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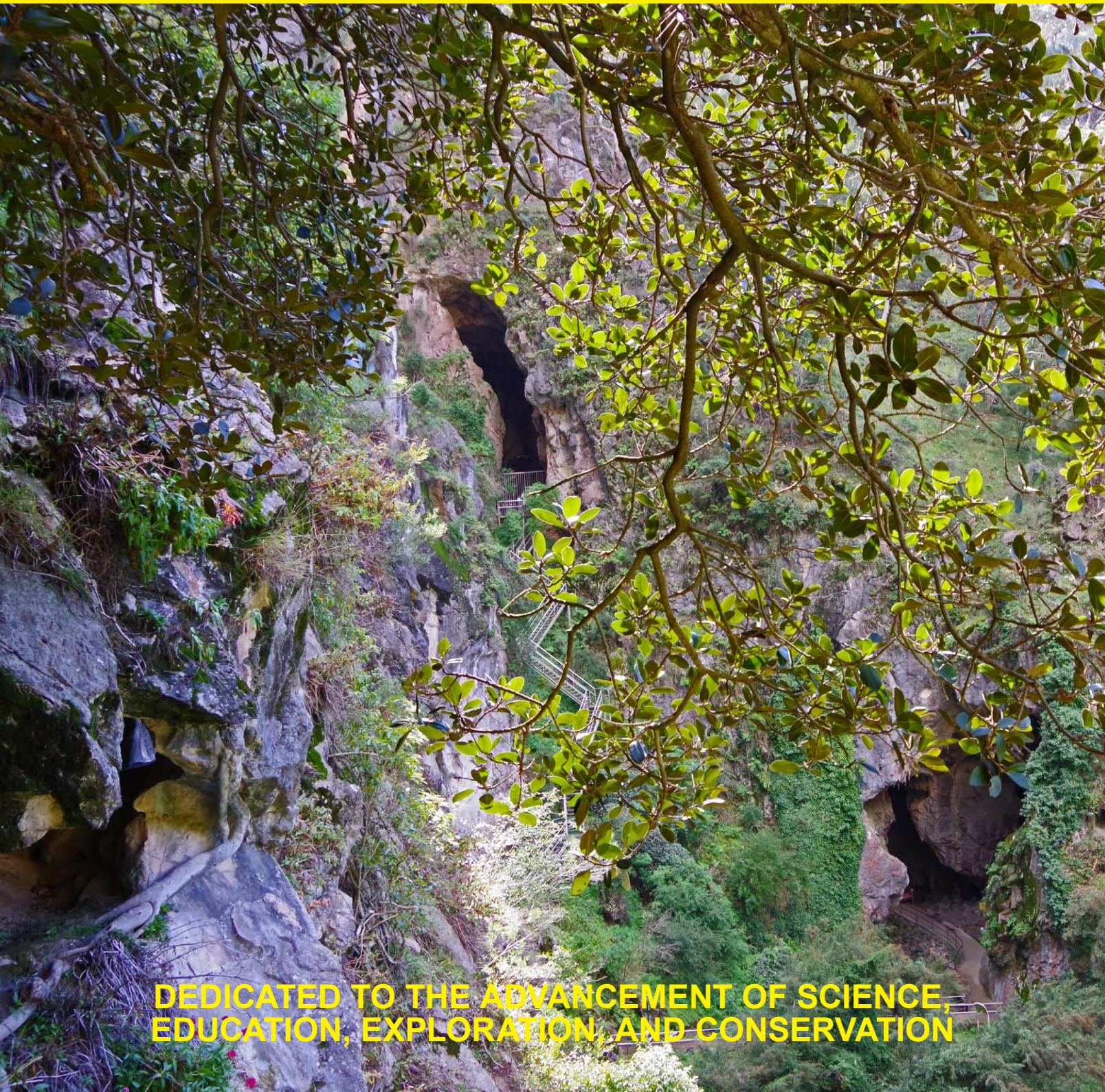

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Front cover: Jenolan Caves, New South Wales, Australia. Visited during the International Congress of Speleology field trip July 2017. Photograph by S. Engel.

EDITORIAL: CHANGES TO THE *JOURNAL OF CAVE AND KARST STUDIES*

Scott A. Engel
Production Editor

In 2011, the *Journal of Cave and Karst Studies* began transitioning to an electronic distribution process. While not all readership agreed with this change, it was done, in part, to address budgetary limitations within the National Speleological Society. But more importantly, it was done to conform with to similar changes being done by peer publications and across the publication industry. As predicted, in the six years since we made this transition, the movement away from print distribution and toward electronic only distribution has continued to expand and evolve across the scientific community and the globe. In that respect, our experiment has been a success, and it is increasingly apparent that a return to full print distribution is unlikely and unnecessary.

With that in mind, this issue represents the next evolution of the Journal as we continue to modernize our production process. As you look through this issue, you will find that we have made two major changes to improve the *Journal.* A brief description of the changes and the reasons behind them are presented below.

Publication Schedule

By transitioning to a predominantly electronic distribution, we have been able to control and reduce costs incurred by the National Speleological Society to produce and distribute individual issues of the *Journal*. As a positive outcome of these cost savings, the Board of Governors has authorized the *Journal* to increase our publication schedule from 3 issues per year to 4 issues per year. The last time the *Journal* had sufficient material and budget to produce 4 issues in one year was 1983. This is an important change that will help to reduce the time between a manuscript submission and publication, allowing current and topical research get out to the readership faster. Because we feel this is such an important improvement, we decided to not wait until the new year to implement this change. You may have already noticed that you did not receive the traditional August issue of the *Journal*. Instead, this issue is dated September, and is the first issue to represent our new quarterly schedule. Going forward, issues of the *Journal* will be published in March, June, September, and December.

Format

With this issue, we are modernizing the *Journal* presentation format. The new format has been designed to improve online viewing and readability, to maintain a professional quality and look that is consistent with peer publications, and to reduce our production time. The most significant change is the transition from a multiple column format to a full page format. This should improve the electronic reading experience by eliminating the need to scroll up and down the page to transition between columns and by making individual pages easier to read at a higher magnification, regardless of the type of device or screen size used for viewing. Additional major changes include use of a more electronic friendly font, left justifying titles and section headers, eliminating the use of small caps to facilitate proper capitalization and italicization of biological names, and simplifying the author information footnotes format.

We, the *Journal* editorial staff, hope these changes will result in an improved experience for both authors and readers of the Journal going forward and will preserve and enhance our mission to promote cave and karst science research and education. We would welcome any comments or suggestions you may have in relation to the new format.

JOIN THE JOURNAL OF CAVE AND KARST STUDIES STAFF

The Journal is seeking qualified volunteers to support our mission of providing a multidisciplinary platform for the publication of peer reviewed research related to caves and karst science. We encourage anyone interested in serving as an associate editor, reviewer, copy editor, or production assistant to contact the Editor-in-Chief, Dr. Malcom Field with your qualifications.

Caroline A. Broderick, Carol M. Wicks, and Randall L. Paylor. Testing the effectiveness of beryllium-7 as a tracer of the movement of sediment over short periods along a cave stream in Hidden River Cave, Kentucky U.S.A. *Journal of Cave and Karst Studies*, v. 79, no. 2, p. 84-88.
DOI: 10.4311/2016ES0115

TESTING THE EFFECTIVENESS OF BERYLLIUM-7 AS A TRACER OF THE MOVEMENT OF SEDIMENT OVER SHORT PERIODS ALONG A CAVE STREAM IN HIDDEN RIVER CAVE, KENTUCKY U.S.A.

Caroline A. Broderick[1], Carol M. Wicks[1,C], and Randall L. Paylor[1]

Abstract

A feasibility study using beryllium-7 (^{7}Be) as a tracer of the movement of sediment through a karstic basin over short timescales of 2 to 3 months was undertaken. If ^{7}Be is an effective tracer of sediment movement, then the average ^{7}Be activity measured on sediment collected from backwater facies after high-flow events would be higher than that measured after periods of low-flow conditions. To test this, sediment was collected along a cave stream in Hidden River Cave, Kentucky, U.S.A. At the most basic level, collected sediment had measureable ^{7}Be activities, so using ^{7}Be to track movement of sediment from the surface into karstic basins is a viable method. In addition, the measured ^{7}Be activities were higher after a period of rainfall and presumably high flow events (> 1 d m^{-1} g^{-1}) than after a period of relatively few rainfall events and presumably low-flow conditions (0.16 d m^{-1} g^{-1}).

Introduction

This study uses isotopic dating of the naturally occurring isotope ^{7}Be ($t_{1/2}$=53.3 days). Other isotopic tracers, such as cesium-137 ($t_{1/2}$ = 30.2 years) and lead-210 ($t_{1/2}$ = 22.2 years), which are used extensively for sediment tracing and budget studies (e.g., Blake et al., 2002), are not useful for short timescales that may be important for studies in karstic systems (Mahler and Lynch, 1999).

^{7}Be forms when low energy neutrons collide with oxygen and nitrogen atoms in the stratosphere (Brost et al., 1991; Junge, 1963). The newly created ^{7}Be atoms circulate in the atmosphere until the ^{7}Be atoms are scavenged by submicron particles that continue circulating in the stratosphere or lower atmosphere as the attached ^{7}Be radioactively decays. Approximately 59% of ^{7}Be atoms decay in the atmosphere, the other 41% of ^{7}Be atoms fall to earth's surface (Brost et al., 1991). Of the ^{7}Be that reaches earth, 94% falls in precipitation and 6% falls as dry deposition.

^{7}Be decays into ^{7}Li through orbital-electron capture (Walsh, 2009) when a proton from the nucleus reacts with one of the electrons from the K electron cloud (e_k) and releases a neutrino (v) and gamma rays as described by Hans (2001): $^{7}Be + e_k \rightarrow {}^{7}Li + v$. Thus, ^{7}Be activity is measured by counting gamma emission that is directly related to decay of ^{7}Be (Corbett et al., 2007; Fabre, 2012).

On the surface of the earth, the ^{7}Be absorbs preferentially on the surface of aluminosilicate particles in soils and sediment. Sediment with measurable ^{7}Be activity indicates that the sediment was exposed to atmospheric deposition, either dry or wet, within three to five half-lives (160 to 270 days; Rotondo and Bentley, 2003). Thus, ^{7}Be has been used in studies of transport of fine-grained sediment in rivers (Sommerfield et al., 1999), Bonniwell et al., 1999) and the erosion of soils (Blake et al., 1999) and in sediment-budget studies (Rotondo and Bentley, 2003; Corbett et al., 2007). Other studies have combined ^{7}Be, ^{137}Cs, and ^{210}Pb for investigation of land degradation and sediment residence times in catchment systems (Brost et al., 1991; Evrard, et al., 2010; Bentley et al., 2014). These cited works provide an understanding of source, transport, and deposition of fine-grained sediments during recharge events. All of those studies investigated transport of sediment over short time scales.

Allochthonous sediment is brought into caves as soil eroded from the surface or as sediment transported into the cave streams by sinking streams during high-flow events (Livesay and McGrain, 1962). Bosch and White (2007) describe the transport of allochthonous sediment into and through karstic basins and the resultant lithofacies: that are diamicton (unsorted, unstratified sediment), thalweg (armor layer), channel (well sorted), and slackwater (carried as suspended load). Previous studies have shown that nearly all of the sediment transport through karstic basins occurs during or immediately following high-flow events (Mahler and Lynch, 1999; Groves and Meiman, 2005). Mahler et al. (1998) state that the best way to calculate the residence times of sediment in karstic basins is through the use of particle tracing. Thus, there is a need for a sediment tracer that tracks sediment movement over short time scales. This study assesses the feasibility of using ^{7}Be as a tracer of the movement of sediment into and through karstic basins with a focus on sediment transported as suspended load and deposited as slackwater facies.

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Description of Study Site

Hidden River Cave (HRC) is within the 900 km^2 Gorin Mill basin in Kentucky, U.S.A. (Fig. 1). The area is part of the Mammoth Cave region and is located about seven miles due east of the National Park boundary. The Gorin Mill basin is characterized by isolated hills (knobs) rising at most 40 m above a karst plain that is pockmarked with numerous shallow (less than 10 m deep) sinkholes. There is no discharge measuring station within the Gorin Mill basin. There are three precipitation stations adjacent to the Gorin Mill basin.

HRC is a fluviokarst system. The humanly accessible entrance to HRC is located in the town of Horse Cave (37°10′34″ N, 85°54′22″ W; Livesay and McGrain, 1962). Water enters the cave as sinking streams in its distal portions (Toomey and Olson, 2008). The cave stream flows along conduits, past the humanly accessible entrance, and continues downstream in the subsurface. Sampling sites were located near to and in the upstream direction from the human entrance.

Field Methods

Samples of sediment from within HRC were collected on April 15, April 29 to May 1, and on May 20 and 21, 2014. These samples were collected at locations near the waterline of the cave stream by using a thin-bladed spatula to carefully scrape the topmost 2 cm of sediment into petri dishes. The area scraped was less than a 100 cm^2. All samples were brought back to Louisiana State University's Department of Geology and Geophysics for determination of ^{7}Be activity, granulometry, and mineralogical composition. Scraping from a limited area to a fixed depth limited the volume and mass of sediment collected. Thus granulometry and mineralogical analyses were limited to a subset of collected samples, whereas, the activity of ^{7}Be was determined for each sample collected.

Laboratory Methods

Upon return to LSU and following the method in Keller et al. (2016), the samples were dried in an oven at 60°C for a minimum of twelve hours, visible organics and large pebbles were removed by hand, and then the remaining sediment was ground. For each sample, the ^{7}Be activity in disintegrations per minute per gram (d m^{-1} g^{-1}) was determined using a Canberra Broad Energy Germanium Detector (BEGE) with processing handled using Canberry GenieData Software. Samples were held in the BEGE detector for a minimum of twenty-four hours so that significant spikes in the gamma-ray spectrum could be identified at the signature 477 keV energy of the decay. The measurements were corrected to account for the decay that occurred since the date of sample collection.

Granulometric analyses were performed using a Beckman-Coulter LS 13 320 laser diffractor. The duration of the laser diffraction runs was five minutes. Pre-processing included sieving the previously dried sample through a 1 µm sieve, rinsing the resultant sample with hydrogen peroxide (0.05 v/v H_2O_2), and deflocculating the sample with 10 mL of 0.05% sodium phosphate (NaH_2PO_4). After pre-processing, each sample was analyzed with the Beckman Coulter laser diffractor for five minutes to collect

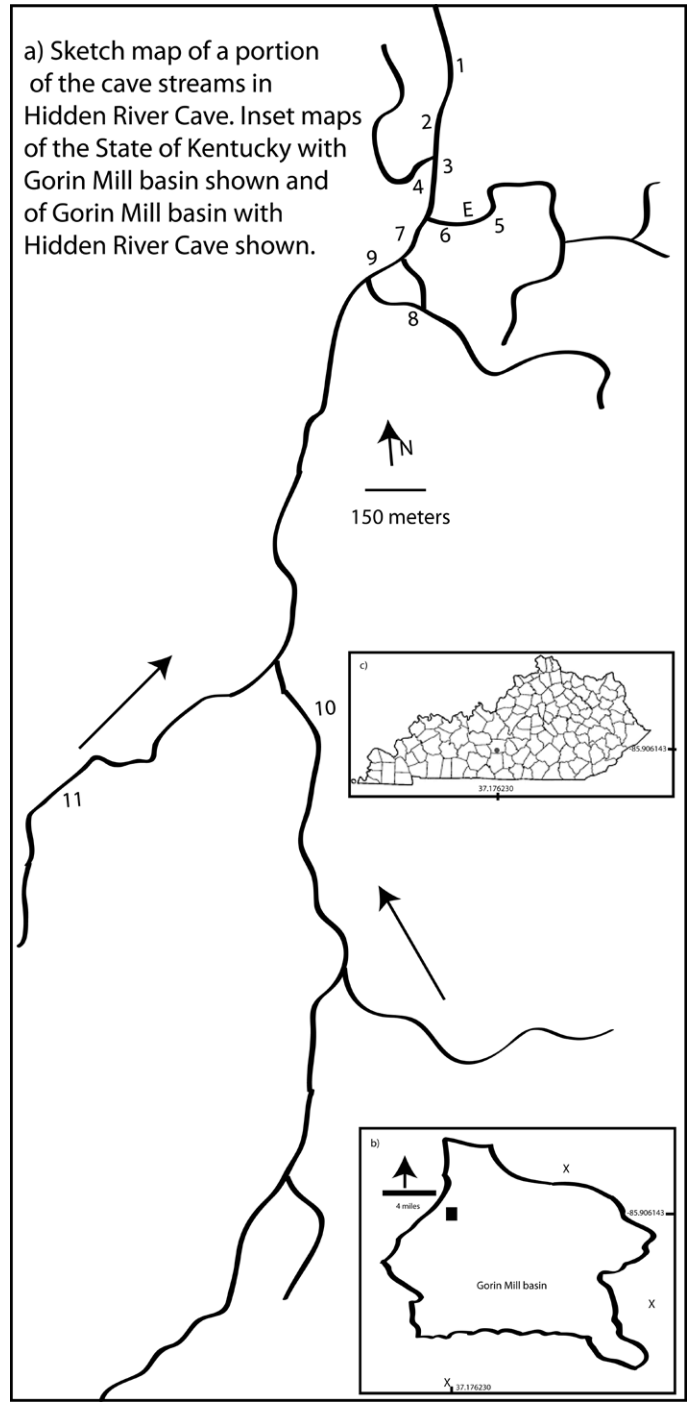

Figure 1. Simplified map showing a portion of the streams in Hidden River Cave that were sampled. The entrance (E), sampling sites (1–11), and flow direction (arrows) are shown (panel a; modified from West, 2013). Location of Hidden River Cave (black square) and nearby precipitation records (×) of the Gorin Mill basin are shown (panel b; Kentucky Geological Society). Location of Gorin Mil basin within the State of Kentucky (panel c).

the mean and the range of grain sizes.

Bulk mineralogical analysis was performed using an Empyrean x-ray diffractometer. Pre-processing included sieving the previously dried samples using a 30 μm sieve, mixing the sample with 10 mL of ethanol, processing in the micronizer and centrifuge, and drying overnight at 60°C (Cook et al., 1975). After pre-processing, samples were analyzed on the XRD by the Reflective Transmission Spinner with the automatic divergence slit between 4° and 70° scans. The scans were done at 0.02 degree increments for 60 seconds. Data were processed through Jade software following standard techniques (Griffin, 1971).

Results

The mineralogical composition of the sediment was 84–88% quartz, 8–12% aluminosilicate clays, and approximately 2% carbonate (Table 1). The mean grain size range from 48 to 77 μm. The ^{7}Be activity ranged from 0.10 to 1.33 d m^{-1} g^{-1}.

As there is no discharge recording location within the Gorin Mill basin, we have had to rely on data collected at nearby locations. This adds uncertainty to our study, because we had to rely on precipitation records to guide our understanding of the timing of low- and high-flow events. Side-by-side duplicate sediment samples were collected on May 29. The ^{7}Be activities of the duplicates were below analytical detection limit; therefore we could not determine variation at the scale at which we collected samples (100 cm^{2}).

Discussion and Summary

Sediment with measurable ^{7}Be activity indicates that the sediment was exposed to atmospheric deposition (either dry or wet) within three to five half-lives (160 to 270 days; Rotondo and Bentley, 2003). As sediment collected from within Hidden River Cave had measurable ^{7}Be activities (Table 1), this sediment had been exposed to atmospheric deposition in the previous 270 days, and that sediment had been at the land surface and transported into HRC within the previous 270 days. This shows that ^{7}Be can be used as a tracer of sediment movement in karstic basins.

There were numerous rainfall events during April and May 2014, including two large events: one occurred on April 28 and the other on May 14 (Fig. 2). These large precipitation events likely resulted in high-flow events that transported

Table 1. ^{7}Be activity and mineralogical composition of the sediment.

Collection Date	Location Sampled	Be-7 Activity, dpm g^{-1}	Quartz, %	Alumino-Silicate Clay, %	Carbonate, %	Grain Size, μm
4/15/2014	3	0.1	···	···	···	···
4/29/2014	2,5	0.28 ± 0.33	85 ± 3.5	11 ± 2.7	1.9 ± 0.16	48 ± 47
4/30/2014	6	0.22	···	···	···	···
5/1/2014	9	0.91	···	···	···	···
5/20/2014	1, 3, 4, 7	0.90 ± 0.56	88 ± 3.3	8.4 ± 2.7	1.8 ± 0.3	73 ± 45
5/21/2014	8, 9, 10, 10	1.33 ± 1.89	84 ± 3.1	12 ± 3.6	2 ± 0.7	77 ± 58

suspended sediment into HRC. The measured ^{7}Be activity in slackwater facies, ranging from 0.22 to 1.3 d m^{-1} g^{-1}, was higher after the two large precipitation events compared to that measured prior to either large precipitation event (0.16 d m^{-1} g^{-1}). Given our null hypothesis that the measured ^{7}Be activity prior to and after high-flow events would be equal and based on a two-sided t-test analysis, the null hypothesis was rejected. This supports our understanding of suspended sediment being delivered into the cave stream by losing streams and deposited as slackwater facies (Bosch and White, 2007). This also lends evidence for the need for tracers to track movement of sediment over short time-scales in karstic basins (Mahler and Lynch, 1999).

Bentley et al. (2014) have reported a relation between ^{7}Be activity and grain size and between ^{7}Be activity and mineralogical composition of the sediment. The sediment

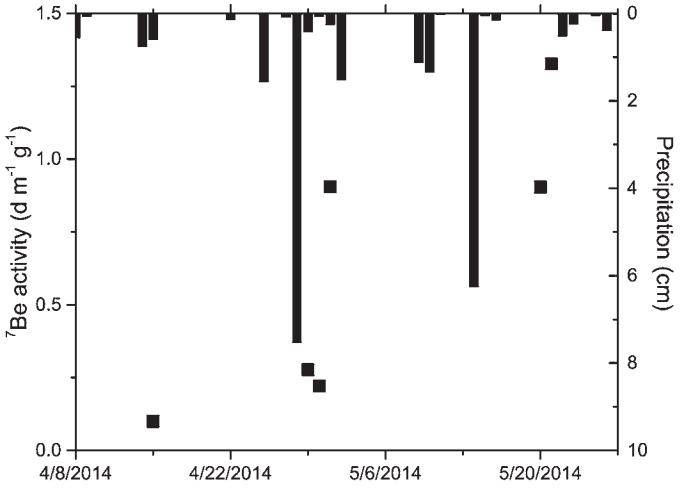

Figure 2. Precipitation (bars) and ^{7}Be activity (closed circles) as a function of time.

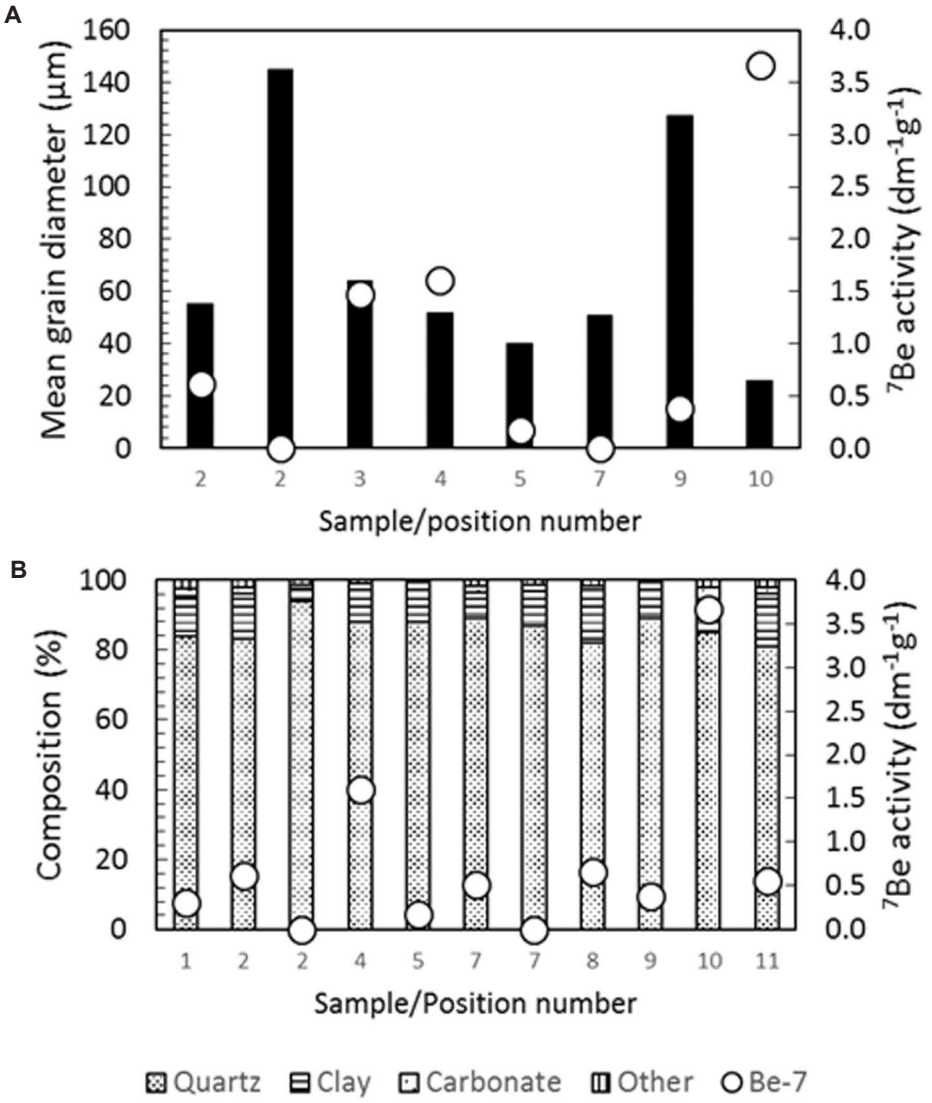

Figure 3. ^{7}Be activity (closed circles) plotted against particle size (bars; panel A) and against mineralogical composition (bars; panel B).

was fine-grained and primarily aluminosilicate in composition. We found no relation between the ^{7}Be activity and the mean, mode, or median grain size of the coarser-grained sediment collected in this study or between the ^{7}Be activity and mineralogical composition (percentage quartz, calcite, or clay) of the calcite-rich sediment collected in this study (Fig. 3). In all cases, correlation coefficients were less than 0.25 and the F-significance levels were greater than 0.15. The coarser and quartz-rich sediment collected in this study might have masked the relations shown by Bentley et al. (2014), who sampled a finer-grained, alumno-silcate rich sediment.

