soil sulfur-enrichment (Chevrier & Mathé, 2007), presence of jarosite suggestive of existence of acidic waters (pH < 4) (Ehlmann & Edwards, 2014; Thollot et al., 2012), Ca, Fe, and Mg-sulfates (Ehlmann & Edwards, 2014) and CO$_2$-rich atmosphere, are factors which coincide with the mineralogy and some physical data presented in this article for the Irazú volcanic caves. Therefore, we consider that our investigated caves can be used as analogue for the Mars environment. Future geomicrobiological analyses on these caves could give us clues in understanding the potential life-forms and geo-environmental interactions from an astrobiological perspective.

CONCLUSIONS

The northwest flank of the Irazú Volcano accommodates three volcanic caves, of which CMI and CMU have been studied in detail. The speleogenetic process that generated these caves is complex and involved tectonic (fault zone), weathering, high temperature degasification, leaching and erosion. The caves are developed in interbedded pyroclastic rocks, affected by hydrothermal alteration.

The studied caves show an extremely high mineralogical diversity, with forty-eight identified mineral species. The secondary mineral assembles of the studied caves are constituted mainly by sulfates, most of them belonging to hydrated acid and sulfates sub-group. Other mineral groups are also present, including native elements and minor halides, oxides and silicates. Thirteen of the mineral species are confirmed as new cave minerals, whereas five others are not confirmed yet by various techniques.

The particular environmental conditions in the studied caves, including location in an active volcano, active faulting, presence of thermal anomalies, interaction of sulfate-rich and acid water from volcanic lake infiltration, and probably rising of geothermal waters somewhere in the history of the caves, are the main factors that control this extreme mineralogical diversity.

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A unique small-scale microclimatic gradient in a temperate karst harbours exceptionally high diversity of soil Collembola

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Abstract: The collapse doline of the Silická ľadnica Ice Cave, 351 m² in area, is a unique phenomenon, a steep microclimate gradient in a low-altitude temperate karst in the Western Carpathian Mts, Slovakia, with a remarkable temperature decrease from the edge of karst plateau towards the doline bottom, which harbours perennial ice deposits. Collembola communities were studied in detail at seven sites along the 117.5 m long gradient slope during 2005–2007. An exceptionally high species richness of soil Collembola was observed, 129 species, which is about 91% of the total species richness generated by Chao1/ACE estimator. Species richness positively correlated with soil temperature at the sites. Among the occupants of the karst doline, 10 were Carpathian or Western-Carpathian endemics, and 21 were cold-adapted (psychrophilic) species with montane or boreo-montane disjunctive distribution. A high number and high abundance of endemic species occurred in the middle zone of the gradient slope. The study further showed that cold and wet karst scree slopes in the transition zone between surface habitats and caves may represent borderline habitats for obligate subterranean species. Communities at cold sites had much steeper rank-dominance curves compared to upper mesophilous and thermophilous sites, thereby documenting the harsh character of this environment. Our results suggest that small-scale microclimatic gradients in a low altitude karst in a temperate zone may serve as a reservoir (source) of exceptional soil fauna diversity, providing important climatic microrefugia for endemic and relict taxa. Karst landforms in the temperate zone with strong climatic inversions may harbour high biodiversity and thus should be central in biodiversity conservation programs.

Keywords: microrefugium, microclimate, ecotone, relict species, species vulnerability

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INTRODUCTION

The diversity of biota can be very high in some ecosystems of the temperate zone. The remarkable biodiversity of mountain regions is considered to be the result of a large variety of natural conditions, the fragmentation of the mountain ridges, diverse geological bedrock and landscape relief and the independent evolution of fauna and flora during historical periods (Kock et al., 2014). This is also well documented for the Carpathians Mountains, which are considered to be a clear hotspot of European diversity for many groups of organisms (Mráz & Ronikier, 2016). Studies focused on species diversity of soil arthropods have indicated high species diversity in heterogeneous environments (e.g., Brose et al., 2003; Tews et al., 2004). Surface karst landforms, the dominant and significant elements of karst regions, are characterized by heterogenous geomorphology, including deep valleys, gorges, sinkholes and collapse dolines that are considered “habitat islands” associated with microclimate and vegetation gradients, which determine high local diversity and endemism of biota (e.g., Bardgett et al., 2005; Raschmanová et al., 2008; Villisics et al., 2011; Bátori et al., 2011; 2012; 2017). The topography, soil microclimate, edaphic factors and structure of the vegetation cover of karst landforms play a crucial role in determining the diversity and abundance patterns of the soil Collembola at sites ruled by the climatic inversion (Raschmanová et al., 2008; 2013; 2016). Due to inverted microclimatic conditions, often with pronounced gradients, deep karst dolines may serve as climatic microrefugia (Rull, 2009) for a wider spectrum of relicts (Bátori et al., 2014), such as cold-adapted (psychrophilic) species with montane,
disjunctive boreo-montane and arctic-alpine distribution ranges. Generally, these habitats are considered buffered environments for psychrophilic forms under ongoing global warming in terms of their ability to sustain a more or less stable microclimate (Dobrowski, 2010; Raschmanová et al., 2013; Bátori et al., 2014; 2017), but in the same way they may also become endangered habitats, given the potential loss of their refugial features with changing conditions on the surface (Wynne et al., 2014). Thus, preservation of these karst landforms and their sensitive soil biota should be a part of proper conservation programs and biodiversity management of natural habitats (Breg, 2007; Raschmanová et al., 2015). Moreover, it was found that heterogeneous environments of temperature and vegetation gradients in karst landforms may be associated with cryptic diversity (Raschmanová et al., 2017). Generally, populations of species with narrow distribution along the gradient slopes are highly vulnerable, and their extinction may lead to the loss of local functional diversity in these ecosystems.