In summary, the goal of this feasibility study was to determine if ^{7}Be was a viable tracer of sediment transported into karstic basins as suspended load and deposited as slackwater facies. The data show that ^{7}Be is a useful tracer. This study was of limited scope due to the small volume of sample that could be scrapped from along the cave stream. In future work, researchers should plan to sample a larger area to collect more grams per location without sampling more than 2 centimeters of depth. Sampling could also be timed to coincide with months of low flow for better control on background activities and high flow for better results on storm-event signals. Even with these limitations, the ^{7}Be method used in this investigation will aid researchers who study movement of sediment in karstic basins.

Acknowledgements

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A. Jacobs, D. Msimang, and E. Venter. First survey of the fungi from the Bakwena Cave in South Africa suggests low human disturbance. *Journal of Cave and Karst Studies*, v. 79, no. 2, p. 89-94. DOI: 10.4311/2016MB0146

FIRST SURVEY OF THE FUNGI FROM THE BAKWENA CAVE IN SOUTH AFRICA SUGGESTS LOW HUMAN DISTURBANCE

Adriaana Jacobs[1], Duduzile Msimang[2], and Eduard Venter[3, C]

Abstract

Currently there are no studies on the microbial karst niche in South Africa. Most of the studies on these environments focus on archaeology and animals that occur in caves. However, there are several caves that are threatened by urban development of the surrounding areas. The Bakwena Cave is such a threatened cave with extensive urban development of the surrounding grassland biome it is located in. The cave is located in dolomite and the entrance is a sinkhole. This cave serves as a permanent roost for a large population of Natal clinging bats *Miniopterus natalensis*. The ecosystem of the cave is driven by the deposition of guano by the bats, as there is no deep penetration of plant debris from the outside. To identify the fungal component of the microbial ecosystem, we sampled guano, soil, and sediment from various areas within the cave over a period of one year. All isolations were performed on low-nutrient medium to restrict the colony growth and to ensure that all culturable fungi were obtained. These isolates were barcoded using the ITS1 gene region to identify them and to establish a baseline of fungi occurring in South African caves. The majority of isolates associated were *Aspergillus* and *Penicillium* species identified in previous studies from cave environments. Some opportunistic pathogens were identified that could have an impact as more people visit the cave due to its close proximity to housing developments. However, currently the cave fits a model that indicates it has a low level of human disturbance. Our study is the first on the cave fungal component in South Africa and provides a baseline that can advise developers and environmental impact assessments on fungal species found in caves.

Introduction

The Bakwena Cave is located to the south of Pretoria in Gauteng, South Africa, in an escarpment of dolomite (Martini and Kavalieris, 1976). It is a publically accessible cave that is located on the perimeter of the Agricultural Research Council Irene campus (S25°53′53.3″ E28°13′19.8″). The cave serves as a permanent roost for Natal clinging bats (*Miniopterus natalensis*) (Durand et al., 2012), while Temminck's hairy bat (*Myotus tricolor*) and Geoffry's horseshoe bat (*Rhinolophus clivosus*) occur intermittently. The cave is irregularly used by amateur speleologists and other groups. Entrance to the cave is by rappelling through a cone-shaped sinkhole to the top of a shaft that has a 5 m ladder fitted to the side. The bottom of the ladder rests on the top of a scree slope that provides access to the large main cavern below (Fig. 1), with several large guano deposits found in the main chamber. The cave system consists of a large main chamber that has three tunnels leading from it. The tunnel located in the most eastern part of the cave extends at a slope for 22 meters to end at the edge of the water table. This cave is one of the few dolomitic caves in South Africa that provide access to the water table (Durand et al., 2012). At the southwestern end of the cave, two tunnels lead to two smaller chambers. The largest of these is a permanent roost of the Natal clinging bat and contains large amounts of guano. The second chamber is accessible through a narrow tunnel. There are no bats roosting in this chamber, and it has a very narrow tunnel that leads from it. The average temperature and relative humidity in the main chamber are 15.4 ± 0.4°C and 93.2 ± 2.7% and for the larger side chamber are 21.1 ± 1.6°C and 100.0 ± 0.6%. The Bakwena Cave was surrounded by large tracts of grassland that serve as foraging area for the bats. Currently the cave is under threat by urban development, and the resulting habitat shrinkage and fragmentation will likely have a negative impact on the bats that utilize the cave as a roost. As more housing is developed close to the cave, there will most probably be more visitors that could increase the levels of fungal-associated diseases.

Karst environments such as those found in the Bakwena Cave host different microbial groups, and the diversity is affected by the energy status of the cave (Groth et al., 1999; Jurado et al., 2008). These microbes occupy a variety of substrates, such as sediments, vermiculations, guano, and decaying organic material that create different ecological niches (Nováková, 2009; Portillo et al., 2008). The cave is surrounded by grass and trees that grow down the entrance slope that was created when the sinkhole formed and results in soil and plant debris being carried into the cave during the rainy and windy seasons. The depth to which this debris penetrates is limited by the percolating effect of the scree slope. The large number of bats, ranging between an estimated 800 and 1850 depending on the season (Durand et al., 2012), roosting in the cave results in large guano deposits that provides a rich niche for the growth of microbes. Global research suggests that the most common cave fungi are Ascomycetes, with Zygomycetes and Basidiomycetes

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being isolated at less abundant levels (Vanderwolf et al., 2013). Most species isolated from caves are soil saprophytes, and the predominant differences reported between studies are introduced by spatial diversity patterns and substrate specificity (Vanderwolf et al., 2013). Karst environments have been considered extreme habitats inhabited by specialized species (Lorch et al., 2013). However, this assumption does not hold true for the mycological component of cave ecosystems, as very few species are considered truly troglobitic (Vanderwolf et al., 2013).

Recent trends in biology are the development of species barcodes and the use of these in ecological studies (Lahaye et al., 2008; Hollingsworth et al., 2009). In mycological phylogenetic analysis, the focus fell on the nuclear ribosomal DNA (nrDNA), as it is composed of highly conserved and variable domains (Woese et al., 1990; Barton et al., 2004). During the last decade large amounts of data were generated for the majority of known fungal species on this region. Therefore, this area was investigated and found to be the most suitable barcoding region for the fungi (Schoch et al., 2012) with the exception of a few genera (O'Donnell and Cigelnik, 1997). The nrDNA encodes for the ribosomal RNA that makes up part of the ribosome (Patwardhan et al., 2014) and is considered as a useful marker for phylogenetic studies (White et al., 1990; Begerow et al., 2010; Eberhardt, 2010). The nrDNA encodes multiple-copy loci and is commonly used, as all organisms except most viruses have ribosomes. An additional feature of the nrDNA region is that it incorporates both coding and non-coding DNA, which have various levels of selection on them due to their conserved and non-conserved nature. These nrDNA are organized in clusters of tandem repeats that consist of the internal transcribed spacer 1 (ITS1), *5.8S rRNA* gene and ITS2. Due to the ITS2 region's functionality in RNA processing, it can be used to discriminate at species and subspecies level and contains unique secondary structures that can be used for alignment of higher taxonomic ranks like genus and order (Song et al., 2012). Thus, this region is the best to use as the DNA barcode marker for identifying both single taxa and mixed environmental isolates (Bellemain et al., 2010, Schoch et al., 2012).

Studies into the microbial niches contained in karst systems are few when compared to large ecological studies from plants and animals. There is also no fungal barcoding project to our knowledge that focuses specifically on karst ecosystems in Africa. The low cost associated with fungal barcoding of a biome or ecosystem provides an interesting opportunity to establish the different distribution patterns of fungi found in karst systems. The possibilities to discover new fungal species increases tremendously when coupled with next-generation sequencing. In this study, we aimed to establish a baseline for fungal species distribution found in a dolomitic cave. We sampled the culturable fungal population of the Bakwena Cave ecosystem over a twelve-month period (2009–2010) to establish a baseline for fungi found associated with this type of karst system.

Materials and Methods

Samples of dry and wet guano were collected aseptically into sterile containers between April 2009 and March 2010 from four sites in the Bakwena cave system (Fig 1). Sites one and two were in the main chamber and three and four in two different side chambers. All the samples were taken from areas that had no indication of human activity such as footprints or other disturbances. Sample 1 was taken from the main guano heap located in front of the shelf that led to the pair of side chambers. A soil sample was also occasionally taken from close to the guano heap. Sample 2 was taken from a guano heap that was used for measuring the accumulation of guano over time by another research group, although no evidence of any disturbance was recorded at each of our visits. This heap was located behind the earthen mound at the bottom of the scree slope that led from the entrance of the cave. Sample 3 was collected from the sediment at the edge of the water table that was reached through the eastern passage. Sample 4 was sampled from the side chamber where the largest roost of bats occurred and that was deemed the most pristine, with the least evidence of human activity because the entrance to the side chamber is difficult to manage. All samples were immediately placed on ice and transported to the laboratory, where samples were stored at 15 °C until processing. The physiological and physiochemical characteristics of the cave sediments, including moisture content, pH, and organic matter of sediments were determined and reported by Durand et al. (2012).

To isolate the culturable fungal fraction from the cave ecosystem, isolations were made from all the collected guano and soil samples. The isolations were done by spreading 5 g of organic material onto potato carrot agar (nutrient poor media) (Crous et al., 2009) plates, amended with 0.02 g/L tetracycline and 0.01 g/L ampicillin to suppress bacterial growth, and then isolating all morphologically unique colonies after seven days of incubation at 20°C. Following sub-culturing on malt extract agar media (Merck) to obtain pure cultures, all isolates were grouped into morphological taxonomic units (MTU) based on colony morphology, i.e. colony color and growth pattern. Single conidium (spore) cultures were obtained for all the isolates to ensure that a single genetic entity was used for further studies. All the single-conidial cultures were deposited in the PPRI collection of the National Collection of Fungi, Plant Protection Research, Agricultural Research Council, Pretoria, South Africa.

DNA was extracted from one representative of each MTU that was grown on potato dextrose agar (Merck) at 25°C for seven days, and mycelium was harvested by scraping it from the agar surface with a sterile spatula. The DNA was

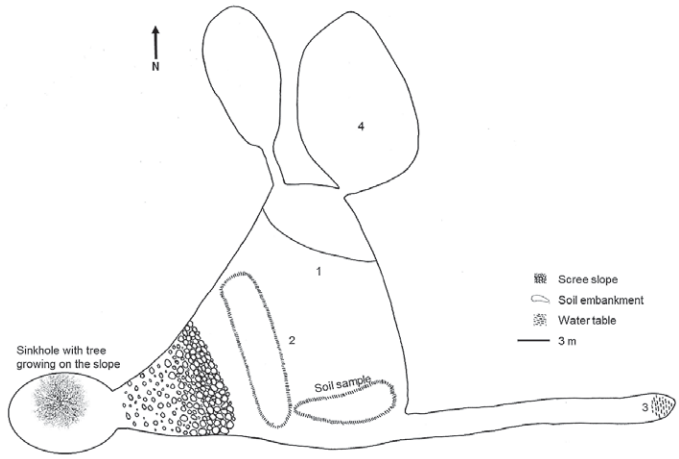

Figure 1. Layout of the Bakwena Cave. The entrance to the cave is located at the top of the scree slope; it is entered by rappelling down a cone-shaped sinkhole and climbing down a ladder that was installed at the top of the scree slope. A side view of the cave layout is available in Durand et al. (2012). Samples were taken from moist and dry guano at the indicated sampling sites. The bat colony was divided into two groups that were located above sampling sites 1 and 2 and in the side chamber where sample 4 was taken. Sample 3 was taken from sediment at the edge of the water table.

isolated using a DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. The extracted DNA was used as template in PCR reactions to amplify the ITS region of the rDNA gene region using the primer set ITS1 (5'-CGAATCTTTGAACGCACATTG-3') and ITS4 (5'-CCGTGTTTCAAGACGGG-3') (White et al., 1990). Amplification of the ITS region from the fungal DNA was performed in 20 µl PCR reaction mix containing 50 ng DNA template, 0. 5 µM of each primer, 0.04 U ExSel *Taq* polymerase (JMR Holdings), 0.25 mM each dNTP, and 1 X ExSel buffer. Amplification was performed on a MyCycler (Biorad) with initial denaturation for 3 minutes at 94°C, followed by 30 cycles of 1 minute at 94°C, 1 minute at 50 °C and 1 minute at 72°C, with a 7 minute final extension at 72°C. The amplicons were separated on a 1. 5 % (w/v) agarose gel submerged in 1 X TAE (40 mM Tris-acetate pH 8.0, 1 mM ethylenediamine tetraacetic acid) buffer for verification and viewed under UV-light. The PCR amplicons were purified using a QIAquick PCR Purification kit.

The MTUs identities were determined by sequencing the ITS amplicons on a 3130xl DNA Analyzer (Thermo Fisher Scientific) using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Thermo Fischer Scientific) with the same primers as those used in the PCR reactions. All of the sequences generated in this study were deposited in GenBank. The ITS1 barcode sequences generated from the MTUs were queried against the NCBI nucleotide sequence and MycoBank databases using BLASTn (basic local alignment search tool, Zhang et al., 2000) to ascertain their closest relationships using a similarity percentage that exceeded 95%.

Results

Our study focused on isolating and characterizing only the culturable fungal species from the Bakwena Cave. Samples were obtained over a period of twelve months during which the temperature and relative humidity were also recorded (Durand et al., 2012). Isolated fungi were separated into 54 morphological taxonomic units (MTU) based on their colony morphology. From these a total of 93 isolates were deposited in the PPRI living culture collection of the National Collection of Fungi over the twelve-month sampling period. These are represented by the accessions numbers PPRI 22065–22077, 22114–22146, 22148–22150, 22635–22638, 22651–22652, 22654–22660, 22663–22665, 22670–22672, 22674–22681, 22683, 22686–22689, 22690–22705.

The ITS barcode amplicons generated for the individual isolates were about 600 bp in size. The BLASTn results of the DNA barcodes generated for 38 MTUs distinguished six genera and 19 species (Table 1) from both databases. The species-level identifications were congruent for 81.5 % of the isolates across the two databases. The majority of the strains belong to genera within the fungal order Eurotiales and the family Trichocomaceae. Genera found in the Trichocomaceae are morphologically characterized by well-developed mycelium that is often brightly colored. The significant genera in the Trichocomaceae include, among others, *Penicillium* and *Aspergillus* (Cannon and Kirk, 2007), with several genera known for their production of toxins that include aflatoxins, ochratoxins and patulins. Five genera that are frequently isolated from karst environments were also represented by our sampling. These were *Penicillium*, *Aspergillus*, *Mucor*, *Candida*, and *Epicoccum (*Vanderwolf et al., 2013*)*. The genus *Bionectria* has been isolated from caves in the USA (Shapiro and Pringle, 2010). Six species that are regularly isolated from karst environments were *Aspergillus versicolor* (Nováková, 2009), *P. chrysogenum* (Nováková, 2009; Vanderwolf et al., 2013), *P. brevicompactum* (Vanderwolf et al., 2013), *P. atramentosum* (Nováková, 2009), *P. pinophilum* (Nováková, 2009)*, and M. circinelloides* (Nováková, 2009). We also isolated two medically significant species, *A. versicolor* and *A. ochraceus* (Bayman et al., 2002; Engelhart et al., 2002). These species have been associated with fungal infections in immune-compromised individuals. *Candida palmioleophila*, a yeast*,* has been previously reported from bat guano sampled in Japan (Sugita et al., 2005).

Table 1. Diversity of fungal isolates identified from the Bakwena cave. Identifications are based on barcode similarities to the MycoBank and NCBI databases. The MTU and NCBI accession numbers indicate the respective PPR culture number of the deposited fungal isolate and the nucleotide sequence deposited at the NCBI. The sampling site is indicated and cross references to sites indicated in Figure 1.

MTU	PPRI Number	MycoBank Identification	Similarity (%)	NCBI Identification	Similarity (%)	NCBI Accession	Sampling Site
71	13380	*A. westerdijkiae*	100	*A. ochraceus*	100	KY465907	Main chamber – Moist guano
103	22141	*P. griseofulvum*	97.8	*P. griseofulvum*	100	KY069890	Water and sediment
96	22142	*P. brevicompactum*	100	*Penicillium sp.*	100	KY069868	Water and sediment
82	22143	*P. griseofulvum*	100	*P. griseofulvum*	100	KY069885	Water and sediment
77	22145	*Aspergillus sp.*	99.6	*A. ochraceus*	100	KY069892	Main chamber – Moist guano
76	22146	*A. westerdijkiae*	99.8	*A. westerdijkiae*	99	KY069864	Main chamber – Moist guano
78	22635	*Mucor flavus*	95.7	*M. flavus*	99	KY069874	Main chamber – Dry guano
97	22636	*P. chrysogenum*	96.8	*P. chrysogenum*	94	KY069876	Main chamber – Moist guano
44	22651	*P. chrysogenum*	100	*P. chrysogenum*	99	KY069888	Main chamber – Moist guano
67	22655	*A. westerdijkiae*	99.8	*A. westerdijkiae*	100	KY069893	Main chamber –Dry guano
54	22656	*P. crustosum*	100	*P. crustosum*	100	KY465908	Main chamber – Dry guano
46	22657	*P. commune*	96	*P. commune*	100	KY069882	Main chamber – Soil sample
28	22658	*P. crustosum*	98.4	*Penicillium sp.*	100	KY069881	Main chamber – Dry guano
59	22659	*P. griseofulvum*	100	*P. griseofulvum*	100	KY069863	Main chamber – Dry guano
49	22664	*P. crustosum*	100	*P. crustosum*	100	KY069880	Side chamber – Dry guano
51	22665	*A. westerdijkiae*	99.4	*A. westerdijkiae*	100	KY069883	Main chamber – Moist guano
36	22671	*C. palmioleophila*	99.2	*C. palmioleophila*	98	KY069869	Main chamber – Moist guano
33	22674	*A. ochraceus*	98.9	*A. ochraceus*	100	KY069887	Main chamber– Moist guano
39	22675	*A. westerdijkiae*	100	*A. westerdijkiae*	100	KY465909	Main chamber – Moist guano
93	22676	*P. chrysogenum*	99.8	*P. chrysogenum*	100	KY069884	Water and sediment
87	22678	*P. atramentosum*	100	*P. atramentosum*	100	KY069875	Main chamber – Dry guano
37	22681	*P. polonicum*	98.4	*P. polonicum*	100	KY069866	Side chamber – Moist guano
8	22682	*Meyerozyma caribbica*	97.5	*Me. caribbica*	100	KY465906	Main chamber – Dry guano
3	22683	*A. westerdijkiae*	100	*A. westerdijkiae*	98	KY069872	Main chamber – Moist guano
86	22689	*E. nigrum*	100	*E. nigrum*	100	KY069867	Main chamber – Moist guano
6	22691	*A. versicolor*	99.8	*A. versicolor*	99	KY069865	Main chamber – Moist guano
24	22696	*Bionectria ochroleuca*	99.4	*B. ochroleuca*	99	KY069895	Side chamber – Moist guano
26	22697	*A. versicolor*	99.6	*A. versicolor*	100	KY069871	Side chamber – Dry guano
42	22698	*A. westerdijkiae*	99.3	*A. westerdijkiae*	100	KY069878	Main chamber – Moist guano
50	22699	*P. brevistipitatum*	100	*P. griseofulvum*	100	KY069894	Main chamber – Dry guano
63	22701	*P. griseofulvum*	100	*P. griseofulvum*	100	KY069877	Side chamber – Dry guano
70	22702	*P. pinophilum*	99.6	*P. pinophilum*	100	KY069886	Main chamber –Dry guano
27	22703	*A. versicolor*	100	*A. versicolor*	100	KY069870	Side chamber – Dry guano
30	22705	*A. westerdijkiae*	100	*A. westerdijkiae*	100	KY069873	Side chamber – Dry guano
20	22935	*A. cretensis*	97.1	*A. ochraceus*	100	KY069896	Main chamber – Dry guano
48	22936	*P. crustosum*	100	*P. commune*	100	KY069879	Side chamber – Dry guano
79	22937	*M. circinelloides*	92.1	*M. circinelloides*	100	KY069891	Main chamber – Dry guano

Discussion

The study of karst systems in South Africa has been limited to macro fauna and flora and their anthropological value. The area surrounding Pretoria has numerous caves that incorporate archaeological sites and are studied extensively. One of these areas, The Cradle of Humankind, was proclaimed as a world heritage site in 1999 and is protected against development. The Bakwena Cave does not fall into such a proclaimed site and is very exposed to and impacted by urban development. To ascertain what the effect of new developments will have on caves such as the Bakwena Cave, the South African Karst Ecology Study Group (SAKES) was established in 2009 (Durand et al., 2012). SAKES set itself the aim to focus on macro and microbiota found in the Bakwena Cave and has focused on the bats, spiders, mites, amphi-

pods, and microorganisms. Microorganisms in most natural systems have been considered ecologically important due to their involvement in carbon cycling as decomposers (Nielsen et al., 2011). In karst ecosystems, they perform a variety of other roles, including constituting the major food sources of non-predacious troglobitic invertebrates. Furthermore, the fungal component acts as parasites of cave insects (Vanderwolf et al., 2013).

The level of fungal species richness within a karst system is affected by the level of disturbance that the system undergoes (Shapiro and Pringle, 2010). Karst systems with medium disturbance levels and medium- to low-impact sites have significantly greater species numbers than more disturbed or very low disturbance sites. Based on the selection criteria proposed by Shapiro and Pringle (2010), the Bakwena Cave can be classified as a low-disturbance cave as only 19 culturable fungal species were obtained. This hypothesis is supported by the fact that there were no additional visits except by our sampling trips recorded to the cave. What was interesting is that only *Penicillium* species were associated with the soil, sediment, and water samples. Of these, only *P. commune* and *P. brevicompactum* were not isolated from any other medium in the cave, which could indicate that they were introduced by other means, most probably through human activity, or they are natural cave-dwelling species. Most of the identified fungi are saprophytes that either utilize dead plant material as food source or, in the case of the Bakwena Cave, bat guano.

Bats can affect the diversity of fungi found in caves through the transportation of spores, deposition of guano, and their carcasses. The guano that bats deposit in cave systems generally hosts the largest fungal diversity of all cave substrates studied (Vanderwolf et al., 2013). In our study, we expected the largest diversity of fungi to be isolated from the guano, compared with soil and sediment. We isolated the majority of species from the main chamber occurring either on moist or dry guano. The difference between the age and state of the guano did not add significantly to the fungal diversity. Several mite families were also identified from the cave, including the Acaridae (*Sancassania* sp.), the Laelapidae *(Laelaps* sp.) and the Uropodoidea (Durand et al., 2012). Two of these are known to feed on fungi (Krantz and Walter, 2009) and may be a contributing factor to the lower number of fungal isolates obtained.

The different genera and species isolated in this study corresponds well to previous karst studies (Nováková, 2009; Vanderwolf et al., 2013; Sugita et al., 2005; Shapiro and Pringle, 2010). Here, we isolated and identified 38 MTUs up to species level. For the majority of these, both databases reported the same species, but for some, only one database returned a species hit. For others, the two databases produced two different identities, due to the limited use of the ITS region for fungal species identification (Cannon and Kirk, 2007; O'Donnell and Cigelnik, 1997). This gene region works well for most fungal species, but in some instances, the region that is amplified does not contain enough phylogenetic signal to effectively separate or identify between closely related species.

A number of factors can influence the culturable fungal fraction obtained from a karst ecosystem (Vanderwolf et al., 2013). These include the isolation methods used, the species of bats occupying the cave, and the level of human disturbance (Shapiro and Pringle, 2010). According to Vanderwolf et al. (2013), the isolation methods used, including growth media and temperature, may also play a role. During this study, we used a nutrient-poor media (PCA) and incubated at 20 °C that should have been sufficient for a large number of genera to be isolated. Shapiro and Pringle (2010) concluded that only fungal cultures obtained at an incubation temperature of 10°C are true cave dwellers, as the rest may present fungal species that utilize the organic material that came from outside the cave. In our study, we sampled mainly from guano, so the association with this substrate is fairly strong. Domsch et al. (2007) states that Eurotiales, especially *Penicilium*, is well adapted for cold temperatures, which may serve as a possible explanation for the dominance in our study. A study on dust collected from homes across the world revealed that the diversity of fungi found outside influences the diversity of fungi found inside (Visagie et al., 2014). This phenomenon might also hold true for fungi found in caves, where an area that is more diverse in its microbial populations will have a more diverse fungal population. The best way to further establish the fungal diversity would be to make use of metapopulation analysis to provide a much more complete picture of the niche.

Conclusion

Ours is the first study of the culturable fungal population of the Bakwena Cave and represents the first study of a fungal cave ecosystem in South Africa. We have identified several fungi that have previously been associated with caves, and this verifies the results that we have obtained. The correlation between our study and other published studies most probably indicates that the cave ecosystem is as healthy as others that have been studied. Our data for the fungal diversity indicate that the Bakwena Cave has experienced only low levels of disturbance until now. It further sets a baseline that can be used to advise on future developments surrounding the cave.

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ORGANIC MATTER ENRICHMENT AFFECTS ARCHAEA COMMUNITY IN LIMESTONE CAVE SEDIMENTS

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Abstract

Caves are unique environments filled with complex microbial communities that have adapted to oligotrophy. Communities of fungi and bacteria are commonly studied in touristic caves or are associated with guano or other sources of organic matter, but the archaeal community is often overlooked in these conditions. Based on this gap in the existing literature, the present study aims to evaluate the effect of a unique in vitro contamination event by organic matter in the archaeal community over the course of one year. For that purpose, samples were collected in Gruta Manoel Ioiô, a limestone cave located in Iraquara, Brazil. The collected samples were transported to the laboratory to undergo an enrichment of 0.25% or 0.5% mixture 1:1 (w/w) of yeast and meat extract. Samplings were collected at 0, 1, 6, and 12 months to evaluate the effects on the archaeal community by polymerase chain reaction followed by Denaturing Gel Gradient Electrophoresis (PCR-DGGE). PCR-DGGE profiles show that Operational Taxonomic Units (OTUs) remained in all samples, but variations were observed among the contaminated and control samples, especially at 6 months. Also, an increase in the number of OTUs was observed in samples that received the addition of organic matter in relation to the control. These OTUs were identified as Euryarchaeota and Crenarchaeota. This study showed that the archaeal community could be impacted by organic matter contamination in caves.

Introduction

Caves are unique natural environments, usually characterized by the absence of light and by oligotrophy (Barton and Jurado, 2007; Simon et al., 2007). Despite those characteristics, caves present a high potential for microbial life that is little known, especially archaea (Gonzalez et al., 2006; Tetu et al., 2013; Barton et al., 2014; Ortiz et al., 2014). These micro-organisms are a monophyletic group of phenotypically diverse prokaryotes. Initially, it was believed that they were found only in extreme environments; however, several articles have demonstrated that archaea probably occur in all environments that support life, including caves (Gonzalez et al., 2006; Chen et al., 2009; Jarrel et al., 2011). This group was found to be important for ecological relationships in several environments, particularly because of their role in biogeochemical cycles (Kudo et al., 1997; Chen et al., 2009; Jarrel et al., 2011).

Microbial life in caves has typically adapted to an oligotrophic environment, but several factors can increase the nutritional offerings inside caves, such as runoff, animal feces like bat guano, and tourism activity (Nieves-Rivera et al., 2009; Nováková, 2009; Borda et al., 2014). However, archaea are rarely studied in such conditions. Studies commonly focus on bacteria and fungi communities (Chelius and Moore, 2004; Gonzalez et al., 2006; Ikner et al., 2007; Chelius et al., 2009; Adetutu et al., 2011), with a few studies involving protozoa (Sigala-Regalado et al., 2011; Garcia-Sanchez et al., 2013). The focus on bacteria and fungi is related to organic matter input that can present problems linked to uncontrolled proliferation of fungi and bacteria, causing risks to the cave itself and to humans (Jurado et al., 2010; Saiz-Jimenez, 2012). In general, fungi, protozoa, and bacteria proliferation in caves is related to the input of organic matter and micro-climatic changes (Bastian et al., 2010; Saiz-Jimenez, 2012). However, little knowledge exists about archaea in caves associated with guano or runoff. The present study in microcosms evaluated the effects of the addition of organic matter on the archaeal community in limestone cave sediments.

Materials and Methods

Gruta Manuel Ioiô is a touristic limestone cave (12°20′9.96″S, 41°33′50.04″W) where the major attraction is the numerous stalactites, stalagmites, and columns. It is located in Iraquara, Bahia, Brazil, near to several others touristic caves in Chapada Diamantina. Unlike Manoel Ioiô, most of these caves were opened earlier for tourism and have a large variety of speleothems that attracted many more tourists resulting in the studied cave having lower numbers of visitors. It is important to mention that in this dry cave there aren't any platforms for visitors; they walk on the floor of the cave itself. The sampling was done in an area closed for tourists. Sediment samples were collected (SISBIO authorization number 38453) approximately 500 m from the entrance. Five subsamples were collected in sterile plastic bags approximately 1 m apart and mixed together to form a composite sample (Marques et al., 2016). Organic matter present in the sediment was less than 5000 ppm.

Microcosms were set up with 75 g of cave sediment in 250 mL Enlermayer flasks. Two concentrations of yeast and beef extract (Acumedia) mixture (1:1) were tested, at 0.25 and 0.5%. They were diluted in 10 mL of distillated water,

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and all experiments were made in triplicate. Flasks with beef extract, yeast extract, and water, but without soil, were used as controls for medium contamination, and additional controls were made with only sediment and distillated water (Marques et al., 2016). All flasks were incubated in the dark for one year at 25 °C, a temperature close to the recorded in the cave's chamber (22°C).

At the days 0, 30, 180 and 365, approximately one gram was collected from each triplicate with a water-washed and fire-sterilized collector. 0.25 g was used for DNA extraction utilizing the MoBio PowerSoil DNA kit (MoBio Laboratories Inc., Carlsbad, CA) according to the manufacturer protocols. PCR was performed in a thermocycler (Eppendorf, Hamburg, Germany) using the archaea-specific primers 1100F (5′- CAC GGG GGG AGT CAG GTA ACG AGC GAG -3′) and 1400R (5′- GTG CAA GGA GCA GGG AC -3′) (Kudo et al., 1997) in a 25 µL reaction mixture containing 0.2 µM of each primer, 0.2 mM of each dNTP, 1.25 U Taq DNA polymerase (Invitrogen, São Paulo, Brazil), 3 mM MgCl2, 1X PCR buffer, and approximately 60 ng DNA.

For Denaturing Gel Gradient Electrophoresis (DGGE), primer 1100F was attached with a GC-clamp (5′- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG G-3′) (Muyzer et al., 1993). After PCR, the samples were subjected to DGGE in a DCode universal mutation detection system (Bio-rad Laboratories, Hercules, CA). Amplicons were electrophoresed on 8% polyacrylamide gel (w/v) (37.5:1 acrylamide:bisacrylamide) in a denaturing gradient of 20–60% [4 M of urea, with 40% (v/v) formamide representing a 100% denaturing gradient]. Electrophoresis was performed in a TAE 1X buffer for 16 h at 70 V and 60°C. The gel was stained with silver nitrate (Marques et al., 2016). The presence and absence of bands were used for a nonmetric MDS and dendrogram analysis. Both analyses were performed on the PAST 3.0 software platform (www.folk.uio.no/ohammer/past/).

Selected bands were aseptically excised from DGGE and eluted in ultrapure water. After 36 h to 48 h at 4°C, the elution bands were stored at −20°C until a new PCR was performed using 1400R and 1100F primers without a GC-clamp (Kudo et al., 1997). PCR products were visualized in 1% agarose gel, purified using a Purelink PCR purification kit (Invitrogen, Brazil), and cloned with a TA cloning kit (Invitrogen, Brazil); all procedures were performed according to the manufacturer's instructions. PCR reaction tests were performed using transformed colonies with M13F (5′-GTA AA ACG ACG GCC AGT-3′) and M13R (5′-CAG GAA ACA GCT ATG AC-3′) primers at the same concentrations of reagents as described above. The PCR conditions used were 30 cycles of 94°C for 20 s, 55°C for 15 s, and 72°C for 60 s. Amplicons were sequenced in an ABI-Prism 3100 genetic analyzer (Applied Biosystems). Sequences were processed and analyzed with the Phrap/Phred/Cross Match software (Ewing and Green, 1998) and the BLASTN tool (Altschul et al., 1990). The phylogenetic tree was inferred using the Neighbor-Joining and Jukes-Cantor methods in Mega7. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown and bootstrap values inferior to 40 were removed.

Results and Discussion

Microbial communities in caves can be seen as geologically isolated from the surface (Barton and Jurado, 2007); however, several factors, such as runoff, guano, and touristic activity, can disturb the microbial community by adding organic matter. Thus, yeast and meat extract were used as a method to evaluate the in vitro effect of organic matter on the archaea community.

The results observed in the community after a simulated contamination event (Fig. 1) show that the archaeal community changed during treatment, but maintained a similar structure of 6 OTUs in all tested conditions, different from what was observed with fungi (Marques et al., 2016). The nonmetric MDS (NMMDS) and Jaccard similarity dendrogram analyses (Fig. 2) better represent these changes, where the controls maintained a similar structure throughout the year. However, the contaminated samples at months 1 and 12 showed a closer proximity to their respective controls than the samples at 6 months. The month 6 sample also presented a more distinct profile with respect to an increase in the OTU number (Fig. 1). Despite small variations in the profiles of each concentration, the contaminated samples group together according to the month (Fig. 1). The similar impacts caused by the two concentrations tested are an indication that these microorganisms are sensitive to an increase in organic matter and that some of them develop better under these conditions. These archaea that were better adapted in contamination experiments must be organotrophic because of the availability of organic matter, but the archaea shared with the controls could be either organotrophic or autotrophic. Curiously, the major changes did not occur immediately in either concentration, as in month 1 the archaeal community showed less change than at 6 months, where major changes were observed (Fig. 1). These changes must have happened because of competition between archaea, bacteria, and fungi for the organic matter. Archaea commonly have a slower metabolism than the other two groups, leading to a slower change, especially in comparison with the observed changes in fungi in such conditions (Marques et al., 2016). It is interesting to note that at 12 months after the single contamination event, the archaeal community appears to return to profiles close to that of the controls (Figs. 1 and 2); however, more time would be required to provide a better comparison of the probable partial recovery of the native archaeal community. These findings are interesting because of several attempts reported in the literature to recover

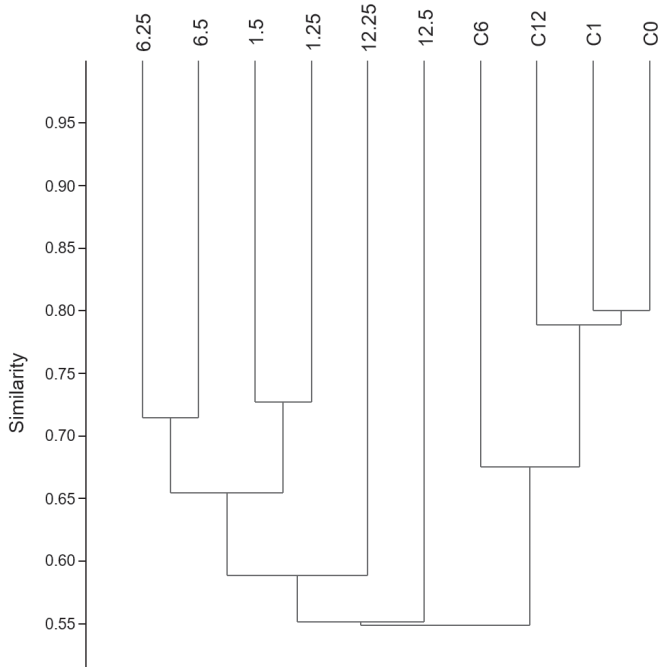

Figure 1. Denaturing Gel Gradient Electrophoresis fingerprint and similarity dendrogram of archaeal 16S rRNA gene from organic-matter-contaminated limestone-cave sediment. "C" indicates control samples, while the number before the decimal point indicates the month of sampling followed by the concentration of organic matter: 25 for 0.25% and 5 for 0.5% of organic matter.

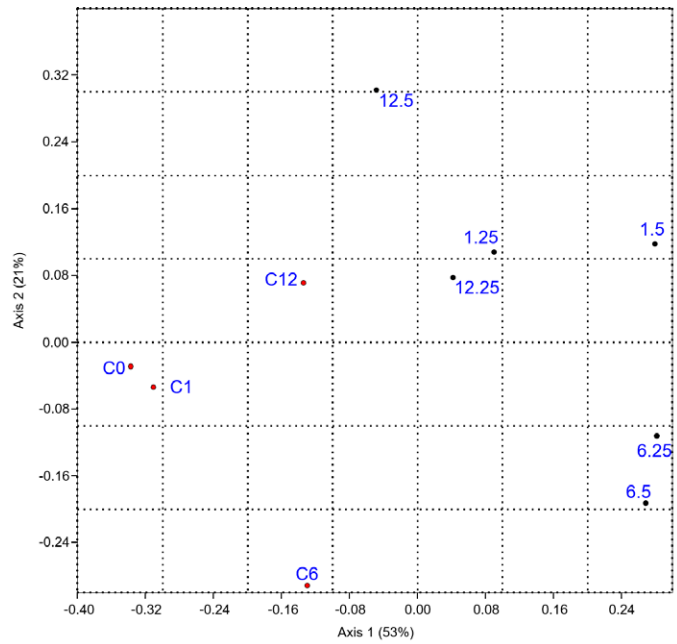

Figure 2. Nonmetric multidimentional scaling (NMDS) ordination of Archaeal 16S rRNA gene Denaturing Gel Gradient Electrophoresis fingerprinting from organic-matter-contaminated cave sediment. The stress value was 0.151 based on Bray-Curtis coefficient distance. Red dots are controls samples, while black dots are experiment samples. Numbers before the decimal point identify the month of sampling, followed by organic matter concentration, 25 for 0.25% and 5 for 0.5% of organic matter.

microbial communities after contamination events (Bastian et al., 2010; Martin-Sanchez et al., 2012) and may indicate that, just as seasonal modifications are found to happen with archaeal communities outside caves based on weather and temperature (Williams et al., 2012; Sher et al., 2013), the same could happen with the archaeal community in caves with respect to organic matter contamination.

Bacteria and fungi have become a major concern in touristic caves because of, among other things, the input of organic matter transported to the cave by touristic activity (Chelius et al., 2009; Martin-Sanchez et al., 2012) and the proliferation of microrganisms, especially pathogenic ones. Because of the lack of known human pathogenic archaea and the general difficulty to culture them, this group is often ignored in such caves. However, recent studies have shown the importance of archaea to a cave's ecosystem (Legatzki et al., 2011; Jones et al., 2014; Ortiz et al., 2014), and it is shown in this study that they suffer major changes over time because of a single event of contamination by organic matter (Figs. 1 and 2). Nevertheless, some considerations need to be noted regarding our experiments. Direct or indirect transport of organic matter is a reality in several caves (Chelius et al., 2009; Jurado et al., 2010; Saiz-Jimenez, 2012) that can drastically change the food web in a cave's ecosystem. These results did not show the real effect in natural conditions, because several others factor also influence contamination events that cannot be easily controlled and mimicked in vitro. One example is that microorganisms will be transported into the caves and among microenvironments, while another is that continuous artificial light will affect the microbial community in touristic caves (Mulec and Kosi, 2009; Cennamo et al., 2012; Saiz-Jimenez, 2012) and could also indirectly affect archaea. In addition to the input of organic matter, animals could start to proliferate in the cavern, prompting major changes in the community (Chelius et al., 2009; Yoder et al., 2009).

Despite these limitations, the use of yeast and beef extract for an in vitro organic-matter study shows that some previously undetected archaea were favored by the contamination event (Fig. 1); these organisms had a low representativeness, less than 1%, which is associated with undetected bands in DGGE (Muyzer et al., 1993), independent of the concentration tested. Considering that there was only one contamination event, these Operational Taxonomic Units could represent potential archaea related with microbial blooms. In caves, such blooms are only associated with bacteria and fungi (Jurado et al., 2010; Saiz-Jimenez, 2012); archaeal blooms have been reported in other environments (Oren and Gurevich, 1995; Fan and Xing, 2016). Phylogenetic analysis of those OTUs identifies them as Crenarchaeota (8 OTUs) and Euryarchaeota (2 OTUs), as shown in Figure 3. Among the eight Crenarchaeota OTUs, three were grouped with soil archaea, one was related to the Nitrosocaldus genus, and four were archaea from a marine environ-

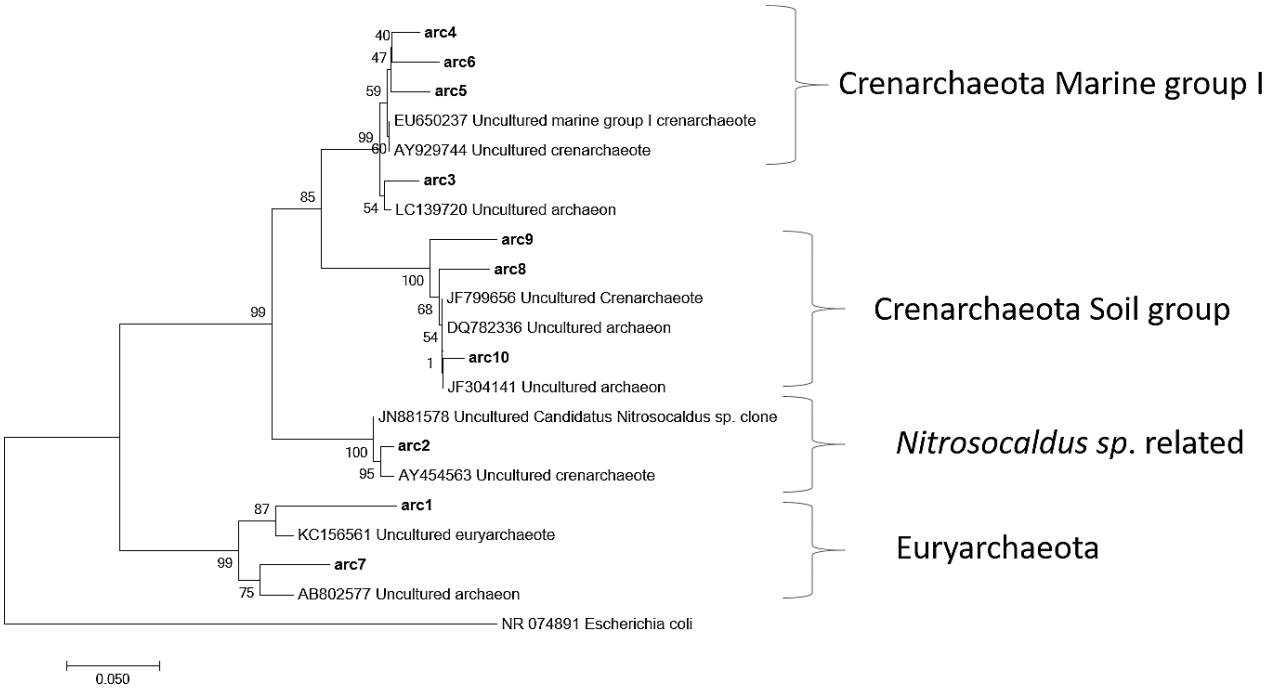

Figure 3. Phylogenetic analysis of archaeal Denaturing Gel Gradient Electrophoresis bands from cave-sediment-contaminated with organic matter.

ment. The Euryarchaeota and Creanarchaeota taxa are dominant in archaea communities in limestone caves (Chelius and Moore, 2004; Chen et al., 2009), including Crenarchaeota from marine environments that have previously been found in caves (Chelius and Moore, 2004).

Conclusions

This work shows the importance of understanding archaeal dynamics in organic matter contamination. Although no human pathogens are known from this group, their ecological importance is unquestionable, and in vitro organic matter input changes the microbial community. The slow change and partial recovery of the archaea community after 12 months could be useful for considering the effect in studies of microbial communities and for recovery of native microorganisms in caves after organic matter contamination events, especially in touristic caves. While we found variation among controls and among times tested, more studies must be done to validate these results, including studies in different caves, different distances from the entrance, and in situ studies in touristic caves.

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SEASONAL CAVE AIR VENTILATION CONTROLLING VARIATION IN CAVE AIR Pco_2 AND DRIP WATER GEOCHEMISTRY AT INAZUMI CAVE, OITA, NORTHEASTERN KYUSHU, JAPAN

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Abstract

To compare the influence of cave air P_{CO_2} and drip rate on the drip water geochemistry, approximately one year of cave air monitoring and sampling of drip water were conducted at Inazumi Cave, Oita, northeastern Kyushu, Japan, from February to December 2014. The monitoring revealed that temperature dependent cave air ventilation controlled distinct seasonal variation in the cave air P_{CO_2} and minor variation in temperature and relative humidity with increasing distance from the cave entrance. In addition to traditional sampling of drip water, novel sampling methods were designed to compare the influence of the P_{CO_2} and the drip rate on the karstic and drip water geochemistry. The chemical analysis indicated that the karstic and drip water instantaneously outgassed CO_2 once in contact with low cave air P_{CO_2}. The drip rate alone, however, had less significant influence on the drip water geochemistry than the P_{CO_2}. The drip water P_{CO_2} was repeatedly lower than cave air P_{CO_2}, and this is probably due to prior calcite precipitation, to a temporal increase of the cave air P_{CO_2} by anthropogenic CO_2, or to storage conditions of the sample; all of which cause alteration of drip water geochemistry from the original state.

Introduction

Stalagmite, a limestone cave deposit, has been recognized as a useful terrestrial paleoclimate archive because the variety of proxies preserved in lamina provides valuable sources for interpretation of climate change. For instance, variations in $\delta^{18}O$ have been interpreted as reflecting variations in meteoric water sources (e.g., Wang et al., 2001), the intensity of Asian monsoon, and amount of rainfall through the time (e.g., Burns et al., 2002; Watanabe et al., 2010; Cai et al., 2010). Moreover, variations in trace elements such as Mg and Sr (e.g., Treble et al., 2003; Johnson et al., 2006) and lamina thickness (e.g., Proctor et al., 2000, 2002) and its texture and fabrics (e.g., Mattey et al., 2008; Boch et al., 2011) have been used for reconstruction of terrestrial paleoclimate.

Although these proxies are regarded as related to meteorological and climatological information outside limestone caves, they might be altered by a kinetic effect, "in-cave processes" including evaporation, CO_2 degassing, and $CaCO_3$ precipitation from drip water (e.g., Hendy, 1971; Mickler et al., 2006), which are controlled by variations in cave air temperature, relative humidity, and P_{CO_2}. Isotopes and geochemistry of the deposited $CaCO_3$ in stalagmites are, therefore, strongly affected by a complex interplay that the drip water has experienced in the cave environment. To understand the complex interplay, cave air monitoring can help to narrow down the variables that affect the isotopic and geochemical signatures of the deposited $CaCO_3$ and the drip water.

Spötl et al. (2005) conducted pioneering work in the Obir Cave, Austria, and reported seasonal variation in the cave air P_{CO_2} as the main variable affecting CO_2 degassing and $CaCO_3$ precipitation from the drip water. Subsequent studies have revealed similar trends (e.g., Banner et al., 2007; Mattey et al., 2010; Boch et al., 2011; Wang et al., 2016), and the variation in P_{CO_2} has increasingly gained attention as an important variable controlling drip water kinetics.

However, some laboratory experiments have demonstrated that drip rate is an important variable affecting the kinetics of the drip water geochemistry. Day and Henderson (2011) conducted a $CaCO_3$ precipitation experiment in a cave analog condition with three different drip rates, slow, moderate, and fast, and reported that the moderate drip rate produced the greatest amount of $CaCO_3$ deposition, suggesting that the drip rate strongly affects the laminar thickness of stalagmites.

Both the cave air P_{CO_2} and the drip rate are important variables controlling the drip water geochemistry and speleothem growth dynamics. Interpreting the influence of the cave air P_{CO_2} and the drip rate on the geochemistry provides better understanding of how the geochemistry is altered in a natural cave environment.

In this study, we compare the influence of the cave air P_{CO_2} and the drip rate on the drip water geochemistry by applying novel sampling techniques to collect drip water in the course of the monitoring period at the Inazumi Cave, Japan. This study and the novel sampling techniques suggest that variation in the cave air P_{CO_2} controls drip water geochemistry more than the drip rate in the Inazumi Cave.

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Site Description

The Inazumi Cave is located at Bungo'ono, Oita prefecture, northeastern Kyushu, Japan (32°54′00.4″N, 131°32′31.8″E). The tourist cave consists of two approximately 300 m long meandering branches from their intersection, Suichu Cave and Shinsei Cave (Fig. 1A). The branches were previously separated, but are currently connected by artificial passages for tourism. The Suichu Cave contains a cave river flowing out of point D to the opening A, and some chambers and passages are partially underwater. There are two openings to Inazumi Cave (A and B in Fig. 1B). Opening B is the entrance point for tourists, while opening A is a river channel with no sidewalk for visitors. This study used the opening B for entering to all passages and chambers of the cave.

The Inazumi Cave is characterized by a history of being completely flooded. Blockage of all cave openings by pyroclastic flow deposited by the Aso-4 eruption occurred ca. 90,000 years ago, resulting in loss of all drain outlets and filling up of the entire cave by river water (Fujii and Nishida, 1999). The pyroclastic flow deposits are now all removed by erosion by the Nakatsuburei River near the Inazumi Cave, and the water level in the cave is low, enabling visits by tourists on the sidewalk. Occasional complete cave flooding has been reported during heavy rain periods. Some erosional features and mud coatings over the whole cave and on numerous speleothems are evidence of previous cave flooding.

Figure 1C presents a hythergraph of Ume, a meteorological station about 15 km away from the Inazumi Cave, indicating mean annual temperature of 14.3°C and mean annual precipitation of 185.8 mm yr^{-1}, ranging from 44.1 in December to 359.2 mm in August for 1981–2010. The climate of this region is characterized by distinct seasonality due to the East Asian monsoon, with higher precipitation and temperatures in summer and autumn and lower in winter and spring. Flooding in Inazumi Cave has been reported mainly during summer and autumn, when many tourists visit Inazumi Cave.