The Silická ľadnica Ice Cave, listed among UNESCO World Heritage Sites, is a unique phenomenon characterized by perennial ice accumulations and a pronounced microclimatic gradient in a collapse doline at the front of the cave. Our previous papers based on one-year study focusing on the doline showed relatively high local species diversity and distinct communities of soil Collembola at individual sites of the doline slope (Raschmanová et al., 2013; 2015). Several recent studies have stressed the importance of cave entrances as a unique and complex ecotope that may harbour high species diversity and endemism of terrestrial invertebrates (Wynne et al., 2014; Prous et al., 2015; Yao et al., 2016).

In the present study we analyzed a larger dataset from the Silická ľadnica Ice Cave originating over a 3-year period. We hypothesized that karst landscape gradients serve as islands of exceptional diversity of soil fauna and provide important microrefugia for endemic and relict taxa. The study aimed to answer the following questions: (1) what is the total diversity and dominance pattern of species in collembolan communities at individual sites along the microclimatic gradient in the collapse doline of the Silická ľadnica Ice Cave, and (2) what is the presumed role of natural small-scale microclimatic gradients in a low-altitude temperate karst in terms of a potential microrefugium and source of local species diversity of the soil fauna. Furthermore, data on local species richness of the unique climatic gradients in karst areas may provide a focus for legislation in term of conservation of rare and vulnerable species, as important components of global biodiversity.

**MATERIALS AND METHODS**

**Site description**

The Silická ľadnica Ice Cave (48°32’58.9”N and 20°30’13.9”E) is located in the Slovak and Aggtelek Karst, Western Carpathian Mts, Slovakia (Fig. 1A). The region is characterized by a mid-latitude karst landscape with a series of karst plateaus at elevations between 400 and 700 m a.s.l., with decreasing elevation from the north to the south, separated by 100–300 m deep canyons and valleys. Dolines (sinkholes), blind valleys and karrens belong among the typical landform features in this region. Mean annual temperature in the area ranges from +5.7 to +8.5°C and annual average precipitation from 630 to 990 mm (Rozložník et al., 1994; Hofierka et al., 2008).

The Silická ľadnica Ice Cave was formed in Middle Triassic Wetterstein limestones of Silica Nappe. The entrance zone of the cave is a steeply inclined spacious cavity with a large opening to the surface (corrosive-collapsed abyss, light hole). In this part, the cave contains perennial ice decorations and permanent floor ice in form of a little glacier. The cave entrance lies at an elevation of 470 m, facing north in a leafy forest. The upper edge of the collapse doline-like depression extends to a height of 503 m a.s.l. This site is thus the lowest-lying perennial ice cave below the latitude of 50 degrees north in the temperate climatic zone. The volume of ice accumulations in the cave ranges between 213 and 340 m³ (Roda et al., 1974). The permanent glaciation of the cave entrance zone is a relatively recent phenomenon induced by collapse of the cave roof dated back ~2000 years (Bella & Zelinka, 2018).

Seven sites were selected along the scree slope representing a natural temperature gradient on a 117.5 m transect line from the bottom of cave mouth to the upper part of the doline, (a surface area of 351 m²). Sites were situated on a scree slope of 5–35° at an elevation of 451–500 m a.s.l. facing south and southwest. For a detailed location of research sites, see Figs 1B–C. Site (1) was 7 m from the edge of permanent floor ice, a specific habitat near the cave entrance with primary soil on stony debris. Site (1) and (2) had pioneer vegetation of mosses and liverworts, shallow soil 3–4 cm deep and soil type lithosol. Site (3) had sparse pioneer vegetation of mosses, liverworts and herbal cover with Urtica sp., soil depth 10 cm at maximum, rendzina soil type. Site (4) was situated in a microdepression on a moderate slope with dense herbal cover and well developed 15 cm deep rendzina soil. Site (5) was hornbeam maple wood near a rock wall on a very moderate slope with dense herbal cover and shallow lithosol. Site (6) was young hornbeam wood on a steep slope with rendzina soil. Both sites (5, 6) had stony shallow soil, 5 cm deep. The last site (7) was cornel-hornbeam wood on the edge of the karst plateau with 10–20 cm thick soil profile, cambisol. The vegetational associations of sites were characterized by Petrášová & Šuvara (2008). At the bottom of doline (sites 1–3) the soil was frozen during winter and early spring and covered by snow in variable thickness and duration.

The sampling design, specifically the number of samples collected at the sites, was applied to prevent potential destruction of the unique and fragile habitats in the collapse doline. A total of 5 replicate soil samples were taken from each site in spring and autumn during the period 2005–2007 (18 May and
Fig. 1. A) Black circle – the collapse doline of the Silická ľadnica Ice Cave; B) Collecting sites in the collapse doline; C) Location of research sites (black circles) on scree slope in the collapse doline (according to Raschmanová et al., 2013) (for site numbers see chapter Methods).