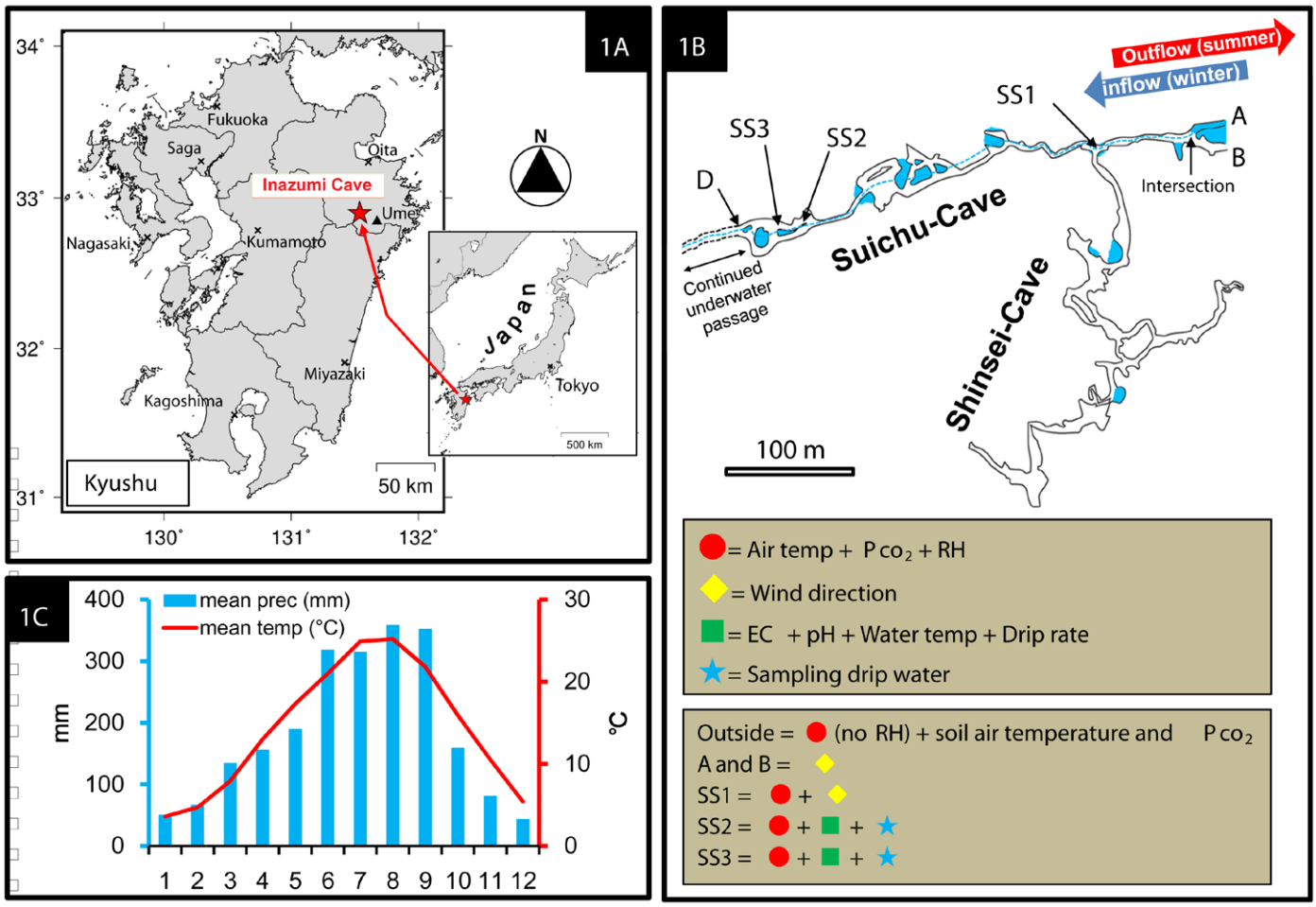

Figure 1. (1A): Location of Inazumi Cave indicated by red star. (1B): Plan view of Inazumi Cave with the locations of monitoring and sampling sites. See symbols below the cave map for details of the monitoring at each site. Light blue-colored parts in the cave map are underwater passages and chambers, and the dashed light blue line is the cave-river channel flowing out of point D in Suichu Cave to the opening A. Blue and red arrows above the opening A and B indicate how the direction of the cave air differed by seasons. (1C): A hythergraph at Ume, the closest meteorological station to Inazumi Cave.

Materials and Methods

Cave air monitoring and drip water sampling were conducted for three days and two nights in Inazumi Cave nearly every month from February to December 2014. No monitoring was conducted in September, but monitoring and sampling of the drip water were conducted at the beginning and end of October instead (OI and OII, respectively, in figures). Cave air monitoring was initiated at 11:00 on the first day and ended at 15:00 on the third day, making 52 hours in total for every monitoring period. As we define daytime and night time as the time periods from 7:00 to 17:00 and from 18:00 to 6:00 respectively, the first day of the monitoring period is shorter than other periods.

Cave Air Monitoring

Cave air temperature, relative humidity and P_{CO_2} were logged at three monitoring sites labeled SS1, SS2, and SS3 (Fig. 1B). The air temperature and P_{CO_2} but not RH outside the cave were also logged. The air temperature and RH were logged using humidity logger LR5001 (accuracy ± 0.5°C from 0.0 to 35.0°C for temperature and ± 5% at 20–30°C / 10–50% for RH). The air P_{CO_2} was logged using a SenseAir portable CO_2 logger (± 20 ppm at 20 °C and 1013 hPa).

Soil air temperatures and P_{CO_2} below the surface of the soil were measured daytime and night time of every monitoring period. An electrode with a silicon tube was installed at 1 m depth below the soil surface about 50 m away from the cave entrance. The electrical resistance value was measured and converted into the soil air temperature. The soil air P_{CO_2} was measured from a silicon tube attached to the electrode using a CO_2 detector (Gastec Corp.).

The cave air direction was visually recorded using smoke from incense sticks at 5, 100, and 200 cm from the floor of the passage, labeled respectively as lower, middle, and upper sections, at SS1 and the openings A and B. The flow for the opening A was recorded at the intersection of the two openings because there was no accessible passage for the opening A (Fig. 1B). Using smoke from incense sticks is a method described by Ohsawa (2009) and Hasegawa et al. (2014).

In-Situ Measurement of Drip Water

The water temperature, pH, electrical conductivity, and drip rate were measured at two dripping sites SS2 and SS3. Water temperature and pH were measured using a pH meter (± 0.01 for pH; ± 0.1 °C for water temperature, D-50 series; Horiba Ltd.). The EC was measured using a compact conductivity meter (± 2% full scale for each range, B-711; Horiba Ltd.). The drip rate was measured using a stopwatch and a 4.9 ml PET tube.

Sampling of Water

Drip water sampling by a novel sampling approach was conducted at sites SS2 and SS3 each day and night time during the monitoring periods. At SS2, a series of three handrails is installed where drip water hits the handrails in order, and the drip water before and after hitting each of the handrails was collected and labeled respectively as SS2U, SS2M1, SS2M2, SS2M3, and SS2L from the top to the bottom. (Handrails are labeled respectively as Handrails 1, 2, and 3 from the top to the bottom; see Fig. 2A-1 for a schematic diagram and the image in Fig. 2A-2). Sampling of SS2M3 only started from May. The drip water is supplied from a vein-shaped and cream-colored stalactite and flows along the stalactite's flank (Fig. 2A-3). Small dome-like and brown stalagmites are formed on each of the handrails at the drip-water contact and gradually become smaller in the sequential order of the handrails (Fig. 2A-1 and 2A-4). Vertical distances between the vein-shaped stalactite and the handrails are as follows: 105 cm between the vein-shaped stalactite and the Handrail 1; 34 cm between the Handrails 1, 2, and 3, and 7 cm between the Handrail 3 and the cave floor.

At SS3, a sandblasted glass tube was installed on a point where the seepage water drips (Figs. 2B-1 and 2B-2). The seepage water before and after hitting the glass tube was collected and labeled respectively as SS3U, SS3M, and SS3L from the top down. This sampling method is similar to site SS2. In addition, the water before dripping was collected with a novel sampling method illustrated in Figure 2B-4 and 2B-5 and labeled as WBD. This method is designed to obtain a purer chemical signature of the water within the carbonate rock matrix above the cave by blocking the water-cave air interaction. Before instrument usage, pre-existing air inside the sampling bag was completely removed, and the silicon tube part was tightly clamped to prevent the sampling bag from collecting ambient air. The rubber part was attached to the tip of the stalactite and the clamp was open, enabling the WBD sample to flow into the bag. After sampling a sufficient amount of the WBD for chemical analysis (ca. 100 ml), the sampling bag was tightly closed, and the rubber part was removed from the stalactite. Note that the water supplied from the stalactite at SS3 is seeping from the stalactite's core, not flowing along the stalactite's flank. All drip water at SS2 and SS3 was collected in 4.9 ml PET tubes with no head space, capped by a silicon plug, and sealed tightly using vinyl tape. All samples were kept in storage in a refrigerator before chemical analysis.

Chemical Analysis

Cations (Ca, Mg, Na, and K) and anions (Cl, F, SO_4, and NO_3) of the samples were measured using ion chromatography (ICS-1100; Dionex Corp.) at the Department of Geology and Mineralogy of Kyoto University. Concentration

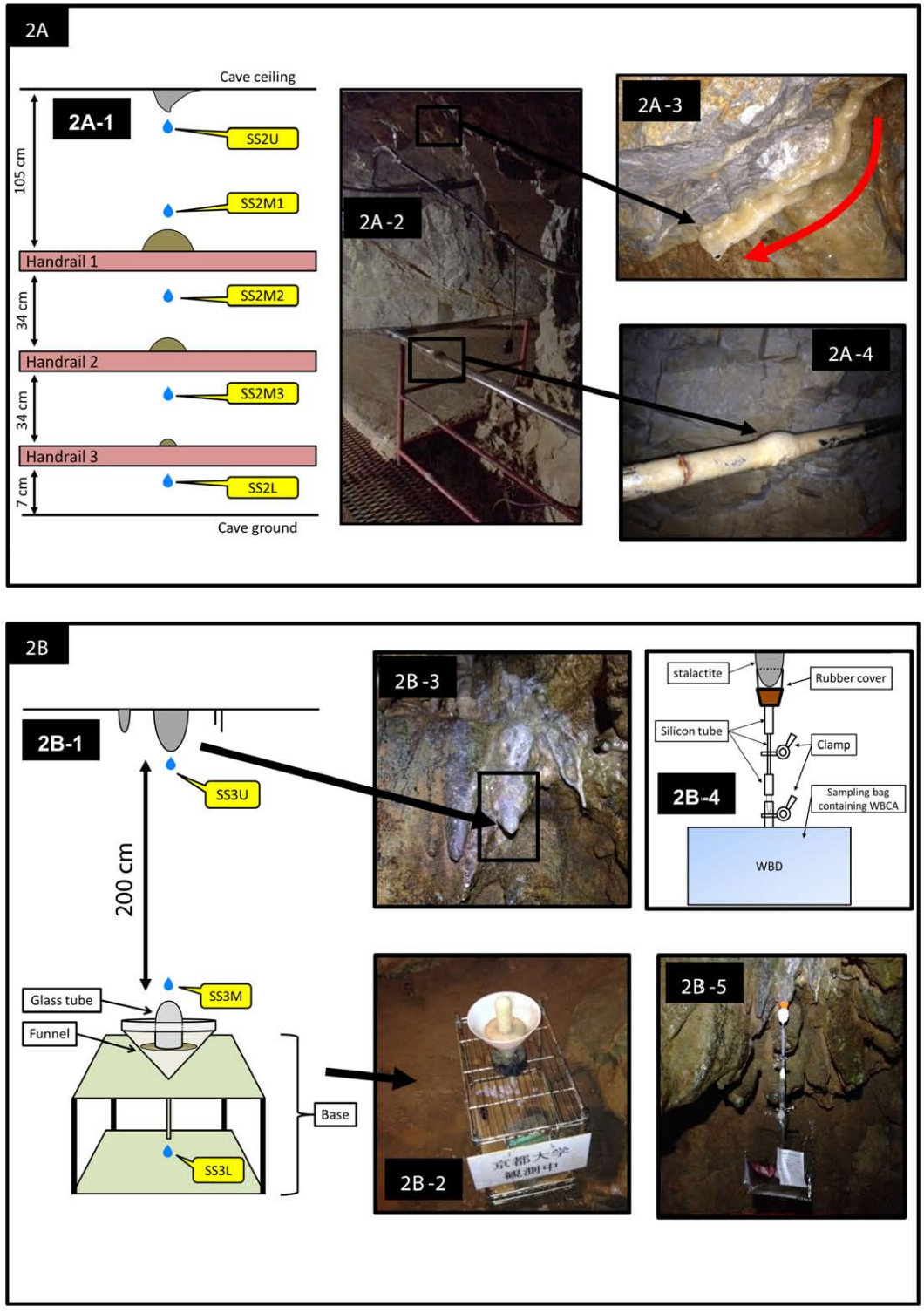

Figure 2. Schematic diagrams and photographs of the novel sampling method of the drip water at SS2 and SS3. (2A-1): A series of three handrails (2A-2) exists at SS2, with the drip water hitting the handrails in the sequential order of the handrails. Samples of water before and after hitting the handrails were collected. The water is supplied from a vein-shaped and cream-colored stalactite (2A-3). The red arrow on the photograph indicates the flow direction of the water fed from the stalactite. Brown dome-shaped stalagmites are formed on each handrail (2A-4), and the size becomes smaller in sequential order of the handrails. Vertical distances between the vein-shaped stalactite and the handrails are the following: 105 cm between the vein-shaped stalactite and the handrail 1, 34 cm between the handrails 1, 2, and 3, and; 7 cm between the handrail 3 and the cave ground. (2B-1): A sandblasted glass tube was placed on the dripping point at SS3, and the water before and after it hit the glass tube was sampled. (2B-2): A photograph of the glass tube at SS3. (2B-3): A photograph of the conical stalactite. The stalactite enclosed by a black square was used for collecting dripping water and the WBD. (2B-4): Schematic diagram of sampling the WBD. (2B-5): A photograph of sampling the WBD.

of HCO_3^- was determined by a spectrometry method described by Mishima et al. (2009). The method is designed for quantitative analysis of HCO_3^- in a small amount (0.1–0.3 mL) of environmental water such as drip water.

Mishima's method uses bromocresol green solution for measuring HCO_3^- in the dip water. The absorption spectra of BCG are varied at different pHs: 445 nm at acidic and 616 nm at basic pH. Combining the Henderson–Hasselbalch equation and Beer and Lambert law produces the following formula

$$\mathrm{pH} = pK_a + \log\left[\frac{\frac{A_{616}}{A_{445}} - \frac{e_{21}}{e_{11}}}{\frac{e_{22}}{e_{11}} - \frac{A_{616}\,e_{12}}{A_{445}\,e_{11}}}\right] \quad (1)$$

(1) where pK_a is the dissociate constant determined by water temperature, A_{445} and A_{616} respectively denote the absorption spectra at acidic and basic pH, ε_{11} and ε_{12} respectively represent the absorption coefficients at 445 nm, and ε_{21} and ε_{22} are those at 616 nm. $\varepsilon_{22}/\varepsilon_{11}$, $\varepsilon_{12}/\varepsilon_{11}$, and $\varepsilon_{21}/\varepsilon_{11}$ in Equation (1) will be constant if the water temperature is constant. These variables can be expressed respectively as a, b, and c, and the Equation (1) can be rearranged to

$$\left[HCO_3^-\right] = \left[H^+\right] - K_a\left[\frac{a - b\left(\frac{A_{616}}{A_{445}}\right)}{\left(\frac{A_{616}}{A_{445}}\right) - c}\right] \quad (2)$$

(2) In Equation (2), $[HCO_3^-]$ and $[H^+]$ respectively represent bicarbonate- and hydrogen-ion concentrations in mg L^{-1}. Equation (2) was used to determine HCO_3^- of the drip water sample.

For obtaining a calibration curve, 0.04 w/v % of bromocresol green solution (Wako Pure Chemical Inds. Ltd.), 0.1 mol L^{-1} HCl and 500 mg/L HCO_3^- standard solutions were prepared from sodium hydrogen carbonate ($NaHCO_3$, Special Grade; Wako Pure Chemical Inds. Ltd.). The 0.1 mol L^{-1} HCl was diluted to 1000 times with de-ionized water to prepare 0.1 mmol L^{-1} HCl solution. Subsequently, 0.38 mL BCG and 10 mL of the diluted 0.1 mmol L^{-1} HCl were aliquoted into seven 12 mL PET tubes. The 500 mg/L $NaHCO_3$ standard solution was diluted with de-ionized water to prepare 50, 100, 150, 200, 250, and 300 mg L^{-1} standard solutions. Of each standard solution, 0.2 mL was added to the solutions containing the BCG and HCl. The mixture containing the BCG, HCl, and HCO_3^- standards were agitated gently and settled for at least 5 minutes until the color stabilized. After 5 minutes, the absorbance ratios A_{616}/A_{445} of the solutions was measured using a spectrophotometer (UV-1800; Shimadzu Corp.). The obtained absorbance ratio was provided using Japan Poladigital's DeltaGraph to obtain the calibration curve. Determining HCO_3^- of drip water samples followed the same procedure: 0.2 mL of the drip water sample was added to the solution of 0.38 mL of BCG and 10 mL of the 1000-times diluted HCl. Then the absorbance of the drip water was measured using the same spectrophotometric method. As equilibrium concentrations obtained using these two methods, Mishima's method obtained 152.2 ± 1.7 mg/L (1σ) and the traditional neutralization titration obtained 154.0 ± 1.1 mg/L (1σ).

The calcite saturation index ($SI_{calcite}$) and the drip water P_{CO_2} were calculated using software (PHREEQC ver. 3; Parkhurst and Appelo, 2013). A $CaCO_3$-precipitation test using a sandblasted glass plate was conducted at different dripping sites of Inazumi Cave, and the $CaCO_3$ precipitated on the glass plate was identified as calcite using Raman spectroscopy at the Department of Geology and Mineralogy of Kyoto University (unpublished data). Therefore, the $SI_{calcite}$, not the $SI_{aragonite}$, was used. No data of water temperature and pH of the WBD samples were obtained because of the unique sampling method. Instead, the temperature and pH of SS3U were used for calculation of $SI_{calcite}$ and drip water P_{CO_2} of the WBD as reference values.

Results

Air Monitoring

Figure 3A presents time series variation in the cave air temperature, RH, and P_{CO_2} at the three monitored sites. The outside air temperature was variable, ranging from −0.5 in March to 31.9°C in July, the lowest and largest values respectively. The air temperature and RH inside the cave gradually stabilized with increasing distance from the cave entrance. The air temperature and RH at SS1 indicated a seasonal variation, ranging from 12.9 to 16.8°C for temperature and from 77.6% to 100% for RH. At SS2 and SS3, the deepest localities of the Suichu Cave, the temperature and RH were stable, maintaining 16 ± 0.2°C and 100 % through all monitoring periods. The cave air P_{CO_2} indicated distinct seasonal variation with increasing distance from the cave entrance, ranging from 371 to 3216 ppm at SS1, from 856 to 7073 ppm at SS2 and from 892 to 6788 ppm at SS3, respectively. The extreme increase of the P_{CO_2} at SS2 and SS3 at the end of monitoring period on February 2014 is due to a long stay of numerous visitors. The outside air P_{CO_2}, on the

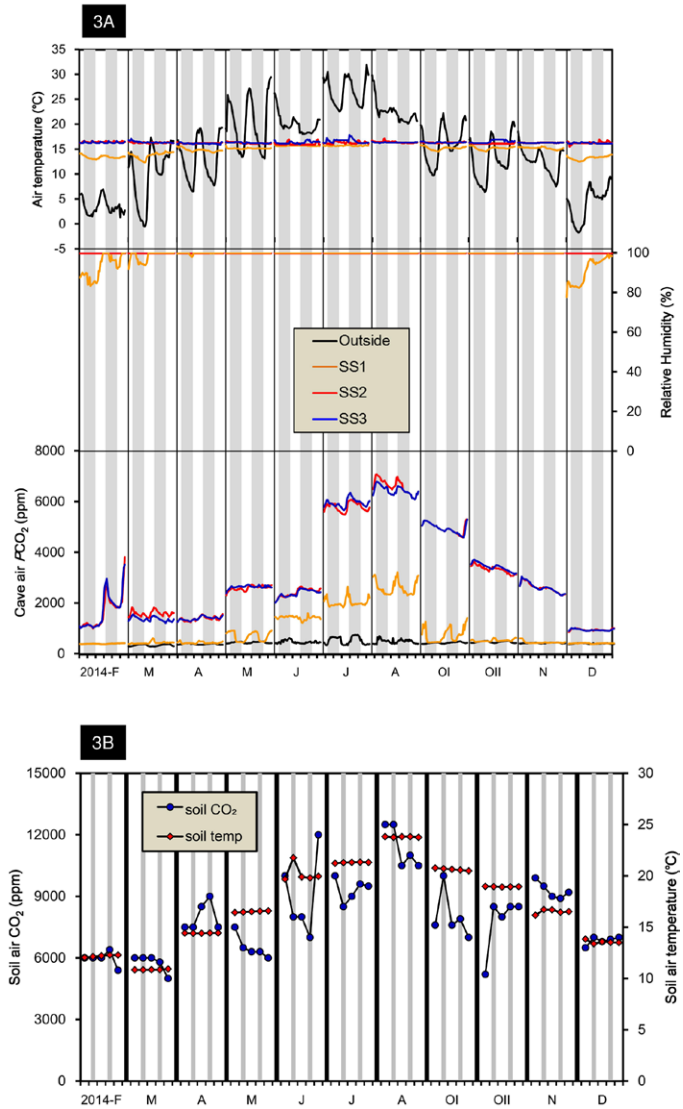

Figure 3. (3A): Time series variation in logged cave air temperature, relative humidity, and P_{CO_2} at sites monitored for 52 hours monthly from February to December 2014. Gray bars indicate night time from 18:00 to 6:00 and white ones indicate daytime from 7:00 to 17:00. Note that RH at SS2 and SS3 always show 100% during the course of this study and that first white bars at each monitoring periods are narrower than others since the onset time of each monitoring was began at 11:00. (3B): Time series variation in measured soil air temperature and P_{CO_2} The gray and white bars are the same as in 3A.

other hand, showed 429 ± 77 ppm through all monitoring periods, more stable than the cave air.

Soil air temperature and P_{CO_2} showed seasonal variation, with high values during the warm season and low values during the cold season (Fig. 3B). The air P_{CO_2} showed irregular diurnal fluctuation, whereas the temperature showed little diurnal variation.

Figure 4A summarizes time series variation in the cave air direction at SS1 and indicates seasonal variation as the overall trend and diurnal variation in April, May, October I and II. While outside air flowed into the cave during the cold season, the air flowed out of the cave during warm period. Smoke gradually diffused from the tip of the incense stick, but showed the same direction at all three recorded sections in the course of this study. No differences in the air flow were found depending on altitude in the cave passage, except for April, when the air flow at lower section moved in the opposite direction than the air in middle and upper sections (Fig. 4). The cave air of all three recorded sections at the opening B, on the other hand, always flowed out of the cave regardless of season, and the smoke diffused in all directions to far from the opening, making it difficult to identify the flow direction. This might be caused by the opening B having a large chamber where cave air was easily scattered in all directions. The cave air flow at the intersection of the opening A and B showed the same pattern observed at SS1. During cold season, in particular, we observed that air was constantly supplied from the opening A, with a narrow passage, suggesting that the cave air ventilation regime in Inazumi Cave is exclusively controlled by opening A's air circulation.

In Situ Measurement of Drip Water

Figures 5A and 5B show time series variations in in situ measurement of drip water at SS2 and SS3, respectively. Water temperature at both of SS2 and SS3 was stable and maintained 16 ± 0.2°C during the course of this research. EC at SS2 and SS3 showed seasonal variation, with high values in the warm season and low values in the cold season. Particularly, in the cold season, EC decreased in the sequential order of the handrails and glass

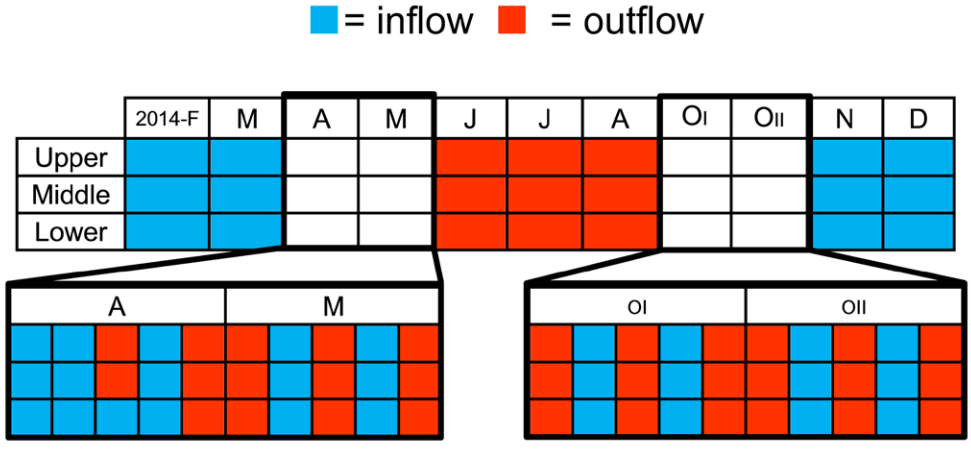

Figure 4. Variation in the cave air direction at upper, middle, and lower sections of the cave passage at SS1 during the monitoring periods. Blue boxes indicate inflow, incursion of fresh air into the cave, and red ones are outflow, release of the cave air to outside. April, May, October I and II showed distinct diurnal variation. The direction of cave air in other months showed the uniform direction at all recorded sections in daytime and night time.