13 November 2005, 18 May and 25 October 2006, 1 May and 6 October 2007), i.e., 210 samples altogether. The samples represented soil cores 10 cm in diameter to a maximum depth of 8 cm (depending on the soil thickness), including litter layer. Soil microarthropods were extracted in a modified high-gradient apparatus (Crossley & Blair, 1991) for 7 days. Collembola were identified to the species level using identification keys, specifically Weiner (1981); Pomorski (1998); Fjellberg (1998, 2007); Bretfeld (1999); Potapov (2001); Smolis & Skarzynski (2003); Thibaud et al. (2004); Dunger & Schlitt (2011); and others. This literature was also used as a source of data on geographic distribution and ecology of individual species. Collembola recorded at sites were classified after Culver & Pipan (2014) into edaphobionts as obligate soil-dwellers, troglobionts as obligate cave-dwellers and generalist species with occurrence in both soil and hypogean environments. Collembola specimens are deposited in collection of P. J. Šafárik University, Košice, Slovakia.

Climatic, soil-microclimatic, and soil-chemical data

Climate dynamics recorded by the nearest meteorological station in the village of Silica during the sampling period are provided in Fig. 2. The basic characteristics of the sites are given in Table 1. Soil temperature at the sites was measured by data-loggers (iButton DS1921G) continually every 4 hours from September 2006 to October 2007 at a depth of...
3 cm and calculated as daily averages; the mean, minimum and maximum values (T\text{mean}, T\text{min} and T\text{max}) were calculated for each site. Three soil samples were taken for gravimetric soil moisture from each site in October 2007. Further details on the microclimatic gradient at the collapse doline of the Silická Jadrnica Ice Cave are provided by Raschmanová et al. (2013). Soil pH was measured potentiometrically using a glass electrode and a reference calomel electrode as active pH in water (pH\text{H}_2\text{O}). Soil organic carbon content (C) and total N were measured according to Králová et al. (1991).

### Community data analyses

Abundance (A), species richness (S), the Shannon diversity index (H') and the Pielou index of evenness (J') were calculated for each site. Differences in soil temperature, moisture and Collembola abundance between sites were tested by nonparametric Kruskal–Wallis ANOVA, with post hoc multiple comparing (Statistica for Windows version 12).

To examine general trends in the Collembola dominance structure at each site, dominance curves were plotted for every site and for the three-year period. Species were ranked by dominance, and the percentage of the total number of individuals belonging to each species was plotted to base 10 logarithmic scale against species rank.

Theoretical species richness was estimated globally and for each site by diversity estimators from sample-based abundance data. By default, the biased-corrected form of Chao1 along with log-linear 95% confidence intervals is used. For those datasets with a coefficient of variation of the abundance distribution greater than 0.5, the larger from the Chao1 classic and ACE richness estimators is recommended (Chao, 1987; Chao et al., 2005; Colwell et al., 2012). Furthermore, the data were analysed using rarefaction procedures that are specifically designed to avoid the potential bias generated by uneven sampling. The estimation

Table 1. Vegetation associations, soil microclimatic and chemical characteristics at sites in the collapse doline.

<table>
<thead>
<tr>
<th>Vegetation associations</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>T\text{mean} [°C]</td>
<td>1.7 ± 2.16a</td>
<td>2.7 ± 2.43b</td>
<td>4.1 ± 3.09c</td>
<td>5.4 ± 4.0d</td>
<td>7.2 ± 5.31e</td>
<td>8.2 ± 4.92f</td>
<td>8.3 ± 4.81f</td>
</tr>
<tr>
<td>T\text{min} [°C]</td>
<td>-2.6</td>
<td>-0.5</td>
<td>-0.5</td>
<td>-0.3</td>
<td>-1.0</td>
<td>-0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>T\text{max} [°C]</td>
<td>5.0</td>
<td>6.3</td>
<td>8.5</td>
<td>11.0</td>
<td>16.3</td>
<td>17.5</td>
<td>17.5</td>
</tr>
<tr>
<td>W [%]</td>
<td>53.65 ± 3.76ab</td>
<td>63.72 ± 3.06a</td>
<td>64.23 ± 2.39a</td>
<td>47.64 ± 5.60ab</td>
<td>36.74 ± 4.19ab</td>
<td>33.17 ± 5.14ab</td>
<td>28.04 ± 3.75b</td>
</tr>
<tr>
<td>pH\text{H}_2\text{O}</td>
<td>7.3</td>
<td>6.9</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>6.5</td>
<td>6</td>
</tr>
<tr>
<td>C/N</td>
<td>28.1</td>
<td>30.9</td>
<td>22.5</td>
<td>21.3</td>
<td>26.3</td>
<td>24.3</td>
<td>21</td>
</tr>
</tbody>
</table>

T\text{mean} – mean soil temperature (from daily averages, September 2006–October 2007) and standard deviation, T\text{min} – daily minimum soil temperature, T\text{max} – daily maximum soil temperature, W – average soil moisture and standard deviation (October 2007), pH/H\text{H}_2\text{O}, C/N soil ratio (June 2009), (for site numbers see chapter Methods). Significant differences between sites (Kruskal–Wallis ANOVA with post hoc multiple comparing) are indicated by different lowercase letters for T\text{mean} [°C] and W [%] separately.
of species richness, rarefaction and extrapolation curves were calculated by the EstimateS software (Colwell, 2013).

RESULTS

Climatic, soil-microclimatic, and soil-chemical data

Based on the climatic data recorded from the nearest meteorological station, the annual air temperature and precipitation means for the sequence of years 2005–2007 were 7.6, 8.1, and 9.1°C, and 65, 57.6, and 54.3 mm, respectively (Fig. 2). Relatively high monthly precipitation was recorded in spring and summer of 2005 and 2006, and in spring and autumn of 2007. The vegetation, soil microclimatic and chemical characteristics documented notable differences at collecting sites along the slope (Table 1). The soil temperature (September 2006 – October 2007) at sites ranged as follows: \( T_{\text{mean}} \), 1.7–8.3°C, \( T_{\text{min}} \), -2.6–0.1°C, and \( T_{\text{max}} \), 5.0–17.5°C. \( T_{\text{mean}} \) was significantly different between sites at \( P < 0.05 \), while sites 6 and 7 represented a homogenous group. Soil moisture ranged considerably between 28.04% (site 7) and 64.23% (site 3); its value at site 7 was significantly lower than at sites 2 and 3, while other differences between sites in this parameter were statistically not significant. The highest soil pH was near the permanent ice (1), and identical pH values were found at sites 3–5 in the middle part of the transect. The cold sites (1, 2) had a higher C/N ratio compared to others, followed by two wood sites (5, 6); lower values of the parameter were recorded at sites with herbal cover (3, 4), and the lowest in the thermophilous wood on the edge of karst plateau (7) (Table 1).

Community data

Average values of abundances (A), species richness (S) and its estimator (Chao1 or ACE) and the diversity indices (H’, J) of communities at the sites are shown in Table 2. The mean abundance of the assemblages at the seven sites ranged from 9,151 to 38,209 individuals m\(^{-2}\). A clearly higher mean abundance was observed near the permanent floor ice (site 1) compared to other sites. Considerably high numbers of the dominant species Ceratophysella sigillata and cold-adapted cryptic species of the genus Folsomia (Raschmanová et al., 2017) in the samples taken at the coldest site (1) in spring led to an extremely high standard deviation of mean abundance. Relatively high values of the abundance were detected at unforested sites (2), in the slope microdepression with herbal cover (4) and in young thermophilous wood (6), followed by thermophilous wood on the plateau (7) and cold site (3) with more or less similar values. The lowest mean abundance was observed in the hornbeam-maple wood on the moistest slope (5), being significantly lower than at sites 2 and 4; other differences were statistically not confirmed.

Table 2. Community parameters of Collembola at sites in the collapse doline over three years (2005–2007).

<table>
<thead>
<tr>
<th>Samples</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A [ind.m(^{-2})]</td>
<td>38209ab</td>
<td>26807a</td>
<td>14718ab</td>
<td>20038a</td>
<td>9151b</td>
<td>173421ab</td>
<td>159871ab</td>
<td>210</td>
</tr>
<tr>
<td>St. dev.</td>
<td>±56629</td>
<td>±23645</td>
<td>±10327</td>
<td>±16025</td>
<td>±4400</td>
<td>±12404</td>
<td>±13597</td>
<td>-</td>
</tr>
<tr>
<td>H’</td>
<td>0.79</td>
<td>1.77</td>
<td>2.59</td>
<td>2.84</td>
<td>3.02</td>
<td>2.64</td>
<td>2.42</td>
<td>-</td>
</tr>
<tr>
<td>J’</td>
<td>0.22</td>
<td>0.47</td>
<td>0.65</td>
<td>0.69</td>
<td>0.73</td>
<td>0.63</td>
<td>0.60</td>
<td>-</td>
</tr>
<tr>
<td>S</td>
<td>35</td>
<td>44</td>
<td>52</td>
<td>61</td>
<td>61</td>
<td>64</td>
<td>57</td>
<td>129</td>
</tr>
<tr>
<td>CI for S</td>
<td>28.2-41.8</td>
<td>38.1-50.0</td>
<td>46.1-57.9</td>
<td>54.6-67.4</td>
<td>52.5-69.6</td>
<td>56.1-71.9</td>
<td>49.3-64.7</td>
<td>121.1-136.9</td>
</tr>
<tr>
<td>Chao1 / ACE</td>
<td>55</td>
<td>53*</td>
<td>57</td>
<td>67</td>
<td>77</td>
<td>77</td>
<td>66</td>
<td>143</td>
</tr>
<tr>
<td>CI for Chao1</td>
<td>40.2-107.9</td>
<td>-</td>
<td>53.0-72.7</td>
<td>62.5-82.0</td>
<td>65.6-118.7</td>
<td>67.7-109.6</td>
<td>59.4-88.6</td>
<td>133.8-167.1</td>
</tr>
<tr>
<td>S[%]</td>
<td>64.2</td>
<td>83.8</td>
<td>92.0</td>
<td>91.6</td>
<td>78.9</td>
<td>83.1</td>
<td>86.8</td>
<td>90.5</td>
</tr>
</tbody>
</table>

A – mean abundance, St. dev. – standard deviation of abundance mean, S – species richness, H’ – Shannon diversity index, J’ – Pielou index of evenness, Chao1 – richness estimator for individual-based abundance data,*ACE – abundance coverage-based estimator of species richness (only for site 2 computed), CI – 95% confidence intervals with lower and upper bounds; S[%] – S expressed as % of richness estimator, (for site numbers see chapter Methods). Significant differences between sites (Kruskal–Wallis ANOVA with post hoc multiple comparing) are indicated by different lowercase letters for A [ind.m\(^{-2}\)].