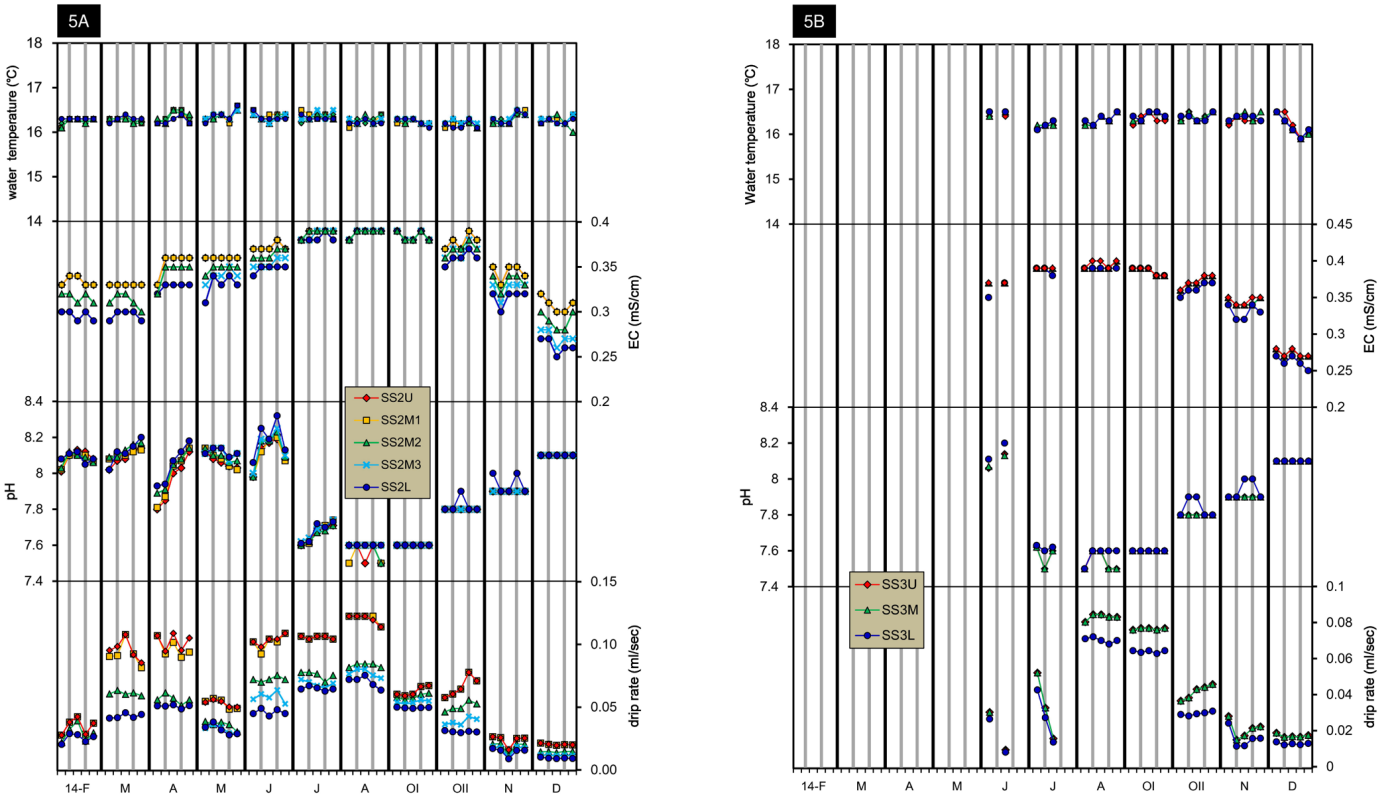

Figure 5. Time series variation in water temperature, EC, pH, and drip rate of the drip water at SS2 (5A) and SS3 (5B). At SS3, there was no supply of drip water from February to May 2014, and the measurement was initiated from June. No data for the WBD were obtained due to the novel sampling method. The white and gray bars are same as Figure 3

tube. The pH was lower in the warm season and higher in the cold season with minor sequential differences. Minor diurnal variation in EC and pH were measured during the course of this study. The drip rate at SS2 from February to July illustrate a slow-and-fast cycle and gradually decreased from August to December. At SS3, the supply of drip water ceased from February to May, was temporarily restored during June and July, and was completely restored in August, with a decreasing trend toward December. The drip rate decreased in the sequential order of the handrails and glass tube; in other words, SS2U and SS3U were the fastest; SS2L and SS3L were the slowest.

Chemical Analysis

Figures 6A and 6B provide time series variations in the chemical analysis of the drip water at SS2 and SS3 respectively. As an overall trend, HCO_3^- and Ca^{2+} at SS2 and SS3 indicated seasonality, with high concentration in the warm season and low concentration in the cold season. The highest HCO_3^- and Ca^{2+} were measured in August, and their concentration were 265.9 mg/L and 89.3 mg/L, respectively at SS2U and 262.4 mg/L and 89.3 mg/L, respectively at the WBD. The lowest were measured in December with concentrations of 55.5 mg/L and 8.0 mg/L at SS2L and 86.0 mg/L and 23.6 mg/L at SS3L. HCO_3^- and Ca^{2+} decreased in the sequential order of the handrails and glass tube during the cold season, with the largest variation in December but minor effects during the warm season. HCO_3^- of the WBD indicated a pronounced difference from SS3U in November and December. The SS2 result in March showed a different pattern from that of other periods, probably because the PET tube was poorly capped with some head space causing undesirable CO_2 degassing while in storage. Other chemical elements including Mg, Na, K, F, Cl, NO_3, and SO_4 were measured and showed no seasonal variation through all monitoring periods.

$SI_{calcite}$ at SS2 and SS3 showed seasonal variation, with high values in the cold season and low in the warm season, but had a positive value ($SI_{calcite} > 0$) all the time except for some of SS2M2, 3, and L in December. The drip water P_{CO_2} at SS2 and SS3 also indicated distinct seasonality, with high values during the warm season and low values during the cold season. It was frequently lower than the cave air P_{CO_2}, with the largest difference in August. Minor diurnal variations in $SI_{calcite}$ and the drip water P_{CO_2} were measured.

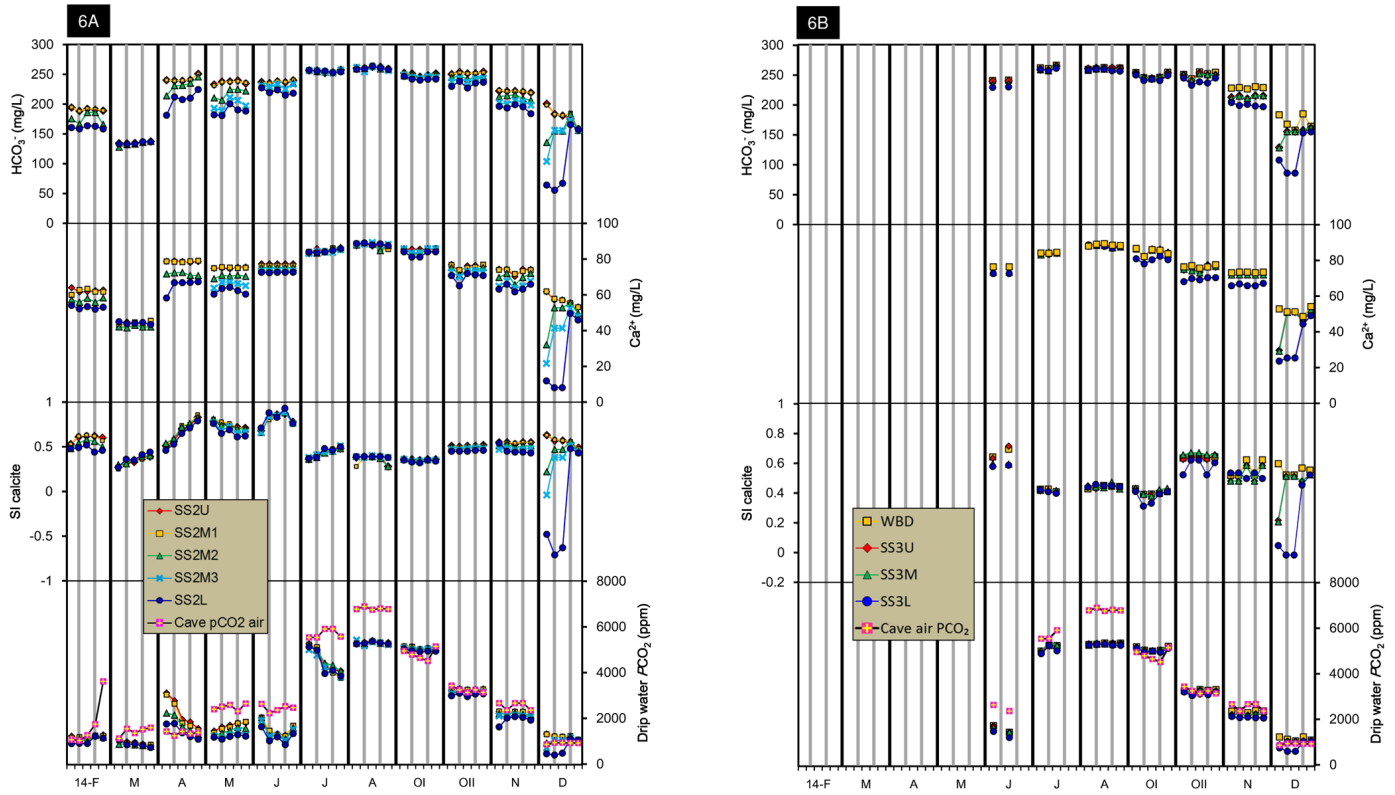

Figure 6. Time series variation in HCO_3^-, Ca^{2+}, $SI_{calcite}$, and drip water P_{CO_2} at SS2 (6A) and SS3 (6B). Note that water temperature and pH of SS3U were used for calculation of P_{CO_2} and $SI_{calcite}$ of the WBD as the reference values. The white and gray bars are same as Figure 3.

Discussion

Cave Air Ventilation

Our cave air monitoring revealed not only distinct seasonal, but also diurnal, air ventilation exclusively controlled by opening A. The diurnal ventilation observed in April, May, and October I and II suggests that the temperature difference between external and internal atmosphere of the cave drives air circulation, hence ventilation, as reported by numerous studies (e.g., Spötl et al., 2005; Kowalczk and Froelich, 2010; Mattey et al., 2010; Boch et al., 2011; Tremaine et al., 2011; Oster et al., 2012; and Hasegawa et al., 2014). The cave air ventilation plays a key role in controlling the variables of the cave environment in the Inazumi Cave. For instance, the outside cold air with low RH and P_{CO_2} flows into the cave when the outside temperature is colder than inside the cave, resulting in a decrease of the cave air temperature, RH and P_{CO_2}. When the temperature outside the cave is warmer than inside the cave, the air direction reverses and inflow turns into outflow, resulting in minor variation in the cave air temperature and RH. In addition, the air P_{CO_2} is increased by the supply of drip-water-origin P_{CO_2} and soil-origin P_{CO_2} percolating down to the cave through fissures and cracks. The decrease of the air temperature, RH, and P_{CO_2} at SS1 is explained by the incursion of outside air, and the increase during warm periods is due to domination by outflow regime and supply of soil-origin and drip-water-origin P_{CO_2}. The air temperature and RH at SS2 and SS3 have shown little variation compared to SS1 during the course of this research. This might be because SS2 and SS3 are farther from the cave entrance and less susceptible to the external air than SS1. Still, distinct seasonal variation in the cave air P_{CO_2} at SS2 and SS3 is the evidence of interaction of internal and external atmospheres of the cave. Varying cave air P_{CO_2}, with stable temperature and RH with increasing distance from a cave entrance, has been reported by Spötl et al. (2005) and Tremaine et al. (2011). Therefore, the Inazumi Cave has a similar air regime to these studies. Based on our monitoring, variation in cave air P_{CO_2} is regarded as the main variable for controlling the drip water geochemistry in the Inazumi Cave, and evaporation and temperature have little effect on the geochemistry.

Cave Air P_{CO_2} vs. Drip rate on Drip Water Geochemistry

The sampling method in this study is designed for determining which of cave air P_{CO_2} or drip rate has a significant effect on drip water geochemistry. If the P_{CO_2} is the main variable, the drip water geochemistry will exhibit either seasonal or diurnal variation following the variation in the cave air P_{CO_2}. If the drip rate is the main variable, the geochemistry will

always decrease in the sequential order of the handrails and glass tube to some extent, because the drip rate always decreases in its sequential order. In order to discuss these variables, we first examine the effect of the P_{CO_2} and then the drip rate.

Cave Air P_{CO_2}

Figure 7 provides the relation between the cave air P_{CO_2} and the drip water geochemistry at SS2 and SS3. HCO_3^- and Ca^{2+} indicate insignificant differences in the sequential order of the handrails and glass tube during high P_{CO_2} periods. As the cave air P_{CO_2} decreases, however, HCO_3^- and Ca^{2+} gradually highlight the decreasing trend and the sequential differences. This might be caused by the incursion of outside air that lowers the air P_{CO_2} and promotes CO_2 degassing and $CaCO_3$ precipitation from the drip-water (Spötl et al., 2005; Banner et al., 2007; Boch et al., 2011). The pronounced differences of the WBD from SS3U are measured when the cave air P_{CO_2} was < 1,500 ppm, indicating that seepage water instantaneously outgasses CO_2 once it encounters low air P_{CO_2}, implying that the WBD contains a more accurate chemical signature within the carbonate rock matrix above the cave than the dripping water. An extremely sharp decreasing trend is found at both SS2 and SS3 when the air P_{CO_2} was < 1,000 in December, implying that the very low cave air P_{CO_2} induces sudden and extreme CO_2 degassing from the drip water. These data are the first report of such an exceptional trend of the drip water geochemistry. Still, because it was measured at both dripping sites SS2 and SS3, it likely implies a general tendency for the drip water geochemistry in very low air P_{CO_2} condition at the Inazumi Cave.

Figure 8 depicts how seasonal ventilation controls cave air P_{CO_2} and the subsequent variation in drip water geochemistry. During cold periods, the incursion of outside air into the cave results in lowering cave air P_{CO_2}, HCO_3^-, and Ca^{2+} and increasing $SI_{calcite}$, hence promoting CO_2 degassing and $CaCO_3$ precipitation. During warm periods, outflow dominates the cave air regime, and soil-origin and drip-water-origin CO_2 flows into the cave, resulting in high air P_{CO_2}. Drip water geochemistry in such environment tends to possess high HCO_3^-, Ca^{2+}, low $SI_{calcite}$, and little CO_2 degassing and $CaCO_3$ precipitation are expected.

Figure 9 shows chemical evolution of HCO_3^- at SS2 and SS3 using the equation of Dulinski and Rozanski (1990). The measured and calculated values of HCO_3^- are the mean values of the drip waters in each monitoring period. Minor differences are found between the measured and calculated values during high cave air P_{CO_2} periods. This could be explained by suppression of CO_2 degassing and $CaCO_3$ precipitation by high air P_{CO_2}. The measured HCO_3^- gradually

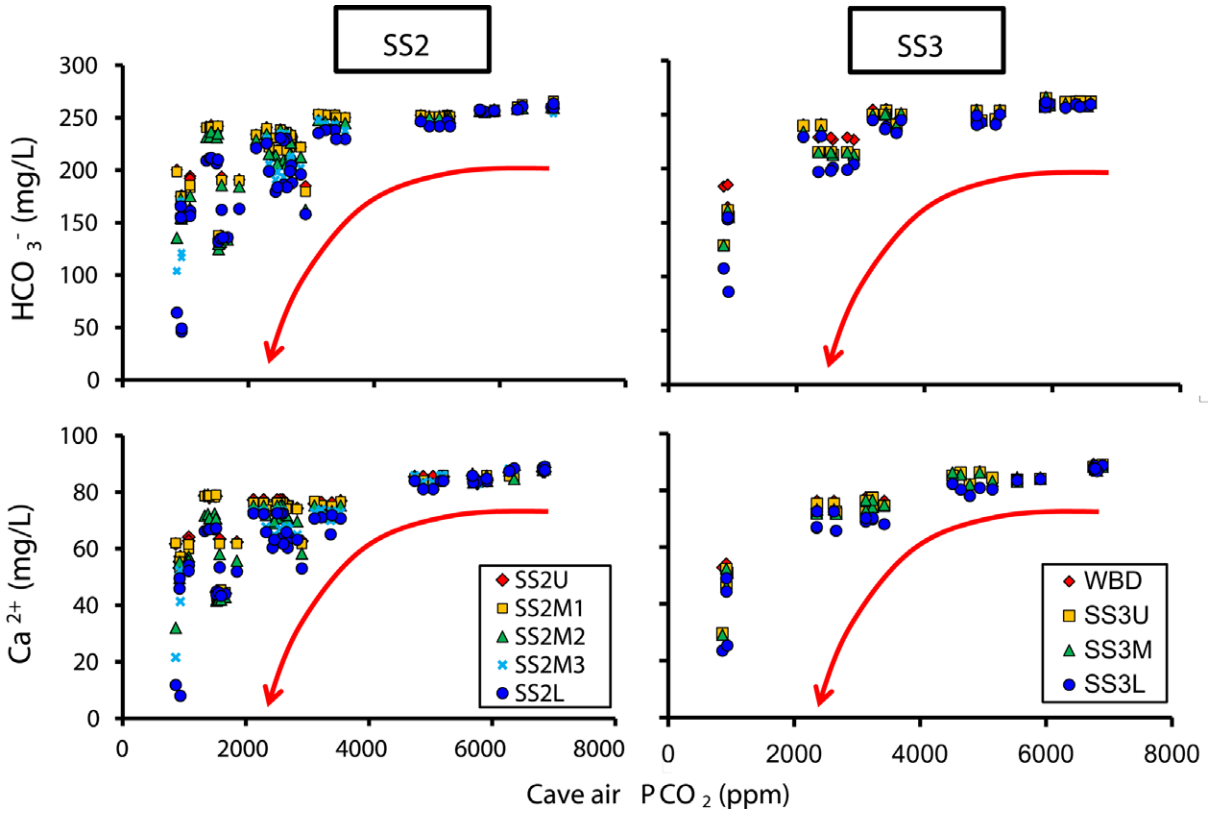

Figure 7. Relationship between the cave air P_{CO_2}, HCO_3^-, and Ca^{2+} at SS2 (left) and SS3 (right). Curved red arrows indicate the decreasing trend of HCO_3^- and Ca^{2+} following the decrease of the air P_{CO_2}.

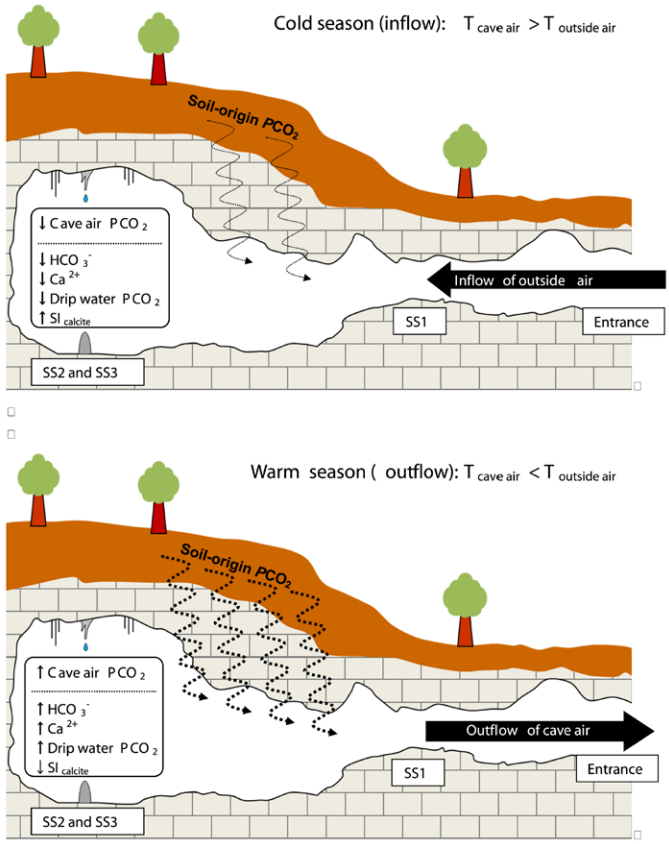

Figure 8. A conceptual diagram illustrating the relationship between seasonal cave air ventilation and drip water geochemistry at Inazumi Cave. The capital T stands for the temperature. Wavy and black dashed line represents the supply of soil air P_{CO_2}. The ventilation, driven by the temperature difference between cave and outside air, controls variation in the cave air P_{CO_2}, resulting in seasonal changes in drip water geochemistry.

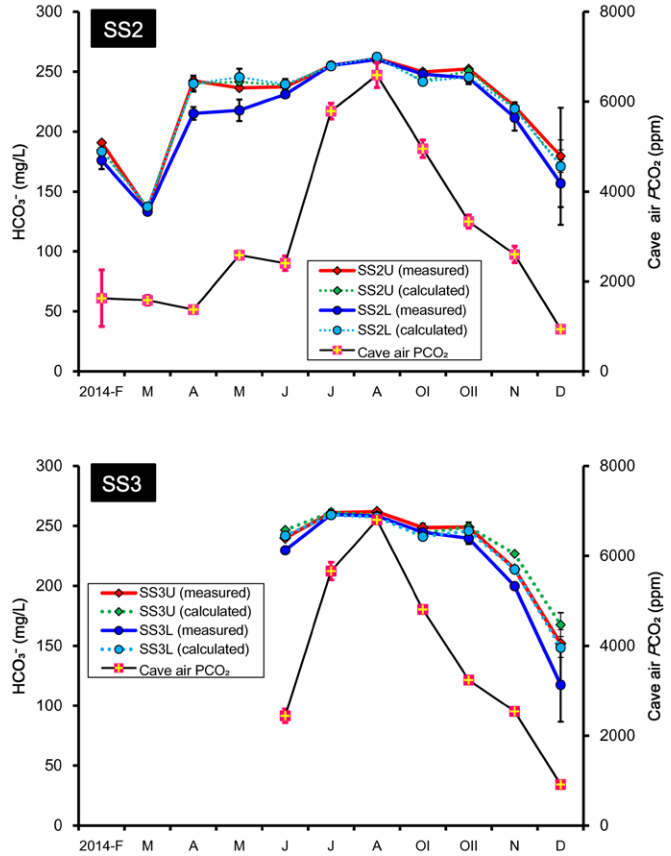

Figure 9. Comparison of the mean values of five samples of measured (solid lines) and calculated values (dashed lines) of HCO_3^- at SS2 (upper figure) and SS3 (lower figure) using the equation formulated by Dulinski and Roanski (1990) with mean values of cave air P_{CO_2} at each monitoring periods.

decreases along with the calculated value as the air P_{CO_2} decreases. In particular, the largest differences between the measured and calculated values are found when the cave air P_{CO_2} is less than 3,000 ppm. This implies that the drip water in the natural setting with low cave air P_{CO_2} is more susceptible to forced CO_2 degassing than expected from the equation of Dulinski and Rozanski (1990). The drip water is considered to achieve chemical equilibrium with the cave atmosphere after it reaches the stalagmite (the handrails and glass tube in this research) (Milanolo and Gabrovšek, 2015). Continuous consumption of HCO_3^- in the sequential order of the handrails and glass tube and the exceptionally large difference of HCO_3^- between the measured and calculated values in December (cave air P_{CO_2} < 1,000 ppm) implies the possibility of forced degassing of CO_2 from the drip water during the low P_{CO_2} season.

The drip water data of P_{CO_2} at SS2 and SS3 frequently showed lower values than the air P_{CO_2} (Figs. 6A and 6B), suggesting no further CO_2 degassing out of the drip water. This tendency is contradictory to the positive values of $SI_{calcite}$ measured at SS2 and SS3 and the formation of the stalactites at SS2 and SS3 and stalagmites on the handrails at SS2. Several possible scenarios are discussed below.

(1) Prior calcite precipitation, or PCP, might have occurred within the carbonate rock matrix above the cave or on the cave ceiling. PCP has been reported as a well-known process in numerous caves and has an effect on drip water geochemistry (Fairchild et al., 2000, 2006; McDonald et al., 2007; Boch et al., 2011; Treble et al., 2015). CO_2 degassing and subsequent $CaCO_3$ precipitation are likely if the percolating water finds any passageways or air pockets with lower air P_{CO_2} within the carbonate rock matrix than the percolating water's P_{CO_2}. Such water possesses lower P_{CO_2} than the air P_{CO_2} when dripping on the stalagmite, and no further CO_2 degassing can be expected. PCP is also applicable to the formation of stalactite and flowstone, resulting in prior consumption of HCO_3^- and Ca^{2+} from the water before dripping and in decreasing the water P_{CO_2}. CO_2 is instantaneously degassed from the seepage water once the water is in contact with the cave air, and the water pH shifts toward basic from acidic (Dreybrodt and Scholz, 2011). The WBD is considered to contain the chemical signature within the carbonate rock matrix above the cave to some extent since the WBD shows higher HCO_3^- and Ca^{2+} than other dripping water at SS3 during the cold season (Fig. 5B). However,

no pH and water temperature of the WBD are obtained due to the applied new sampling technique. An improvement of the sampling method will provide the possibility of measurement of these variables and investigation of the influence of PCP on the drip water geochemistry.