A total of 33,500 individuals of Collembola belonging to 129 species were collected at the sites during the sampling period (Appendix). Juvenile forms of the families Entomobryidae and Tomoceridae were not considered as separate species/taxa. High diversity indices were observed at sites 4–6, somewhat lower at unforested site (3) and at the top of plateau (7) (Table 2). The lowest values were recorded at cold sites with lithosol and a very shallow soil layer (1, 2) that hosted communities with highly abundant species C. sigillata, Folsomia sp., Protophorura aurantica and P. armata. The total species richness of Collembola at sites along the gradient (1–7) positively correlated with the mean and maximum soil temperatures: \( T_{\text{mean}} (r = 0.77; p < 0.05) \) and \( T_{\text{max}} (r = 0.88; p < 0.01) \). There were no significant correlations between environmental factors (\( T_{\text{mean}}, T_{\text{min}}, T_{\text{max}}, W \)) and other community parameters (A, H’, J).
A total of 143 species with CI 133.8–167.1 was found for all sites along the gradient, indicating that about 90.5% of the species richness was recorded in this study. The greatest difference between observed (S) and estimated richness was at cold site (1): 35 (CI 28.2–41.8) and 55 (CI 40.2–107.9) species, respectively; thus, only 64.2% of the estimated species richness was recorded in this case. On the other hand, a slight difference between both parameters was observed at unforested site (3), i.e., 52 and 57 species, with 92.0% of the estimated species richness. Sites (5) and (6) had a high estimated species richness, both with 77 species, followed by the site in the slope microdepression (4) and the thermophilous wood on the plateau (7), with 67 and 66 species, respectively.

The majority of species recorded have large distribution ranges: cosmopolitan, Holarctic, Palearctic, and European distribution. Altogether, 21 were montane species, 10 of which were Carpathian/Western Carpathian endemics (Appendix). Two Western Carpathian endemics of subterranean fauna, *Megalothorax carpaticus* and *Pygmarrhopalites agtelekiensis*, were recorded at cold site (2). The proportion of cold-adapted species to overall species richness at sites and their abundance to overall community abundance are provided in Figs. 5A–B. The highest richness of these species was recorded in the middle (4) and upper zones (5, 6) of the gradient (9–11 species), being lower at other sites along the gradient slope (6–7 species). Their proportion in abundance provided a considerably different picture: abundance decreased with temperature increase at slope sites (-0.93; p < 0.01). The highest abundance of psychrophilic species was found at the coldest site (1) due to the occurrence of the very
abundant species *C. sigillata*. On the other hand, a very low abundance of these forms was observed at the thermophilous forest sites (5–7). A high number and dominance of Carpathian/Western Carpathian endemics was recorded in the middle zone of the gradient (4), whilst their lower number and dominance were found at thermophilous (5–7) and cold sites (2–3).

Overall, we found 102 species of edaphobionts (increasing trend in numbers of species from the doline bottom to the plateau edge), 1 troglobiotic species and 24 generalist species. Two generalists were more abundant, namely *Protaphorura armata* and *Plutomurus carpaticus*, distributed at cold sites of the slope (1–4). The only troglobiont with marked troglomorphic characters was *Pygmarrhopalites aggtelekiensis*.

Fig. 5. A) The share of cold-adapted/psychrophilic to other species at sites over a three-year period (2005–2007) (for site numbers see chapter Methods), Sc – species richness of cold-adapted species, So – species richness of other species; B) Average abundances of cold-adapted species and others, Ac – mean abundances of cold-adapted species, Ao – mean abundances of others.

**DISCUSSION**

**Diversity and abundance patterns along microclimatic gradient**

Biodiversity can be quantified in many ways; however, species richness and single-index diversity (e.g., Shannon–Wiener) remain the most commonly used metric parameters to cover the alpha diversity of communities (Fleishman et al., 2006). In the present study species richness showed a strong positive correlation with soil temperature, as high species richness and also diversity indices were observed at sites in the middle and upper part of the scree slope. In contrast, low species richness and diversity indices were recorded at cold sites with a very shallow lithosol at the bottom of doline.

Species richness estimators and rarefaction curves are reliable and meaningful techniques for calculating the overall number of species used as an essential objective for conservation and management of natural habitats (Schneider & Culver, 2004; Budlé et al., 2005). Such attempts are very useful, especially for studies covering small areas, where more intensive sampling could cause habitat destruction. We found that the upper four sites of the doline slope had greater estimated species richness compared with the rest of the sites, as indicated by estimators and rarefaction curves of cumulative species richness. Saturation curves for individual sites did not reach an asymptote, indicating that the species inventory during the three years was still incomplete, ranging from 64 to 92%. The coldest site in the gradient had the lowest proportion of detected species and was associated with continual addition of species with random occurrence during the sampling period.

The rank-dominance curves documented the differences in the structure of Collembola communities between sites of the slope. Curves of the two cold sites were much steeper than the rest of the sites, indicating lower diversity and high dominance of a few species. The dominance structure at cold sites was thus unbalanced, showing low evenness due to the mass occurrence of the cold-adapted species *C. sigillata* and the cryptic species *Folsomia* sp. and *P. armata*. In contrast, communities at other sites of the gradient had more evenly spaced dominance among species. Moreover, the low standard deviations of mean abundances documented the even distribution of species abundance in soil samples, thus reflecting habitat homogeneity.

The highest abundances of Collembola and the lowest species richness and diversity indices were recorded at the two coldest sites of the inversion slope, thus reflecting a general trend of diversity and abundance of soil biota that is inversely related to latitude (e.g., Petersen & Luxton, 1982; Giller, 1996; Gaston, 2000; Bardgett et al., 2005). Collembola colonizing habitats with an extremely cold climate, such as polar tundra regions, have generally the higher abundances (145,000–245,000 ind. m$^{-2}$) and lower species richness (13–32) compared to temperate forests (Hale, 1966; Fjellberg, 1976; Petersen, 1982; Uvarov & Byzova, 1995). Thus, we may generalize that small-scale microclimatic gradients in karst landforms, characterized by distinct soil microclimatic and vegetation conditions, are analogous to the latitudinal patterns of biomes from deciduous forests to tundra.

**Exceptional diversity of Collembola in a unique karst landform**

The collapse doline of the Silická fajnica Ice Cave is a unique phenomenon with a strongly inverted microclimate gradient within a small area, leading...
to distinct Collembola communities (considerable species turnover along the gradient) and considerably high alpha diversity. The local biodiversity of natural karst landforms in the landscape have been greatly overlooked. Several studies pointed out that diverse geomorphology determines the richness of the plant and animal communities. This is especially true for a karst landscape evolved on limestone bedrock that is characterized by diverse environmental conditions with apparent microclimatic gradients in dolines, ravines, sinkholes and deep valleys (gorges) that may be considered as natural “habitat islands”, the key habitats for wider spectrum of endemic species and relicts (e.g., Ložek, 1972; Breg, 2007; Raschmanová et al., 2008; Černatič-Gregorič & Zega, 2010; Villisics et al., 2011; Bátori et al., 2014; Su et al., 2017). Our study documented that the small area of the collapse doline of the Silická ľadnica supports exceptional diversity of soil Collembola. Only half of the total number of species was recorded during the first year 2005 (Raschmanová et al., 2015). Observations longer than one-year and effective and non-destructive soil sampling designs may reveal the real biodiversity of these unique karst landforms. Soil sampling over three years resulted in a very high species richness, i.e., 129 species altogether, including six species new for science and two first records for Slovakia. This number was close to the estimated species richness (143 species), showing that we recorded 91% of estimated species richness during the study. The Central European mountains harbor a unique and major component of biological diversity in the temperate zone (Köck et al., 2014; Mráz & Ronikier, 2016). For instance, Stach (1959) recorded 134 species in the High Tatras Mountains in Poland, Nosek (1969) 211 species in the Low Tatras Mountains in Slovakia, Čuchta & Shrubových (2015) 209 species in the Bohemian Forest in the Czech Republic, Smolis & Skarzynski (2003) 118 species in the Beskid Niski Mts in Poland, and Weiner (1981) 191 species in the Pieniny Mts in Poland. However, an appropriate comparison of Collembola species numbers between the different studies is difficult due to the non-uniform sampling schemes (number and size of soil samples, size of the study area and length of the sampling period). Finally, our results suggest that karst surface landforms harbour considerable alpha diversity that may reach over 100 Collembola species. Despite the high biodiversity documented for heterogenous karst habitats, its substantial part may be still underestimated due to the cryptic diversity that has been revealed in some Collembola genera (e.g., Porco et al., 2012; Zhang et al., 2014; Katz et al., 2015). It was found that cryptic diversity is underestimated in terms of global species richness (Cicconardi et al., 2013) and rarely included in conservation programs (Scriven et al., 2015), although cryptic species may be seriously threatened in temperature gradients of karst landforms (Raschmanová et al., 2017). Based on this study and the previous studies conducted in the Slovak Karst, which covers 362 km² (Kováč et al., 1997; Kováč, 1999; Kováč et al., 2005; Raschmanová et al., 2008; 2015; 2017), this karst region comprises ~257 collembolan species occupying various soil habitats.

**Importance of the microclimatic gradient in preserving cold-adapted/psychrophilic species in karst landforms**

In Central Europe cold-adapted species often have disjunctive distributions at high altitude habitats in mountains, but also at low altitudes, where their isolated populations are restricted to specific habitats, such as cold scree slopes (Růžička, 2011) and the bottoms of dolines, deep ravines and sinkholes in karst areas with a prominent inversion of microclimate (Raschmanová et al., 2008; Vilisics et al., 2011). Montane species and species with a boreo-montane disjunction range shared 16% of total richness. The numbers of cold-adapted species increased at sites from the doline bottom to the plateau edge (except two upper sites), whereas their mean abundances showed an opposite trend. Psychrophilic species may be considered as rare at lower altitudes, where they have fragmented distribution ranges and usually also low abundances (e.g., Gaston, 1997). These species are particularly vulnerable to habitat changes and disturbances resulting in the decline of their distribution range and population size (Fattorini et al., 2013). Three psychrophilic species were abundant at cold sites, namely *C. sigillata, P. armata* and the cryptic species *Folsomia* sp., being known to prefer soils that tend to be moist and cold (Zettel & Zettel, 1994; Pomorski, 1998; Raschmanová et al., 2017). *C. sigillata* and *Folsomia* sp. were abundant at the bottom of the doline in spring, when the soil there is usually frozen and covered by snow. *Superodontella tyverica* was recorded exclusively at an extremely cold site near the floor ice, in primary soil on stony debris with mosses and liverworts. It was previously known only from forest soils of mountain and subalpine ecosystems in Ukraine (Karps, 2009), and this is the first record of this species in Slovakia. On the other hand, some montane species, such as *Smithinurina bimaculatus* and two species with boreo-montane distribution, namely *Micranurida granulata* and *Lepidocyrtus szeptickyi*, were recorded exclusively at warm sites; however, the latter two species in very low abundance.