(2) The anthropogenic CO_2 temporarily increases cave air P_{CO_2} (e.g., Frisia et al., 2011). The extreme rise of the cave air P_{CO_2} in February 2014 is a typical result caused by a large number of visitors (Fig. 3A). Few tourists visited in other months during the course of this study, and the influence of human activity seems to be negligible. However, if many tourists had visited to the cave before the initiation of the monitoring, the anthropogenic CO_2 would remain in the cave for some time, contributing temporal increase of the air P_{CO_2}. The water P_{CO_2} data in such a case will be lower than the air P_{CO_2}. The Inazumi Cave is a tourist cave with large numbers of visitors in summer and autumn, and the largest gap of P_{CO_2} between drip water and cave air in August would be attributable to the visitors origin P_{CO_2}.

(3) Storage condition of sample could cause undesirable effects of CO_2 degassing from the drip water in the PET tube. If there is any small amount of air present in the PET tube or any chance where drip water contacts with external low-P_{CO_2} air, CO_2 will be stripped out of the drip water, resulting in lower P_{CO_2} than cave air P_{CO_2} when measured in the laboratory. We consider the March chemical analysis result influenced by storage artifacts. Such drip water possesses lower HCO_3^-, Ca^{2+}, and P_{CO_2} than its original state.

Sampling artifacts can be prevented by careful sample storage, however, additional air monitoring is necessary to investigate the influence of PCP and visitors on the drip water geochemistry.

Drip Rate

The drip rate decreases in the sequential order of the handrails at SS2 and the glass tube at SS3 in each monitoring period, indicating that SS2L and SS3L are permanently slow. This infers that waters at SS2L and SS3L are exposed to the cave air for longer times and that they experience thinning and widening of the water film more than other sampling sites. Such water will contain a low concentration of HCO_3^- and Ca^{2+} because of continuous CO_2 degassing and $CaCO_3$ precipitation. However, HCO_3^- and Ca^{2+} at SS2 and SS3 in July and August display only minor sequential variation between the handrails. HCO_3^- and Ca^{2+} in April and June show distinct variation in the sequential order of the handrails, even though the drip rate is similar to the one in July and August. Moreover, the drip rate at SS3 in June and July shows a rapidly decreasing trend, but HCO_3^- and Ca^{2+} show only minor correspondence to the drip rate. This implies that the drip rate alone has less significant influence on the variation in the drip water geochemistry than the cave air P_{CO_2}.

Table 1 presents a comparison of the correlation between the drip water geochemistry, drip rate, and cave air P_{CO_2}. The air P_{CO_2} shows higher correlations with HCO_3^- and Ca^{2+} than the drip rate as the overall trend. As a common feature, the correlation of both the air P_{CO_2} and drip rate increases in the sequential order at SS2 and SS3. Particularly, the correlation with the drip rate at SS2M3 and SS2L is significant. This might be caused by a decreasing trend of the drip rate from August to December corresponding with a decreasing trend of HCO_3^- and Ca^{2+}. However, it is unusual to show partially high correlation. If the drip rate strongly affects the water geochemistry, then all sampling sections should show high correlations similar to SS2M3 and SS2L. In addition, HCO_3^- and Ca^{2+} at SS2 in April and June show distinct sequential variation, even though the drip rates during these periods are similar to those in July and August when HCO_3^- and Ca^{2+} show no minor sequential difference. Some previous studies have shown low correlation between the drip rate and drip water geochemistry, for instance, at the Obir Cave in Austria (Spötl et al., 2005) and the Crag Cave in Ireland (Baldini et al., 2006). Banner et al. (2007) conducted $CaCO_3$ precipitation experiment in limestone caves in Texas, USA

Table 1. Linear correlation coefficients for drip-water geochemistry, cave air P_{CO_2} vs the drip rate at SS2 and SS3. All correlation-coefficient values $p < 0.001$. Note that no data of the drip rate were obtained for the WBD due to the novel sampling method.

	HCO_3^-		Ca^{2+}	
Drip Water Sampling Stations	**Cave Air P_{CO_2}**	**Drip Rate**	**Cave Air P_{CO_2}**	**Drip Rate**
SS2U	0.66	0.33	0.73	0.37
SS2M1	0.66	0.34	0.73	0.38
SS2M2	0.77	0.53	0.79	0.56
SS2M3	0.83	0.84	0.86	0.85
SS2L	0.8	0.67	0.79	0.71
WBD	0.82	···	0.9	···
SS3U	0.84	0.64	0.87	0.7
SS3M	0.84	0.64	0.87	0.7
SS3L	0.82	0.62	0.87	0.68

and demonstrated that the $CaCO_3$ deposition rate correlates more with the cave air P_{CO_2} than the drip rate. Based on the previous studies above and our data from the Inazumi Cave, variation in the cave air P_{CO_2} can be regarded as the main determinant controlling drip water geochemistry.

Conclusions

Both diurnal and seasonal cave air ventilation were observed at the Inazumi Cave, and the ventilation can be attributed to the temperature difference of external and internal atmosphere of the cave, which is exclusively controlled by the opening A's air-circulation regime.

Ventilation played a key role controlling variation in cave air temperature, RH, and P_{CO_2}. While air P_{CO_2} showed remarkable diurnal and seasonal variation at all monitored sites, temperature and RH were stable at increasing distances from the cave entrances. This indicates that the cave air P_{CO_2} is the main variable that controls the variation in the drip water geochemistry at the Inazumi Cave, while temperature and evaporation have minor effects.

A novel sampling technique at SS2 and SS3 indicated seasonal variation in the cave air P_{CO_2}, not drip rate, was the main variable controlling the seasonal variation in HCO_3^- and Ca^{2+}, and that the karstic water instantaneously degassed CO_2 once in contact with low cave air P_{CO_2}.

The drip water P_{CO_2} repeatedly showed lower values than the cave air P_{CO_2}, and possible reasons are prior calcite precipitation, anthropogenic CO_2, or storage condition of samples. Additional cave air monitoring is required to address the impact of PCP and the anthropogenic CO_2 on the drip water geochemistry.

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COMPARISON OF BACTERIAL AND ARCHAEAL COMMUNITIES FROM DIFFERENT HABITATS OF THE HYPOGENIC MOLNÁR JÁNOS CAVE OF THE BUDA THERMAL KARST SYSTEM (HUNGARY)

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Abstract

The Molnár János Cave is part of the northern discharge area of the Buda Thermal Karst System, and is the largest active thermal water cave in the capital of Hungary. To compare the prokaryotic communities, reddish-brown cave wall biofilm, black biogeochemical layers, and thermal water samples from the phreatic mixing zone of the cave were subjected to three investigative approaches, scanning electron microscopy, cultivation, and molecular cloning. According to the SEM images, multilayer network structures were observed in the biofilm formed by iron-accumulating filamentous bacteria and mineral crystals. Cultivated strains belonging to Aeromonadaceae and Enterobacteriaceae were characteristic from both water and subaqueous biofilm samples. The most abundant molecular clones were representatives of the phylum Chloroflexi in the reddish-brown biofilm, the class Gammaproteobacteria in the black biogeochemical layer, and *Thiobacillus* (Betaproteobacteria) in the thermal water samples. The reddish-brown biofilm and black biogeochemical layer's bacterial communities proved to be somewhat more diverse than that of the thermal water. The archaeal 16S rRNA gene clone libraries were dominated by thermophilic ammonia-oxidizer *Nitrosopumilus* and *Nitrososphaera* phylotypes in all three habitats. Considering the metabolic characteristics of known species related to the detected clones, it can be assumed that these communities may participate in the local sulfur and nitrogen cycles and may contribute to microbial mediated sulfuric acid speleogenesis.

Introduction

Under the residential area of Budapest, the capital of Hungary, extensive hypogenic cave systems can be found (Leél-Őssy, 1995). They are part of the Buda Thermal Karst System at the northeastern margin of the Transdanubian Central Range (Fig. 1). In addition, they are located on the border of confined and unconfined carbonate aquifers that serve as the discharge zone of thermal water with different origins and temperatures (Mádl-Szőnyi and Tóth, 2015). In the system, a southern (Gellért Hill) and a northern subsystem (Rózsadomb) have been separated in terms of the flow systems and types of discharged water. Furthermore, major differences can also be observed in the cave-formation processes (Erőss et al., 2012).

Molnár János Cave belongs to the northern (Rózsadomb) discharge area (Fig. 1), and it is the largest active thermal water cave in Europe (Goldscheider et al., 2010). Discharges of lukewarm (20–35°C) and warm to hot (40–65°C) springs are characteristic for this area, and corrosion proved to be the dominant cave forming process (Erőss et al., 2012). The lukewarm springs tend to have lower than 1000 mg L^{-1} total dissolved solids, while the hot waters contain more than 1200 mg L^{-1} (Papp, 1942). Bodor et al. (2015) proved that in the Rózsadomb area infiltration from precipitation does not affect the physical and chemical parameters of the discharged waters. First Erőss et al. (2008) published the characteristic chemical data of the cave's spring water (pH: 6.94; T: 21.7°C; EC: 932 µS cm^{-1}), and later Bodor et al. (2014) found that the temporal variability of the parameters of the spring cave water was negligible.

The chemical characteristics of such hypogenic cave waters are influenced by the type of host rock and the composition of the released gases (Kumaresan et al., 2014). In hypogenic cave systems, microbes can be found in different mineral deposits, streams, snottites, or on cave walls and corrosion residues. Based on the 16S rRNA gene-sequence analysis, members of the phylum Proteobacteria are frequently the most abundant in these habitats. Sulfur-oxidizing bacteria belonging to the classes of Beta-, Gamma- and Epsilonproteobacteria can play an important role in sulfuric acid speleogenesis (Engel, 2011).

In the speleogenesis of Molnár János Cave, microbiologically enforced corrosion was also assumed (Erőss, 2010; Borsodi et al., 2012). In a preliminary study, Firmicutes-related aerobic and anaerobic bacteria, probably participating in local iron and sulfur transformations, were found to be predominant besides Proteobacteria in the iron-rich reddish-brown biofilm formed on the cave wall of the cave (Borsodi et al., 2012).

In the Molnár János Cave, prokaryotes inhabit different habitats, such as the cave walls, where reddish-brown subaqueous biofilm developed, and the black biogeochemical layer can be observed at distinct places on the cave walls under phreatic conditions and in the cavities of the cave, which is almost completely filled with thermal water. As the

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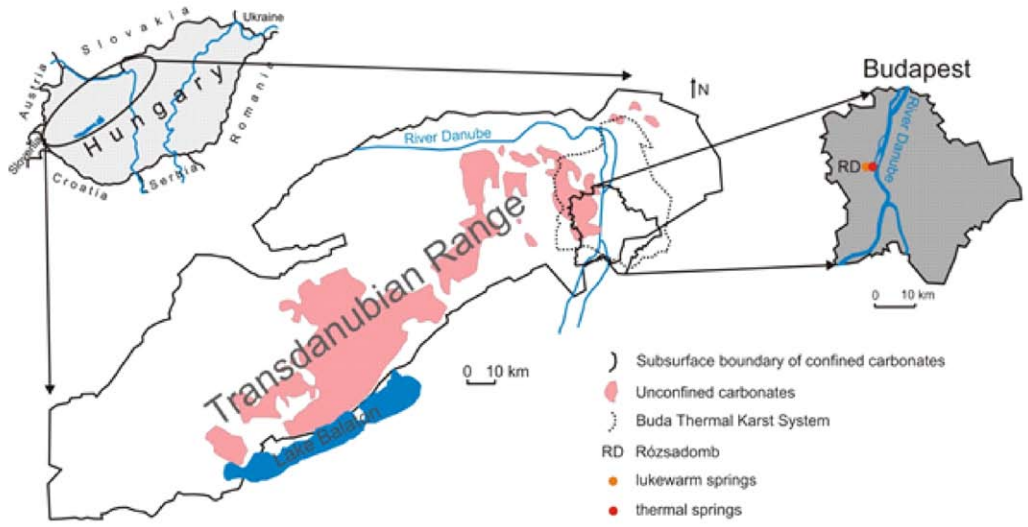

Figure 1. The location of Buda Thermal Karst and Rózsadomb in the Transdanubian Range, Hungary.

cave is located under a heavily populated area of the capital of Hungary, anthropogenic effects may also have an influence on the composition of the cave microbiota.

The aim of the present research was to gain information about the structure of the prokaryotic communities inhabiting the thermal water, the subaqueous biofilm, and black biogeochemical layers found in the Molnár János Cave mixing zone. To get a more detailed picture about the morphological and genetic diversity of bacterial and archaeal communities, SEM, cultivation, and molecular cloning methods were applied.

Materials and Methods

Cave Description and Sampling

The caves of the Rózsadomb are located in the northern part of the Buda Thermal Karst System (Fig. 1). Chambers can be found mostly at the boundary of the Szépvölgy Limestone and the Buda Marl, and the most frequent minerals are calcite ($CaCO_3$) and gypsum ($CaSO_4 \cdot 2H_2O$) (Leél-Őssy, 1995). Currently, the Molnár János Cave is nearly 6 km in length, and the explored maximum depth of the cave is 100 meters under the Rózsadomb (Erőss, 2010). The host rock of the cave is marl, sometimes with considerable (up to 80%) carbonate content. Considering the increasing number of cave divers that regularly visit the Molnár János Cave, the direct human impact on the cave ecology cannot be negligible. The temperature of the cave's water is 23–26°C near the surface, and ~19°C at 25–35 m depth in the cave due to the mixing of thermal and lukewarm components (Erőss et al., 2008).

Samples for the microbiological examinations were collected from the Molnár János Cave in December 2012. The sampling process was conducted in collaboration with divers. Submerged reddish-brown biofilm (MJB) and black biogeochemical layer (MJD) samples were collected with the help of sterile scalpels, while thermal water (MJW) filling the cave was collected using sterile bottles. According to the divers, the black biogeochemical layers are sporadically observed throughout the whole submerged cave system. At the time of sampling, the measured water temperature, pH, and conductivity values were 20.1°C, 6.9 and 1006 µS cm^{-1}, respectively. Relatively high sulfate (121 mg L^{-1}) and sulfide (6.9 mg L^{-1}) concentrations were measured in the cave's water; similar to the values reported by Anda et al. (2015). The total organic carbon and the total nitrogen concentrations of the samples were 1.4 and 1.8 mg L^{-1}. Concentrations of NH_4^+-N, NO_3^--N, and NO_2^--N were less than 0.01, 1.7, and 0.007 mg L^{-1}, respectively. Other detected physical and chemical parameters are reported by Dobosy et al. (2015).

Scanning Electron Microscopy

For scanning electron microscopy, mucilaginous reddish-brown biofilm samples were filtered onto 0.2 µm polycarbonate filters (Millipore). All subsequent steps were the same as previously described (Anda et al., 2014, 2015).

Cultivation

For cultivation, we used tenfold serial dilutions from the biofilm and thermal-water samples using sterile water and then plated onto R2A (DSMZ Medium 830) and *Sphaerotilus-Leptothrix* ferrous sulfate (DSMZ Medium 803) media (www.dsmz.de/?id=441). The R2A medium was chosen because, like cave environments, it also contains relatively small amounts of organic compounds as compared to other media. It provides the possibility for the cultivation of slow-growing oligotrophic bacteria. The ferrous sulfate medium was chosen for cultivation because results of the previous

studies indicated that bacterial iron oxidation may play an important role in the speleogenesis of the Buda Thermal Karst System (Borsodi et al., 2012; Anda et al., 2014, 2015). Following a 14-day incubation at 20°C, discrete bacterial colonies with different morphology were isolated.

Bacterial DNA Extraction and PCR Amplification

The community DNA was isolated using Ultra Clean Soil Kit (MO Bio Inc., CA, USA) according to the manufacturer's instructions, checked in agarose gel (1%) stained with ECO Safe Nucleic Acid Staining Solution (Avegene, Taiwan), and visualized by UV excitation. DNA of pure cultures was isolated using G-spin Genomic DNA Extraction Kit (iNtRON Biotechnology, South Korea) according to the manufacturer's instructions. The primers and temperature protocol used for bacterial PCR and the construction of 16S rRNA clone libraries was the same as previously applied by Anda et al. (2015).

Identification of Bacterial Strains and Molecular Clones

PCR products were grouped according to their Amplified Ribosomal DNA Restriction Analysis pattern produced with enzymes *Msp*I and *Bsu*RI (Fermentas, Lithuania) as described by Anda et al. (2015). The identification of ARDRA group and sequencing reaction were performed as previously described by Anda et al. (2015). Taxonomic relationships of the sequences were determined using the EzTaxon database (Kim et al., 2012) and Basic Local Alignment and Search Tool (BLAST) program (Altschul et al., 1997).

The 16S rRNA gene sequences (on average 800 to 900 bp long) were submitted to the GenBank under accession numbers LN998831-LN998895 for the MJBB (Molnár János reddish-brown biofilm Bacteria) clones, LN998896-LN998926 for the MJDB (Molnár János black biogeochemical layer Bacteria) clones, LN998927-LN998931 for the MJWB (Molnár János thermal water Bacteria) clones, LN998932-LN998939 for the MJBA (Molnár János reddish-brown Archaea) clones, LN998940-LN998947 for the MJDA (Molnár János black biogeochemical layer Archaea) clones, LN998948-LN998960 for the MJWA (Molnár János thermal water Archaea) clones, LN998961-LN998974 for the MJB (Molnár János reddish-brown biofilm) strains, and LN998975-LN998982 for the MJW (Molnár János thermal water) strains.

Results

Scanning Electron Microscopic Observations

The SEM analysis (Fig. 2A-F) revealed a multilayer network architecture of microbial biofilm formed by filamentous bacteria and other cells, together with mineral crystals and organic matter matrix. The structurally complex biofilm was ordered in layers (yellow arrows) and interconnected (red arrows) with filamentous bacteria together with EPS (Fig. 2C). Intertwined filamentous bacteria similar to groundwater-fed, iron-rich microbial mats (Schieber and Glamoclija, 2007) and wastewater biofilms (Felföldi et al., 2015) were also observed. On some micrographs, taken with the vCD detector, very small spheroid shaped precipitates (Fig. 2C-D) and filamentous bacteria (Fig. 2F) can be seen much brighter (white) compared to their environment. The brightness means that the precipitates carried particles containing elements of higher atomic number, such as heavy metals. The elements of greater atomic number may indicate iron compounds accumulated by microbes as previously shown by EDX analysis in an electron microscopic study on the iron-rich biofilm of the Rudas-Török spring cave in the Buda Thermal Karst (Borsodi et al., 2012). It is also apparent that some parts of the biofilm were covered by a transparent substance consisting of elements of small atomic number (C, H, O, N) that could be the residue of the biofilm extracellular polymeric substance. The EPS covered surface of filamentous forms is clearly visible in the greater magnification of Figure 2E.

Cultivation-Based Bacterial Diversity

Altogether 66 bacterial strains were isolated from the biofilm (MJB) and water (MJW) samples using two different media (Table 1). The bacterial strains were identified as representatives of phyla Proteobacteria, Firmicutes, and Actinobacteria based on 16S rRNA analysis. From the ferrous sulfate and R2A media 9 and 8 genera were identified, respectively (Table 1). Among them representatives of the genus *Aeromonas* were found on medium R2A from the MJB sample. In the case of the MJW sample, representatives of the genera *Raoultella*, *Enterobacter*, and *Citrobacter* (Enterobacteriaceae) were cultivated from both media. Among the bacterial strains, representatives of the genus *Brevibacillus* (Firmicutes) and genera *Janibacter* and *Micrococcus* (Actinobacteria) were also cultivated from the MJB sample.

Molecular Clones of Bacteria

The bacterial 16S rRNA gene based clone libraries containing 200 members were created from each of the three samples. Different numbers of phyla were detected per sample: 3 from the water (MJWB), 6 from the black biogeochemical layer (MJDB), and 9 from the reddish-brown biofilm (MJBB). The relatives of the following phyla and classes were found Betaproteobacteria, Gammaproteobacteria, Nitrospirae, Chloroflexi, Firmicutes, Deltaproteobacteria, Ac-

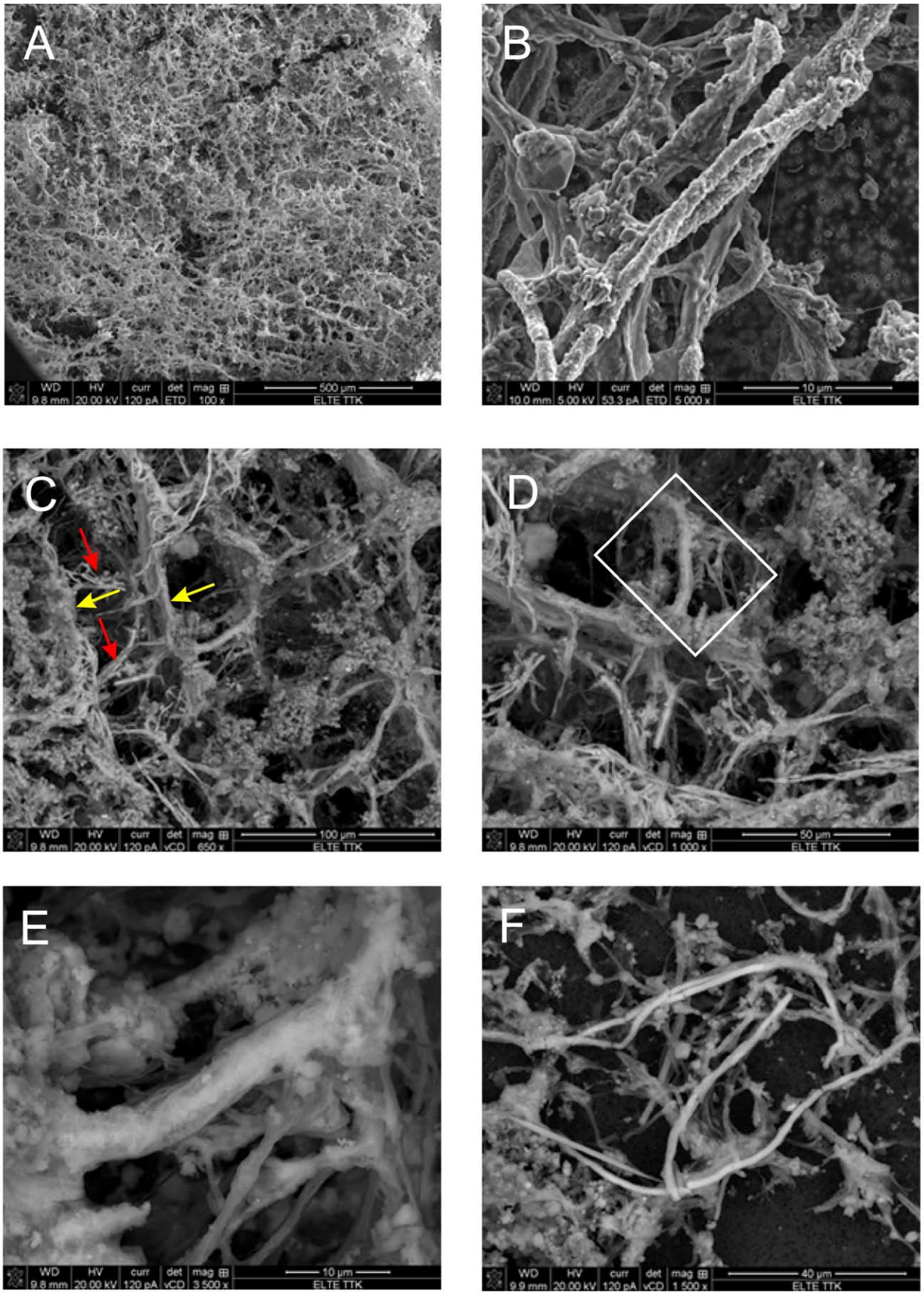

Figure 2. Scanning electron micrographs of naturally grown biofilm samples from Molnár János Cave. Scale bar (A) 500 µm, (C) 100 µm, (D) 50 µm, (F) 40 µm, (B, E) 10 µm.

idobacteria, Alphaproteobacteria, Actinobacteria, Epsilonproteobacteria, Gemmatimonadetes, Spirochaetes, and Verrucomicrobia (Fig. 3). Among them representatives of the class Betaproteobacteria and the phylum Nitrospirae could be identified in all the three samples.

The MJWB clone library was dominated (96.7%) by phylotypes belonging to the class Betaproteobacteria. These molecular clones showed the highest sequence similarity (95.42%) to the genus *Thiobacillus*. In the MJBB clone library, representatives of the phyla Chloroflexi (33.6%) were the most abundant. Within this phylum, phylotypes (12.5%) related to the order Anaerolineales were identified. In the MJDB clone library, the dominance (29.1%) of the genus *Methylohalomonas*-related (Gammaproteobacteria) clones was found. The second most abundant group of the molecular clones showed the highest sequence matching (95.88% and 95.33%) with the widespread species of *Nitrospira moscoviensis* (Ehrich et al., 1995) and *Nitrospira calida* (Lebedeva et al., 2011).