**Endemic and relict species at sites in the collapse doline**

Endemic species are considered to be the most valuable and vulnerable components of soil biota and have the highest conservation value (e.g., Deharveng et al., 2000; Nitzu et al., 2018). Carpathian and Western Carpathian endemics shared 8% of overall richness. Two endemics, *Endonura dudichi* and *Pumilinura loksai*, were distributed in the middle and upper zone of the gradient slope in relation to their known association with thermophilous forest soils (e.g., Raschmanová et al., 2008; Smolis, 2008). Climate relict populations of various terrestrial taxa were more widely distributed in the temperate zone in the past, when climatic conditions were more conducive to a greater range (Culver & Pipan, 2009; Habel, 2010;
Hampe & Jump, 2011). Typical subterranean species, referred to as climate relicts, occupied cold sites at the bottom of the doline, namely Megalothorax carpaticus and Pygmarrhopalites aggtelekiensis, which are Western Carpathian endemics. M. carpaticus (a facultative cave species) is a eutroglophilous species known from various caves of alpine karst or cold caves situated at lower elevations, including cave entrances (Kováč et al., 2016). Based on the wide distribution in both karst and pseudokarst caves of the Carpathian Mts. and the slightly developed troglomorphic characters (morphological adaptations to subterranean environment), M. carpaticus may be considered as younger, glacial relict. The species was also recorded in the Gombasecká Cave, which belongs to the same cave system with the Silická Íadnica Ice Cave. In contrast, the troglobiont P. aggtelekiensis (an obligate cave species) shows very well developed troglomorphic traits and a distribution limited to caves in a few central and southern karst regions of the Western Carpathians; thus, it is presumably a descendant of the older lineage with Tertiary origin, considered to be a climate and phyletic relict (Kováč et al., 2016).

It is important to note that soil habitats (aphotic environment close to the surface) themselves are the kind of the shallow subterranean habitats which are inhabited with characteristic subterranean fauna, including both generalists and troglobionts (e.g., Pipan et al., 2011; Culver & Pipan, 2014; Rendoš et al., 2016; etc.). Generally, subterranean fauna is adapted to the stable microclimatic conditions of the subterranean environment (e.g., Culver & Pipan, 2009). In fact, the sites at the bottom of the collapse doline represent a transition habitat (ecotone in the sense of Prous et al., 2015) between the surface and subterranean environments and is characterized by relatively high soil moisture and stable cold soil temperature throughout the year, documented by low differences between soil temperature maxima and minima. P. aggtelekiensis as an obligate cave species is probably able to migrate between deep scree layers at the bottom of doline and the cave, where it has been found in greater numbers (Kováč & Papáč, unpublish.). The occurrence of P. aggtelekiensis in the soil at a cold site in October 2007 was probably linked with the exceptionally wet climate in this season, with extreme monthly precipitation. The external climatic conditions in this part of year (8°C) were thus similar to the internal climate of this cave, where unglaciated spaces have an annual air temperature of 6.8°C (Roda et al., 1974). Furthermore, these climate conditions (6.8–8°C) are analogous to the internal climate of most local caves (e.g., Kováč et al., 2016). This is the first occurrence of this troglobiont species outside of caves.

Cold and wet scree sites at the karst doline bottom have favourable conditions that may constitute a borderline habitat for obligate subterranean species. Generally, bare and forested scree habitats represent a unique habitat with their own fauna, but they also play the role of an ecotone, in which both epigean and obligate subterranean forms thrive (e.g., Ortúñ et al., 2013; Rendoš et al., 2016; Mammola et al., 2017).

### Anthopogenic and environmental biodiversity threats

The negative effect of anthropogenic activities and climatic change on soil biodiversity has become a major topic in conservation biology (Harte et al., 1995; Rusek, 1996; Addo-Bediako, 2000; Habel et al., 2010; Alatalo et al., 2015). For instance, deforestation of the karst landscape may profoundly affect climatic gradient environments by the temperature increase and decrease of soil moisture content. Many experimental field studies in polar and tundra habitats have shown that simulated temperature increase, or periods of drought, resulted in lower abundances and/or richness of Collembola (Tsiafouli et al., 2005; Briones et al., 2009; Petersen, 2011; Alatalo et al., 2015). The effect of climate change on soil organisms may be different at the population and community levels (e.g., Convey et al., 2002; Convey & Wynn-Williams, 2002; Daly et al., 2010; Alatalo et al., 2017). Hágvar & Klanderud (2009) concluded that collembolan species with a life cycle longer than one year were rather resistant to climate change during long-term warming experiment in subarctic habitats. The same climatic change may have a different effect on soil fauna in habitats of different climatic zones, biomes and ecosystems (Rusek, 1996; Hodkinson et al., 1998; Sjursen et al., 2005). Růžička et al. (2015) concluded that the microclimate of some low-altitude cold talus ecosystems in the temperate zone may be sufficiently resistant to an increase of mean annual atmospheric temperature from global warming by 3°C.

Thus, scree deposits on mountains slopes function as climatic microrefugia for cold-adapted species.