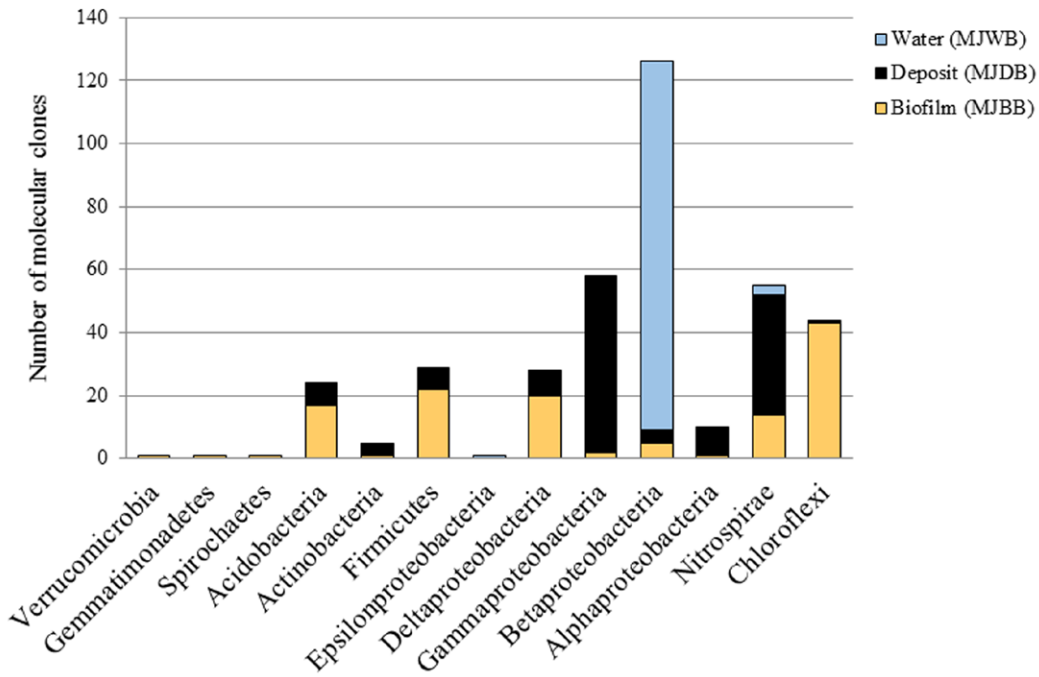

Figure 3. Taxonomic classification of 16S rRNA gene sequences of the bacterial clone libraries from the Molnár János Cave.

Molecular Clones of Archaea

Three archaeal 16S rRNA clone libraries were also constructed from the reddish-brown biofilm (MJBA), the black biogeochemical layer (MJDA), and the water (MJWA) of Molnár János Cave. Each clone library contained 100 members. The 16S rRNA gene sequences of the representative clones belonged to the phyla Euryarchaeota and Thaumarchaeota (Fig. 4). In all three libraries, the phylum Thaumarchaeota (MJWA: 68.2%, MJBA: 97.85%, MJDA: 100%) was the most abundant. Members of the phylum Euryarchaeota were only found in the case of the MJBA (2.15%) and MJWA (31.8%) samples. In the MJWA clone library, phylotypes were closely related to the "*Candidatus* Nitrososphaera gargensis" and *Nitrososphaera viennensis* (with 97.36% and 97.79% sequence similarities).

In both clone libraries (MJBA, MJDA) from the surface associated samples, representatives of "*Candidatus* Nitrososphaera gargensis" (MJBA: 35.48%, MJDA: 16.7%), *Nitrososphaera viennensis* (Stieglmeier et al., 2014) (MJBA: 32.3%, MJDA: 46.6%), and "*Candidatus* Nitrosoarchaeum limnia" (MJBA: 29.03%, MJDA: 23.3%) were the most abundant.

Discussion

The Molnár János Cave, belonging to the Rózsadomb discharge area of the Buda Thermal Karst System, is the largest active thermal water system in Europe where microorganisms can be studied. This study looked at three ecological niches within the cave system: thermal water, subaqueous black biogeochemical layer, and reddish-brown biofilm on the subaqueous cave wall. The Proteobacteria was one of the most abundant bacterial phylum in the studied habitats of the cave, as revealed both by cultivation and molecular cloning. However, the dominant members of the classes Gamma- and Betaproteobacteria showed different distributions according to the sample types and methods used for examinations. The presence of the genus *Aeromonas* was identified among the strains isolated from the reddish-brown biofilm (Table 1). This biofilm growth pattern differs from previous studies that found *Aeromonas* species to be common members of freshwater planktonic communities (Langó et al., 2002). The occurrence of these indicator organisms in the

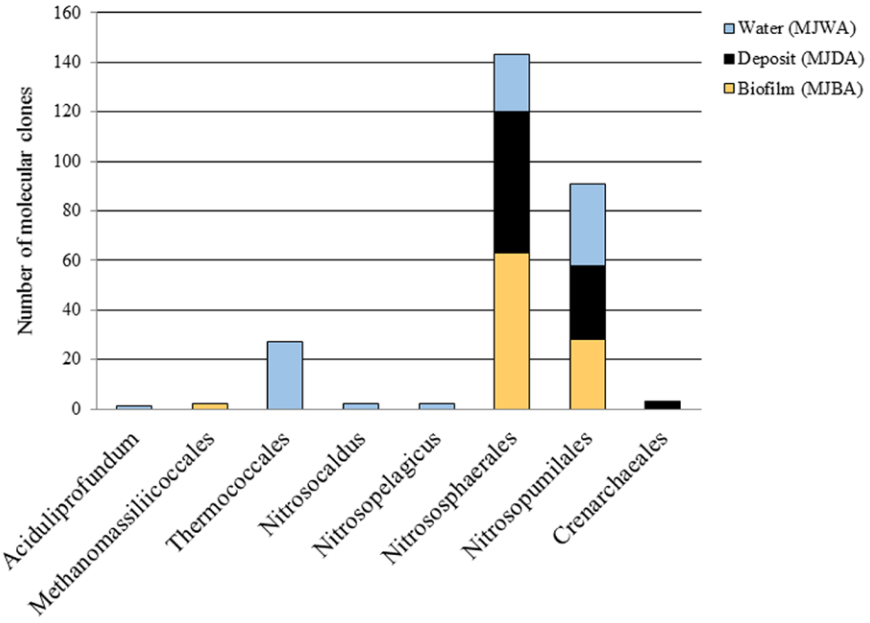

Figure 4. Taxonomic classification of 16S rRNA gene sequences of the archaeal clone libraries from the Molnár János Cave.

Table 1. Cultivated strains from the thermal water and reddish-biofilm samples inhabiting the Molnár János Cave. Notes: R: R2A medium, I: ferrous-sulfate medium, B: reddish-brown biofilm sample, W: thermal water sample.

Taxonomic Identification	Representative Strain (Accession Number)	Similarity % (bp)	Origin of the Sample	Media	
				R	I
Alphaproteobacteria					
Brevundimonas bullata (D12785)	MJW-S108 (LN998978)	100% (0/1090)	W	+	
Caulobacter henricii (AJ227758)	MJB-S139 (LN998974)	98.50% (10/667)	B		+
Paracoccus yeei (AY014173)	MJW-S104 (LN998975)	99.78% (2/914)	W	+	
Betaproteobacteria					
Xylophilus ampelinus (AF078758)	MJB-S116 (LN998968)	97.78% (21/944)	B		+
Variovorax boronicumulans (AB300597)	MJB-S128 (LN998972)	98.83% (10/853)	B		+
Vogesella alkaliphila (HE819389)	MJB-S123 (LN998970)	98.64% (13/955)	B		+
Gammaproteobacteria					
Aeromonas veronii (X60414)	MJB-S102 (LN998961)	99.71% (3/1044)	B	+	
	MJB-S104 (LN998962)	100% (0/1010)	B	+	
	MJB-S108 (LN998965)	100% (0/966)	B	+	
Citrobacter freundii (ANAV01000046)	MJB-S137 (LN998973)	99.80% (2/983)	B		+
Citrobacter youngae (AJ564736)	MJW-S106 (LN998976)	99.78% (2/915)	W	+	
	MJW-S123 (LN998981)	99.79% (2/931)	W		+
Enterobacter ludwigii (AJ853891)	MJW-S107 (LN998977)	98.76% (10/806)	W	+	
	MJW-S122 (LN998980)	99.24% (8/1047)	W		+
Pantoea dispersa (DQ504305)	MJB-S107 (LN998964)	98.67% (14/1054)	B	+	
Raoultella terrigena (Y17658)	MJW-S109 (LN998979)	99.39% (5/822)	W	+	
	MJW-S126 (LN998982)	99.43% (6/1050)	W		+
Firmicutes					
Brevibacillus fluminis (EU375457)	MJB-S119 (LN998969)	97.96% (20/981)	B		+
Brevibacillus ginsengisoli (AB245376)	MJB-S125 (LN998971)	97.41% (27/1042)	B		+
Actinobacteria					
Janibacter terrae (AF176948)	MJB-S105 (LN998963)	99.90% (1/992)	B	+	
Micrococcus yunnanensis (FJ214355)	MJB-S113 (LN998966)	99.81% (2/1046)	B		+
	MJB-S114 (LN998967)	99.90% (1/991)	B		+

cave water possibly suggests human influence, because their growth is facilitated by organic matter input (Araujo et al., 1989). The water of Molnár János Cave is currently thought to be supplied by karst and basinal fluids according to the numerical flow simulation (Mádl-Szőnyi and Tóth, 2015). Nevertheless, in the caves of Buda, the role of sewer leakage cannot be excluded under the residential area (Virág et al., 2009; Bergmann et al., 2011). In this study, representatives of Enterobacteriaceae (Gammaproteobacteria) were identified in the water from the cave, but presence of fecal coliforms was not verified by molecular or culturing methods, indicating that the infiltration from the surface was negligible. Members of the family Enterobacteriaceae with facultative anaerobic, Gram-negative, non-spore-forming, rod-shaped cells have been found in the caves of the Slovak Karst, too. In that case correlation was found between biological pollution and agricultural activity (Seman et al., 2015).

The similarity values of the partial 16S rRNA gene sequences of molecular clones to previously described species were below the (>97%) identification level (Stackebrandt and Goebel, 1994) in almost all cases. The sequence analysis of the representative molecular clones (selected by ARDRA grouping) resulted in the closest matches (with 87% to 99% sequence similarities) mainly to uncultured clone sequences from different environmental sources such as caves, thermal springs, soils, and sediments (Hansel et al., 2008; Lin et al., 2012; Ivanova et al., 2013).

In the water clone library (MJWB), a high proportion (94.2%) of molecular clones was found to be related to *Thiobacillus* species (Betaproteobacteria). The genus *Thiobacillus* consists of chemolithotrophic sulfur-oxidizing bacteria with diverse metabolisms that are a common constituent of sulfuric freshwater, marine, and subsurface habitats (Kelly and Wood, 2000). The bacterial sulfur metabolism is significant in different caves, so sulfur-oxidizing bacteria have a crucial role in sulfuric acid speleogenesis. During their metabolism, as a by-product of sulfide oxidation, the bacteria produce sulfuric acid that dissolves carbonates (Engel, 2011; Kumaresan et al., 2014). In the water of Molnár János Cave, relatively high amounts of sulfide were measured, similar to that of the thermal water of Városliget-II well, another member of the Buda Thermal Karst System. The phylotypes related to the species of *Thiobacillus aquesulis* were the most abundant according to the 16S rRNA gene sequences (Anda et al., 2015). By comparing these results against the *Thiobacillus*-related representatives from these habitats (MJWB-C7 and VLWB-C7), a 99% sequence matching was found, demonstrating the dominance of the same phylotype in both Buda Thermal Karst System communities.

In the black biogeochemical layer clone library (MJDB), the majority of the clones (29.1%) was associated with the genus *Methylohalomonas* (Gammaproteobacteria), which is distantly related (90.18% sequence similarity) to the moderately halophilic and obligate methylotrophic *M. lacus*. Previously Chen et al. (2009) described the dominance of methylotrophic organisms from the Movile Cave, Romania. The facultative methylotrophic bacterium *Methylobacterium radiotolerans* has also detected from the thermal water of the T6 karst well of Harkány Spring, located in southwestern Hungary. It indicates that biological metabolism of methane may also be important in these karst cave environments (Miseta et al., 2012).

The reddish-brown biofilm clone library (MJBB) was dominated by bacteria belonging to the phylum Chloroflexi. The filamentous bacteria were observed in the SEM images. Among them, the order Anaerolineales was identified in the highest proportion (37.2% of the phylum Chloroflexi). Members of the phylum Chloroflexi, such as *Bellilinea caldifistulae* (Yamada et al., 2007), are also prevalent in the biofilm developed on the inner surface of an outflow pipeline of the Városliget-II thermal well (Anda et al., 2015). *Chloroflexus* spp. were also identified from hypogenic caves, such as the Lower Kane Cave in Wyoming, Frasassi Cave System in Italy, and Cueva de Villa Luz in Mexico (Engel, 2007). In addition, several bacterial clones from the reddish-brown biofilm library showed high sequence matching (81.03-94.07%) with different thermophilic sulfate reducing species of the genera *Desulfuromonas*, *Desulfomonile* (Deltaproteobacteria), *Thermanaeromonas* (Firmicutes) and *Thermodesulfovibrio* (Nitrospirae). Sulfur and sulfate reducing Deltaproteobacteria (including the genus *Desulfuromonas*) have also detected in the biofilm of the Rudas-Török spring cave and Diana-Hygieia thermal spring, in the southern area of the Buda Thermal Karst System (Borsodi et al., 2012; Anda et al., 2014). During a previous study of the reddish-brown biofilm of Molnár János Cave, the majority of the molecular clones were related to thermophilic aerobic and anaerobic bacteria belonging to phylum Firmicutes (Borsodi et al., 2012). The members of this phylum (representatives of orders Clostridiales and Thermoanaerobacterales) were confirmed to be significant in this clone library, as well.

Studying the role of the ammonia and nitrite-oxidizing bacteria and ammonia-oxidizing archaea in cave environments and thermal springs has become a research hotspot recently (Chen et al., 2009; Tetu et al., 2013; Ortiz et al., 2014). Thermophilic nitrite-oxidizing bacteria (genus *Nitrospira*) often appear in different spring caves (Marks et al., 2012; Anda et al., 2014). In the black biogeochemical layer of Molnár János Cave, the phylum Nitrospirae was the second most abundant group. The molecular clones showed the highest sequence matching (95.88%) with the species *Nitrospira moscoviensis* described from a corroded iron pipe (Ehrich et al., 1995). Previously, the phylum Nitrospirae was also found to be dominant in the water and biofilm of another thermal spring of the Buda Thermal Karst System (Anda et al., 2014). Besides the nitrite-oxidizing bacteria, ammonia-oxidizing archaea also play an important role in the nitrogen cycle of different karstic environments (Ortiz et al., 2014; Stahl and de la Torre, 2012; Bartossek et al.,

2012). Among the ammonia oxidizing prokaryotes, chemolithoautotrophic ammonia-oxidizing archaea are dominant, and these microorganisms can be found in a wider range of habitats, such as hot springs and acidic soils, than ammonia-oxidizing bacteria (Stahl and de la Torre, 2012). The functional and taxonomic composition of the speleothem metagenome of Kartchner Caverns (Arizona, USA) and the Nullarbor Plain aquatic Weebubbie Cave, Australia, shows a dominance of ammonia-oxidizing archaea belonging to the phylum Thaumarchaeota. The Thaumarchaeota are recognized to be dominant players in global nitrification (Stahl and de la Torre, 2012). In the case of the Kartchner Caverns, Thaumarchaeota reads were classified as *Nitrosopumilus maritimus* (13%) and *Nitrososphaera gargensis* (4.7%). The former is an ammonia-oxidizing marine archaeon (member of group I. 1a), while the latter can often be found in terrestrial environments (Ortiz et al., 2014; Tetu et al., 2013). Representatives of *Nitrososphaera gargensis* were also found in high proportion in the Molnár János Cave (brown biofilm 33 clones, black biogeochemical layer, and water 15 clones) samples.

Conclusions

Prokaryotic communities participating mainly in the sulfur cycle of Molnár János Cave located in the Buda Thermal Karst System showed habitat dependent distribution. The reddish-brown biofilm formed on the subaqueous cave wall was dominated by anaerobic bacteria belonging to the filamentous Chloroflexi and sulfate reducing Deltaproteobacteria, while aerobic sulfur-oxidizing *Thiobacillus* (Betaproteobacteria) were the most abundant in the water of the cave. In addition, *Aeromonas* and the family Enterobacteriaceae were also detected here, indicating human impact. In the black biogeochemical layer, the majority of the phylotypes were associated with methylotrophic species. On the contrary, phylotypes related to ammonia-oxidizing archaea (*Nitrosopumilus* and *Nitrososphaera*) and nitrite-oxidizing bacteria (Nitrospirae) were found to be abundant in all three habitats. Therefore, it can be assumed that nitrogen biotransformation may also be an important microbial process beside the sulfuric acid speleogenesis in the studied hypogenic cave.

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J. Czerwik-Marcinkowska, W. Pusz, and P. Zagożdżon. Cyanobacteria and algae in an old mine adit (Marcinków, Sudety Mountains, southwestern Poland). *Journal of Cave and Karst Studies*, v. 79, no. 2, p. 122-130. DOI: 10.4311/2016MB0116

CYANOBACTERIA AND ALGAE IN AN OLD MINE ADIT (MARCINKÓW, SUDETY MOUNTAINS, SOUTHWESTERN POLAND)

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Abstract

In the interior of an old mine adit in Marcinków, southwestern Poland, during spring, 35 species of phototrophic microorganisms were found on the graphite schists in a corridor 110 m from the entrance to the adit. There were no visible life forms, but wall patches that were brownish, yellowish, grayish, or reddish surrounded by colorless mucilaginous sheaths were found. Cyanobacteria and algae were only observed in the lab cultures after three months of incubation. We identified 22 species of cyanobacteria, 12 species of green algae, and 1 xanthophyte. Extremely low species diversity of phototrophs in the old mine adit is caused by no light, high humidity, and chemical, substrate composition.

Introduction

Mine adits and caves are natural laboratories to study the mineralogy and biology of the subsurface (Spear et al., 2007), and they are considered an extreme environment for autotrophs and heterotrophs that need light for photosynthesis (Ivarsson-Norbäck et al., 2013). Moreover, Wynn-Williams (2000) stated that extremely harsh environmental conditions for photosynthetic organisms, such as drastic temperature changes, severe water deficit, and low intensity of the photon flux, act as a powerful limiting and selective factor. According to Hernández-Mariné and Canals (1994), Ducarme et al. (2004), Poulíčková and Hašler (2007), and Lamprinou et al. (2009, 2012), caves and adits have a special microclimate and ecological conditions, determined usually by three factors: light, humidity, and temperature. Microorganisms influence mineral deposition by bioprecipitation, biomineralization, and alteration of the rock substrate (Spear et al., 2007). Abandoned mines offer a variety of subterranean microclimates similar to those in natural caves (Tuttle and Taylor, 1994) and provide suitable conditions for the growth of algae, cyanobacteria, and other microorganisms.

Mine reclamation achieves, after a long time, a level similar to the cave environment (Sheffield et al., 1992), but Ňancucheo and Johnson (2012) suggest that mining of metals and coal can adversely affect the environment. Among the most widely documented are the mine-impacted water bodies, characteristically acidic (sometimes extremely) and containing elevated concentrations of iron, other metals, and sulfates. All these constitute an extreme environment that is hostile to most life forms (Johnson, 2009). In most mine adits and caves, indigenous organisms are exclusively microbial and predominantly prokaryotic, whereas eukaryotic microorganisms include acidophilic and acid tolerant species of microalgae, fungi, yeasts, protozoa, and rotifers (Baker et al., 2004; Das et al., 2009; Chlebicki et al., 2014).

The biodiversity of acidophilic and acid tolerant algae is relatively limited (Novis and Harding, 2007). Microalgae reported to be actively accumulating metals in highly acidic environments include some Chlorophyta (*Chlamydomonas acidophila, Dunaliella acidophila, Klebsormidium acidophilum*), Heterokontophyta (*Ochromonas* sp.), and Euglenophyta (*Euglena mutabilis*). Subaerial cyanobacteria and algae are photoautotrophic and photoheterotrophic microorganisms occurring in various natural habitats, as well as in environments changed by human activities and mining (Shtina et al., 1985).

Development of aerophytic algae in caves and adits depends, among other things, on pH of water, available water type (seeping or standing), and substratum (Martinčič et al., 1981; Chang and Chang-Schneider, 1991). In our study, water is one of the essential factors for the aerophytic algae and cyanobacteria colonization and growth. Generally, the presence of running or seeping water accelerates growth of aerophytic algae in caves and adits. Strong ecological differentiation of particular species in their relation to liquid water and to mineral composition of substratum is relevant (Peksa and Škaloud, 2011). Aerophytic algae can survive in the environmental only when humidity is high enough (Mulec and Kosi, 2009).

Microorganisms in caves were quite intensively studied in many countries in Europe and around the world (e.g., Pattanaik et al., 2007; Smith and Olson, 2007; Martínez and Asencio, 2010; Czerwik-Marcinkowska and Mrozińska, 2011; Vinogradowa et al., 2011; Czerwik-Marcinkowska et al., 2015). However, data on photoautotrophs inhabiting graphite schists in mine adits are very scarce (Ňancucheo and Johnson, 2012). According to our study, the old mine adit in Marcinków is an especially unfavorable environment for the existence of microorganisms, mainly due to absence of light, hence our study was aimed to determine the diversity of subaerial cyanobacteria and algae, and the influence of the main environmental variables on their development.

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Materials and Methods

Adit Description

Marcinków is a small settlement known since Middle Ages as a center of mining activity (Stysz and Mączka, 2009; Zagożdżon and Zagożdżon, 2009), currently almost completely depopulated. It is located about 9 km east of Bystrzyca Kłodzka, on the western slopes of the so-called Krowiarki hills, close to Śnieżnik massif in the Sudety Mountains. The mining operations from the fifteenth century to the beginning of seventeenth century were concentrated on the exploitation of lead ores containing silver. Intensive exploration works and exploitation of Pb, Ag, and Cu were carried out in the eighteenth century. After World War II (1948–1965), exploitation of uranium ores and searches for graphite deposits were conducted here (see Don, 1988; Madziarz and Sztuk, 2004; Stysz and Mączka, 2009). In Marcinków, there are remnants of seven adits of different ages and states of preservation (Stysz and Mączka, 2009). The examined adit (called adit number 2, 3, 4, or 5 or Middle Uranium Adit in various sources) is located on the geographical coordinates 50°17′1.7″ N and 16°46′53″ E; its entry is situated slightly above the valley bottom. This old mine adit was used for uranium mining in 1950 (Stysz and Mączka, 2009). Its length is about 180 m, average height 2 m, and width varies between 1.7 and 2.2 m. Graphite and mica schists with garnets and opaque minerals (magnetite, pyrite), occur in the adit, also dripstone with ferruginous sinters (straws, stalactites, draperies). The corridors are dark and only penetrated by low-intensity light at the entrance, but at the sampling site there was no detectable light. Humidity is high (almost 90%) due to the more or less regular supply of water from crevices.

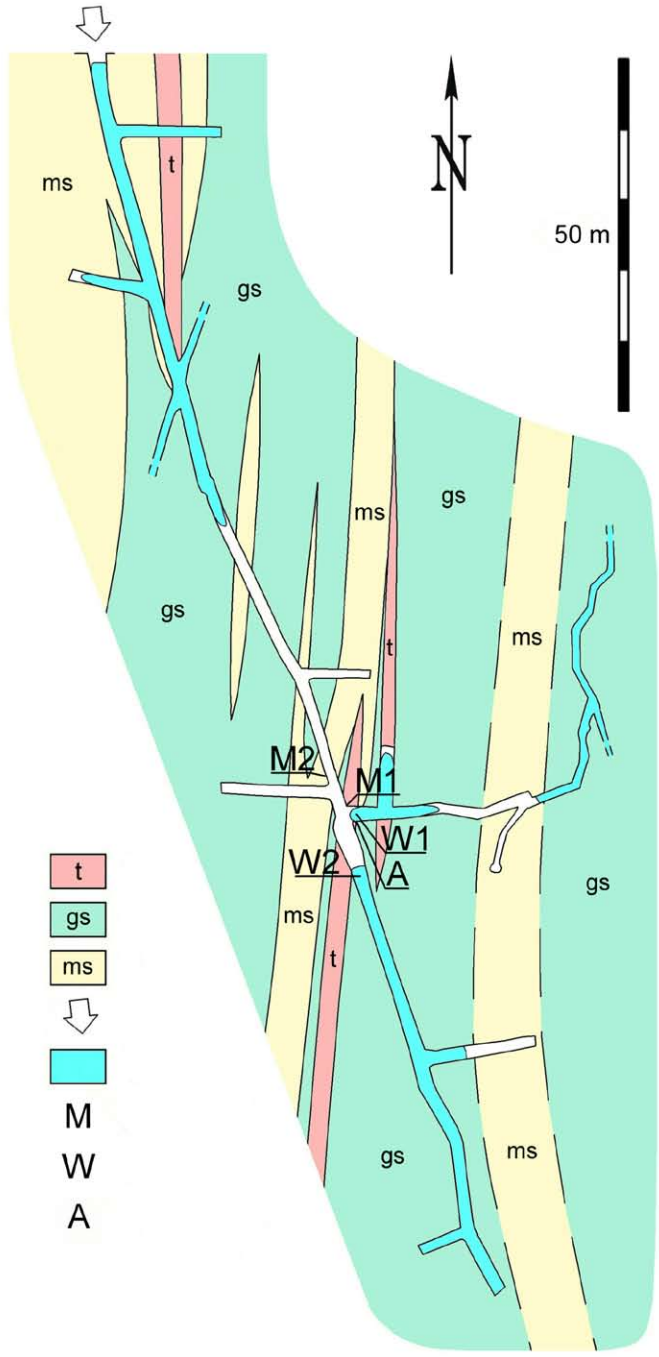

Figure 1. Locations of microorganisms (A, detail in Fig. 2), mineral substratum and water sampling sites in old mine adit in Marcinków. (t) tectonic zones; (gs) graphite schists; (ms) mica schists; (arrow) entrance of adit; zones of stagnant water marked with blue color; (M1, M2) mineral substratum sampling sites; (W1, W2) water sampling sites.