Finally, there is the reasonable threat to the floor ice at the cave entrance of Silická Íadnica Ice Cave. This is documented by an increasing trend in the annual air temperature means observed by the local meteorological station in the period 2010–2016 (between 7.7 and 10.1°C). In addition, Fendeková et al. (2018) reported marked temperature increases (warming) in the whole territory of Slovakia in 2011–2015 based on long-term climate observations carried out from 1981. The Silická Íadnica Ice Cave is indeed a unique natural phenomenon in a temperate zone with perennial ice accumulations at the cave entrance and a profound climatic gradient along the slope from the bottom of the collapse doline to the edge of the karst plateau. Long-term observations are needed in order to better understand what the response of the local soil fauna communities will be to climatic changes. It is at least necessary to maintain primeval forests in the surrounding karst landscape to moderate the potential impacts of climate change on the microclimatic gradients in landforms and their associated biota (Bátori et al., 2014).

### CONCLUSIONS

First, our study documented that the local (alpha) diversity of soil Collembola in a karst landform with a pronounced climatic gradient may be very high in the temperate zone, thus serving as a reservoir of exceptional soil fauna diversity. Small-scale
microclimatic gradients within the diverse karst relief are reflected by the presence of endemic species and psychrophilic forms, some of them considered to be relicts surviving in isolation in the same area from colder periods. Such conditions lead to local species differentiation and evolution of cryptic diversity that highlights the biological value of natural temperature gradients in karst landforms and the importance of their conservation. Second, the cold and wet part of the scree slope in a collapse doline serves as an ecotone between subterranean (cave) habitats and the surface environment that is supported by the occurrence of troglobiotic species. Third, cold microhabitats at the doline bottom will be under threat of global climate warming; therefore, these habitats should be central in conservation priorities and management of natural habitats. Finally, karst landforms with distinctly inverted climatic gradients are excellent areas for long-term monitoring of climate change and its effect on the alpha diversity of biota.

ACKNOWLEDGEMENTS

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Exceptional diversity of soil Collembola in temperate karst

International Journal of Speleology, 47 (2), 247-262. Tampa, FL (USA) May 2018


Appendix. List of soil Collembola at sites in the collapse doline, their mean abundance (ind.m⁻²) during 2005–2007 and their ecological and geographical characteristics.

<table>
<thead>
<tr>
<th>Abbrev.</th>
<th>Group</th>
<th>Species</th>
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<td>Allacma fusca (L., 1758)</td>
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<td>Caprinea marginata (Schött, 1893)</td>
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<td>Deuteraphorura cebennaria (Gisin, 1956)</td>
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**EN, M**

| E | e | Orthonychiurus rectopapillatus | (Stach, 1933) |
| W | G | Oncopodura crassicornis | Shoebotham, 1911 |
| E | e | Orchesella bifasciata | Nicolet, 1842 |
| E | e | Orchesella flavescens | (Bourlet, 1843) |
| C | e | Parisotoma notabilis | (Schäffer, 1896) |
| EN,M | G | Platotumaris carpaticus | Rusek et Weiner, 1978 |
| H | e | Pogonogathellus flavescens | (Tullberg, 1871) |

| u | e | Proisotoma sp. juv. | - |

| W | G | Protaphorura armata | (Tullberg, 1869) |
| W | e | Protaphorura aurantiaca | (Ridley, 1880) |
| U | e | Protaphorura cancellata | (Gisin, 1956) |
| P | e | Protaphorura campata | (Gisin, 1952) |
| U | e | Protaphorura gisini | (Haybach, 1960) |
| U | e | Protaphorura pannonica | (Haybach, 1960) |
| U | e | Protaphorura subarmata | (Gisin, 1957) |
| U | e | Protaphorura subuliginata | (Gisin, 1956) |
| P | e | Protaphorura tricampata | (Gisin, 1956) |
| E, P | e | Pseudanurophorus boernerti | Stach, 1922 |
| H | e | Pseudachorutes corticiculus | (Schäffer, 1896) |
| P | e | Pseudachorutes dubius | Krausbauer, 1898 |
| P | e | Pseudachorutes parvulus | Börner, 1901 |
| SE | e | Pseudachorutes palmiensis | Börner, 1903 |
| P | e | Pseudachorutes subrussell Tullberg, 1871 |
| C | e | Pseudisotoma sensibilis | (Tullberg, 1876) |
| U | e | Pseudosinella sp. 1 |
| U | e | Pseudosinella sp. 2 |
| U | e | Pseudosinella albida | (Stach, 1930) |
| E | e | Pseudosinella horak Rusek, 1985 |
| U | G | Pseudosinella thibaudi | Stomp, 1977 |
| E | e | Pseudosinella sanguinolenta | (Schille, 1908) |
| E,EN | e | Pumilinura loksai | (Dunger, 1973) |
| E,EN | T | Pygmarrhapolites agtelekiensis | Stach, 1945 |
| E | G | Pygmarrhapolites bifidus | Stach, 1945 |
| H | G | Pygmarrhapolites pygmaeus | (Wankel, 1860) |
| E | e | Sminthurinus aureus | (Lubbock, 1862) |
| BM | e | Sminthurinus bimaculatus | Axelson, 1902 |
| W | e | Sminthurinus elegans | (Fitch, 1863) |
| M | e | Sminthurinus gisini | Gama, 1965 |
| SE | e | Spathulosminthurus guthriei | (Stach, 1920) |
| C | e | Sphaeridia pumilis | Krausbauer, 1898 |

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Exceptional diversity of soil Collembola in temperate karst
International Journal of Speleology, 47 (2), 247-262. Tampa, FL (USA) May 2018
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