Sampling

The old mine adit was sampled 25 May 2015. One surface sample was taken from a 1 m^2 area of wall (Fig. 1). Four samples (S1, S2, S3, S4) were taken 110 m from the adit entrance. They consisted of biofilms brownish, yellowish, grayish, or reddish surrounded by colorless mucilaginous sheaths (Fig. 2). About 2 to 3 grams of each sample were scraped from walls using a scalpel, placed into labeled sterile plastic bags, and transported the next day to the lab not on ice. In the lab, material from each sample was examined under a light microscope (Jenamed 2) and aseptically placed into Petri dishes with fresh agarized (1%) nutrient Bold's Basal Medium (Bischoff and Bold, 1963) and cultured at 20 °C in a 12-h light/12-h dark cycle at 3000 $\mu Em^{-2}\ s^{-1}$ lx provided by 40 W cool fluorescent tubes. A microscopic study of cultures began from the first appearance of cyanobacterial and algal growth during three months of cultivation. The gradual development of microorganism was observed. All species were identified in living states using a light microscope. The cells for transmission electron microscopy were processed according to Massalski et al. (1995), and photomicrographs were taken with a TESLA BS 600. The following taxonomic treatises were used: Anagnostidis and Komárek (1988), Komárek and Anagnostidis (2005), Ettl and Gärtner (1995). Ecological characteristic of cyanobacteria and algae are according to John et al. (2011), and Whitton (2012).

The description of the substratum is based on two analyses of chemical composition of rock and iron sinters beside

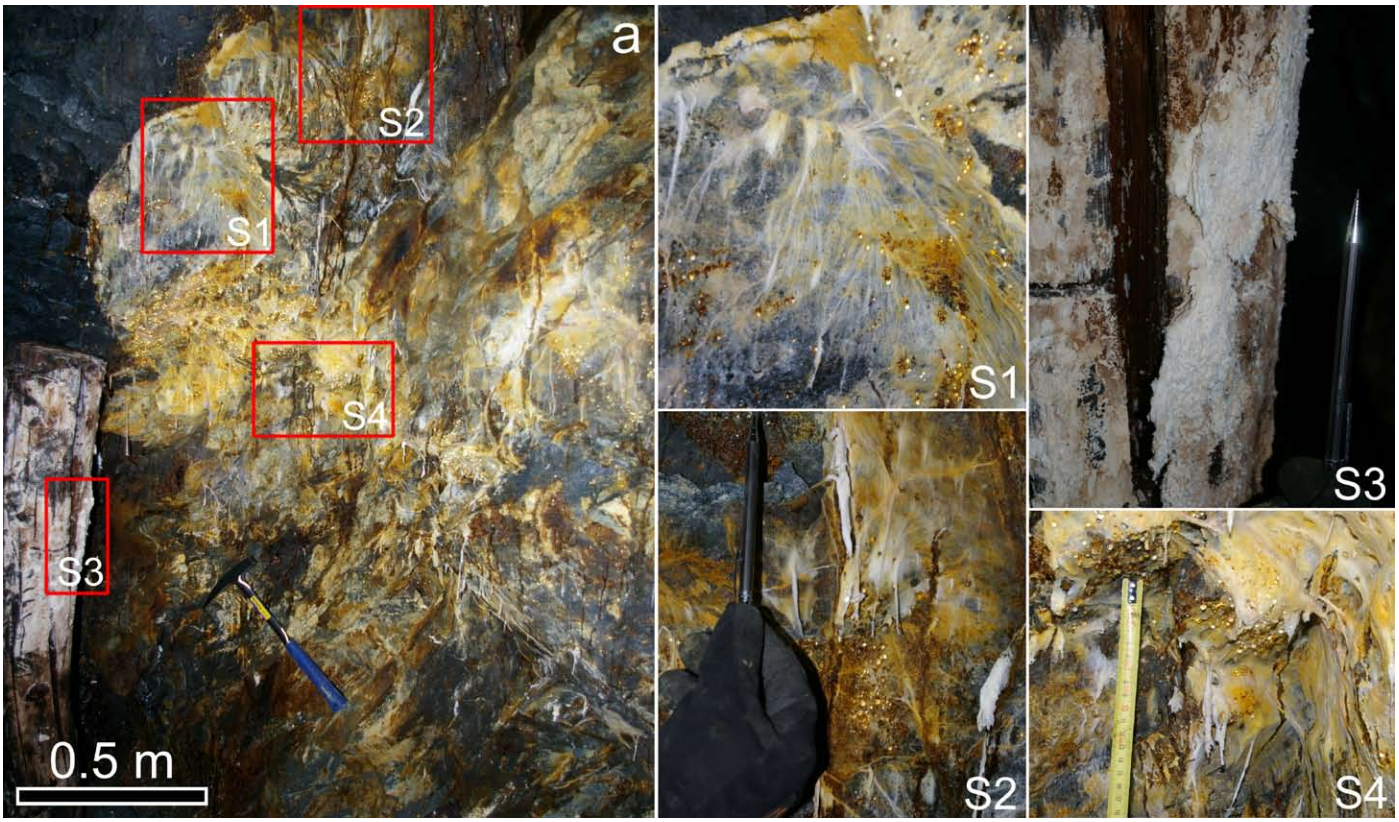

Figure 2. Algological sampling sites (S1, S2, S3, S4) in old mine adit in Marcinków, colored patches of microorganisms on cell wall: (a) map shows location of sampling sites in cartographic scale; (S1–S4) photos present enlarged details of four sites. All biological samples were collected from the 1 m^2 area.

the microorganisms sampling site (A in Fig. 1)). The analyses were carried out using XRF, GFAAS, and ICP – MS methods, and gravimetric analysis to determine the LOI value. Sample M1 was taken from a wall located 110 m in from the entrance (Fig. 1 – M1). Sample M2, to determine the chemical composition of iron sinters, covering large parts of walls and roof, was taken from several points located about 190–210 m in from the entrance of adit (Fig. 1 – M2). The analyses were done with ICP – OES and IC methods (anions), spectrophotometric method (nitrites), FIA (sulfides), and potentiometric method (pH).

Data Analysis

The species occurrence was determined on a scale of 0 to 1, and for the data collected on four studied sites were subjected to statistical analysis (Hammer et al., 2001). With the PAST program, frequency of species and diversity of samples were calculated and significances of differences between these parameters were determined. The Kruskal-Wallis test for species frequency and the diversity t test for species diversity were performed. Correspondence analysis was done to determine variations in data on species occurrence. Detrended correspondence analysis for the data set of species ecological characteristic was carried out. Canonical-variate analysis (CVA) for three taxonomic groups to identify and isolate ecological characteristics of each group was performed. The Monte Carlo permutation test was done to determine significance of diversity in the species data collection. All the above analyses were carried out using the software package Canoco 5.0 (Ter Braak and Šmilauer, 2012).

Results

In total, 22 species of cyanobacteria, 12 species of green algae, and one xanthophyte were documented (Table 1). On the wall in the old mine adit, it was observed that microorganisms were arranged in patches that were brownish, yellowish, or grayish in color and surrounded by colorless mucilaginous sheaths. Seventeen species of coccoid and five filamentous cyanobacteria were found. The genus *Gloeocapsa* was the most frequently occurring (four species), followed by *Cyanosaccus, Leptolyngbya* (three species each), then by *Aphanothece, Chroococcus*, and *Pleurocapsa* (two species each). The *Gloeocapsa* species found here indicate that growth of these taxa on the graphite schist walls were the most optimal. Cyanobacteria from Chroococcales, Synechococcales, Nostocales, and Pleurocapsales groups were found (Figs. 3, 4). Among the Chlorococcales order, *Aphanocapsa, Aphanothece, Asterocapsa, Chroococcus, Cyanosaccus*, and *Gloeocapsa* were present. In studied samples, 12 green algae species were found, among which

Table 1. Locations of subaerial cyanobacteria and algae at old mine adit in Marcinków. Numbers show sampling sites. Information regarding ecology are from the authors own observations (color sheath/cell) and from the literature: John et al. (2011) and Whitton (2012).

Taxa	Sampling Site	Color Sheath/Cell	Ecology
Cyanobacteria			
Aphanocapsa muscicola	1, 2, 3	brownish or greyish	terrestrial
Aphanothece saxicola	2, 4	reddish or yellowish-brownish	on wet rocks
Aphanothece sp.	3	greyish	terrestrial
Asterocapsa sp.	4	brownish	terrestrial
Chroococcus spelaeus	1, 2	yellowish-brownish	terrestrial
Chroococcus sp.	2	yellowish	terrestrial
Cyanosaccus aegeus	3, 4	yellowish or yellowish-brownish	terrestrial
Cyanosaccus sp.	1, 2	yellowish	terrestrial
Cyanosaccus atticus	2	greyish, bluish	euendolithic, carbonate substrates
Gloeocapsa biformis	3, 4	orangish or yellowish-brownish	chasmoendolithic
Gleocapsa nigrescens	3	greyish	epilithic
Gloeocapsa novacekii	2, 4	yellowish or yellowish-brownish	epilithic
Gloeocapsa rupicola	1, 2, 3	reddish	epilithic
Gloeothece palea	2, 4	pale yellowish or yellow-brownish	on wet rocks
Hassalia sp.	1	bluish-greyish	terrestrial
Leptolyngbya carnea	1, 2	yellowish-brownish	terrestrial
Leptolyngbya sp.	3	yellowish-greyish	terrestrial
Leptolyngbya sp.	1, 4	brownish-greyish	terrestrial
Pleurocapsa minor	4	yellowish-brownish	on rocks among other algae
Pleurocapsa sp.	4	yellowish	on wet rocks
Scytonema mirabile	3	yellow-brownish	on moist soil
Scytonema sp.	2, 3	bluish-greyish	on moist soil
Chlorophyta			
Chlorella miniata	2, 3	greenish-bluish	subaerial
Chlorella vulgaris	3	greenish	subaerial
Chlorella sp.	4	greenish-bluish	subaerial
Chlorococcum humicolum	4	greyish	neutral to acid soils, porous acidic rocks
Gloeocystis vesiculosa	3, 4	greenish-brownish	terrestrial
Gloeocystis sp.	2, 3	greyish	terrestrial
Kirchneriella sp.	2, 3	yellowish	on wet rocks
Stichococcus bacillaris	1, 2, 3, 4	yellowish-rownish	bright patches on soil, stone walls, and other damp surfaces
Stichococcus minor	3, 4	yellowish-greenish	terrestrial
Stichococcus sp.	1, 2, 3, 4	greenish-greyish	terrestrial
Stichococcus sp.	1, 4	greenish	terrestrial
Pseudococcomyxa simplex	2, 3	yellowish-blackish	on wet soil
Heterokonthophyta/Xanthophyceae			
Xanthonema exile	2, 4	yellowish-blackish	chasmoendolithic

the most frequently encountered were members of the genera *Stichococcus* (4 species), followed by *Chlorella* (3 species) and *Gloeocystis* (2 species). Whereas green algae species, such as: C*hlorococcum humicolum, Kirchneriella* sp., and *Pseudococcomyxa simplex* were found only once. A chasmoendolithic (organisms growing in rocks fissures) yellow-green algae (Xanthophytes), namely *Xanthonema exile*, was only found at two sites.

Species frequency in the sampling sites was on average 0.5; only in the S1 site were the species less numerous (0.25). These differences were not statistically significant. Similar properties also involved the Shannon diversity index for the studied samples, but the test showed significant differences due to the low diversity of the first site (Fig. 5). As evidenced by the analysis of species distribution in the correspondence-analysis ordination space, the gradient differentiating the occurrence of species along the first axis can be interpreted as an increasing light gradient (Fig. 6). Phototrophs species: *Asterocapsa* sp., *Chlorella* sp., *Chlorococcum humicolum*, *Pleurocapsa minor*, *Pleurocapsa* sp., group in the right part of the ordination diagram, whereas the microorganisms, in the left part, indicate a reduction

in demand for light, even at photon flux densities lower than the photosynthetic compensation point. Detrended correspondence analysis was also done on the basis of the studied samples ecological properties (Fig. 7). The species from wet rocks and soils were distributed along the first ordination axis, whereas on the second ordination axis were most of the remaining species. All the studied species were placed into three taxonomic groups. The environmental data was correlated with taxonomical affiliation. The canonical-variate analysis result distinctly indicates the dissimilarity of the adaptation features among the three groups (Fig. 8).

The adit rock represents an altered (weathered) mica schist, strongly crushed during tectonic movements. A high content of SiO_2 (57.20%) and Al_2O_3 (16.33%) is typical for a silicate rock. Other significant components are oxides of Fe (Fe_2O_3: 8.10%) and of Mg (3.17%) and K (about 3.73%). The most important trace elements are Ba (382 ppm), Rb, V, Zr, and Cr (about 135 to 170 ppm each). The only main element of iron sinters was iron oxide (Fe_2O_3), representing 65.30% of a sample M2, except silica (1.92%), all other components occurring in amounts from 0.32 to less than 0.01%. The sample showed a very high value of loss on ignition of 32.79%. The most common trace elements are Mo (144 ppm), Zn (103 ppm), Co (58 ppm) and Cu (50 ppm). There were differences in the chemical composition of mine-water samples collected from sites close to each other and representing various water types. The first sample (W2) was a fresh water with a slightly acidic nature (pH = 6.4), with total hardness of 101 mg L^{-1} $CaCO_3$ (carbonate hardness: 52.5 mg L^{-1} $CaCO_3$, and non-carbonate hardness: 48 mg L^{-1} $CaCO_3$) and total mineralization of about 200 mg L^{-1}. A second sample (W1) was stagnant water in a zone of extraction of sulfide ores and had a very low pH (3.7), hardness at the level of 128 mg L^{-1} $CaCO_3$ (entirely non-carbonate hardness), and total mineralization about 285 mg L^{-1}.

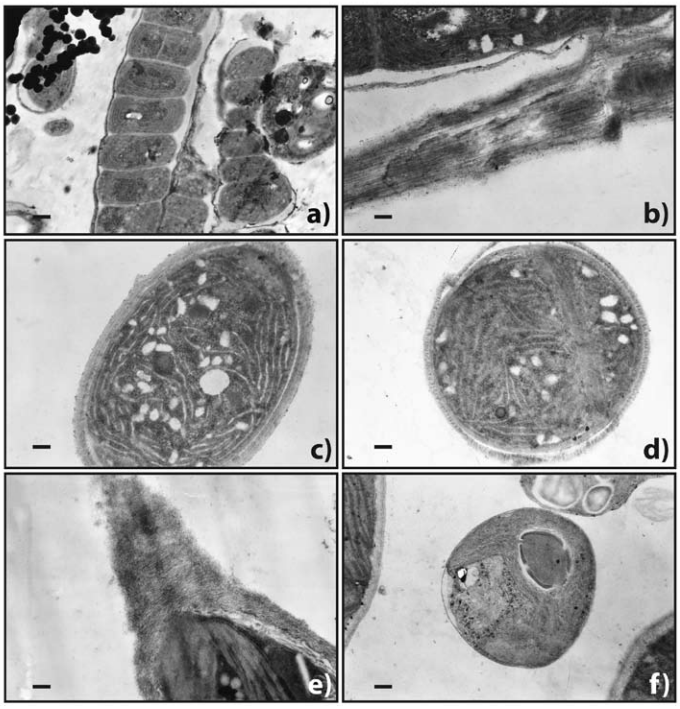

Figure 3. Electron micrographs of an ultrathin section of the biofilm including cyanobacteria and green algae from mine adit in Marcinków (a) Filaments of *Leptolyngbya* sp. show cells in different developmental stages within the same aggregate. (b) transverse section of cyanobacteria cell-wall layers with bacterium inside the cavity in the cell wall, and the electron dense material. (c-d) *Pleurocapsa minor*. (e) transverse section of green algae cell wall in mucilage outside the cell wall. (f) cross section of *Chlorella vulgaris* cell. Scale bar: 5 µm.

Discussion

The majority of cyanobacteria have mechanisms for occurrence under extreme environmental conditions – in deserts, in the Antarctic region, or under and within rocks, as well as in caves (Lukešová, 1993; Flechtner, 1999). They are very resistant to low oxygen levels, very high or very low temperatures, and poor light (Mannan and Pakrasi, 1993). Moreover, cyanobacteria exhibit very strong phenoplasticity, hence a variety of characteristics under pressure of environmental factors. Most cyanobacteria sheaths appeared colored because of pigments present acting as filters to diminish the amount of incident light (Krumbein and Potts, 1978).

In the old mine adit in Marcinków, species belonging to the order Chroococcales were the most abundant cyanobacteria documented at all sampling sites. However, coccoid species were more abundant in darkness than filamentous taxa, but in Vinogradowa et al. (1998), the proportion of coccoid to filamentous forms decreased as irradiance got less. The genus *Gloeocapsa*, the most commonly encountered cyanobacteria in the adit, has also been recorded in many other caves in Europe: from Poland (Czerwik-Marcinkowska and Mrozińska, 2011), Slovenia (Mulec et al., 2008), and Russia (Mazina and Maximov, 2011), while in Greece (Lamprinou et al., 2009) and Spain (Urzi et al., 2010) the diversity of this species was relatively lower. *Chroococcus* are also common in caves (Cennamo et al., 2012; Czerwik-Marcinkowska, 2013). Species from *Scytonema* genus are considered to be one of the most dominant aeroterrestrial cyanobacteria (Pattanaik et al., 2007). Colonies of *Aphanocapsa muscicola* present in the studied mine adit were also found in caves and on dry rocks in Israel (Vinogradova et al., 1998, 2009, 2011). Similarly, *Aphanocapsa saxicola* occurring in S2 and S4 sites in the studied mine adit was also observed in terrestrial habitats of Israel, such as on dry rocks of north and south facing slopes, in soil samples of terra rossa, in soil crusts on loess, and in sand on shallow slopes (Vinogradova et al., 1995).

It is interesting that in the old mine adit Oscillatoriales were not observed; usually representatives of this group play a significant role in cavernicilous microorganisms. According to Mulec et al. (2008), coccoid forms tolerate low light

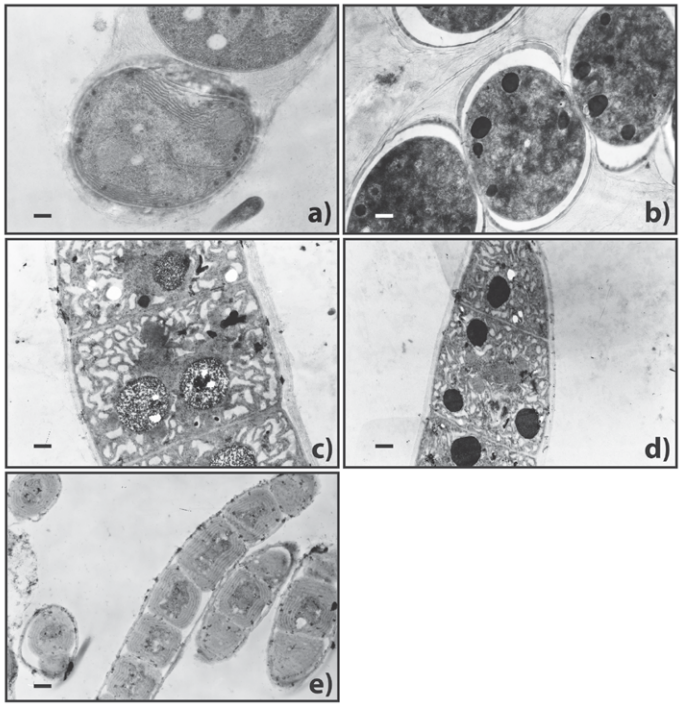

Figure 4. Electron micrographs of cyanobacteria filaments in studied adit (a-b) *Leptolyngbya* sp. from sampling site S1 (c-d) *Scytonema* sp.; apex of a filament; from sampling site S2 and S3 (e) *Leptolyngbya* sp. from sampling site S4. Scale bar: 10 µm.

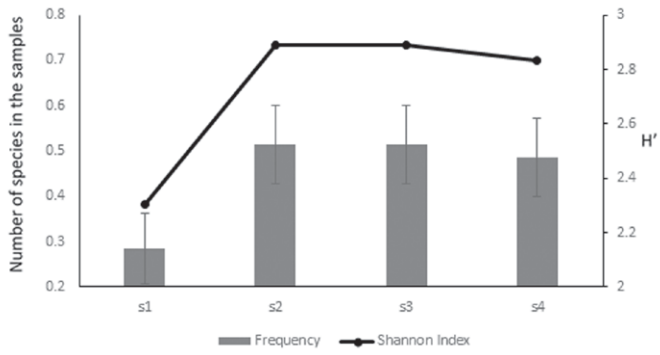

Figure 5. Percentage of total species (± SE, column diagram, left side) and diversity index Shannon-Wiener (H′, line diagram, right side) in studied samples (S1, S2, S3, S4). The frequency differences between samples are not statistically significant (Kruskal-Wallis test, $p > 0.05$) but the H′ differences are statistically significant (diversity t tests, $p < 0,05$).

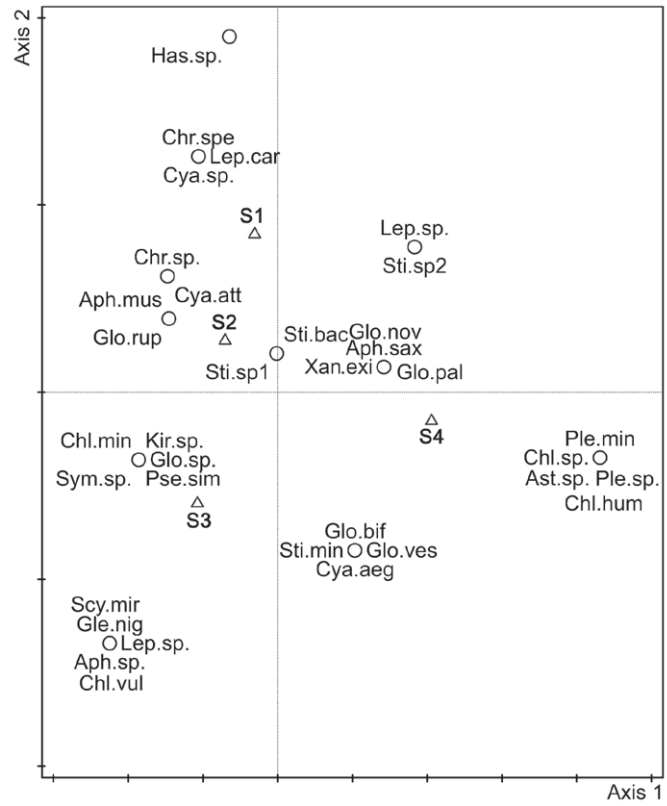

Figure 6. Analysis of the compatibility (correspondence analysis) of species occurrence data in studied samples (S1, S2, S3, S4 representing algological sampling sites). Eigenvalue of first ordination axis was 0.48. Abbreviations of species names consist of three letters of a generic name and three letters of a species name. The complete names of species are listed in Table 1: *Aphanocapsa muscicola* (Aph. mus); *Aphanothece saxicola* (Aph. sax); *Aphanothece* sp. (Aph. sp.); *Asterocapsa* sp. (Ast. sp.); *Chlorella miniata (*Chl. min); *Chlorella vulgaris* (Chl. vul); *Chlorella* sp. (Chl. sp.); *Chlorococcum humicolum* (Chl. hum); *Chroococcus spelaeus* (Chr. spe); *Chroococcus* sp. (Chr. sp.); *Cyanosaccus aegeus* (Cya. aeg); *Cyanosaccus* sp. (Cya. sp.); *Cyanosaccus atticus* (Cya. att); *Gloeocapsa biformis* (Glo. bif); *Gleocapsa nigrescens* (Gle. nig); *Gloeocapsa novacekii* (Glo. nov); *Gloeocapsa rupicola* (Glo. rup); *Gloeocystis vesiculosa* (Glo. ves); *Gloeocystis* sp. (Glo. sp.); *Gloeothece palea* (Glo. pal); *Hassalia* sp. (Has. sp.); *Kirchneriella* sp. (Kir. sp.); *Leptolyngbya carnea* (Lep. car); *Leptolyngbya* sp. (Lep. sp.); *Leptolyngbya* sp. (Lep. sp.); *Pleurocapsa minor* (Ple. min); *Pleurocapsa* sp. (Ple. sp.); *Scytonema mirabile* (Scy. mir); *Scytonema* sp. (Sym. sp.); *Stichococcus bacillaris* (Sti. bac); *Stichococcus minor* (Sti. min); *Stichococcus* sp1 (Sti. sp1); *Stichococcus* sp2 (Sti. sp2); *Pseudococcomyxa simplex* (Pse. sim); *Xanthonema exile* (Xan. exi).

intensity most successfully among cyanobacteria, although their proportion in speleocommunities is very high. Mulec et al. (2008) suggest that the oscillatorians trichomal species adapt better to conditions with constantly low values of light density compared to nostocaceans. Adaptation mechanisms of cyanobacteria allow them to more successful acclimatization to aphotic environments, thus it can be supposed that extremely low diversity of microorganisms in old mine adit is caused by the cumulative effects of stress factors such as no-light, graphite schists substrate, and high humidity. The majority of cyanobacteria species found in the mine adit were recorded in similar environments in Poland (Czerwik-Marcinkowska and Mrozińska, 2011). Green alga *Chlorella vulgaris* present in the studied adit, occurs besides in caves, on wet soils, tree bark, wet rock surfaces, and walls in the Tyrolean Alps, also on volcanic soil in Japan and Himalayas (Ettl and Gärtner, 1995). Experiments on axenic cultures grown in an inorganic medium carried out by Kol (1966) showed that several algal strains can tolerate the complete absence of light; and furthermore, some algal strains showed intensive development even under such conditions. Many microorganisms from caves and adits have the ability to adapt to the ecological conditions and are aerophytes, terrestrial forms, and the edaphon.

Acid mine drainage is a phenomenon commonly associated with mining activities throughout the world (Novis and

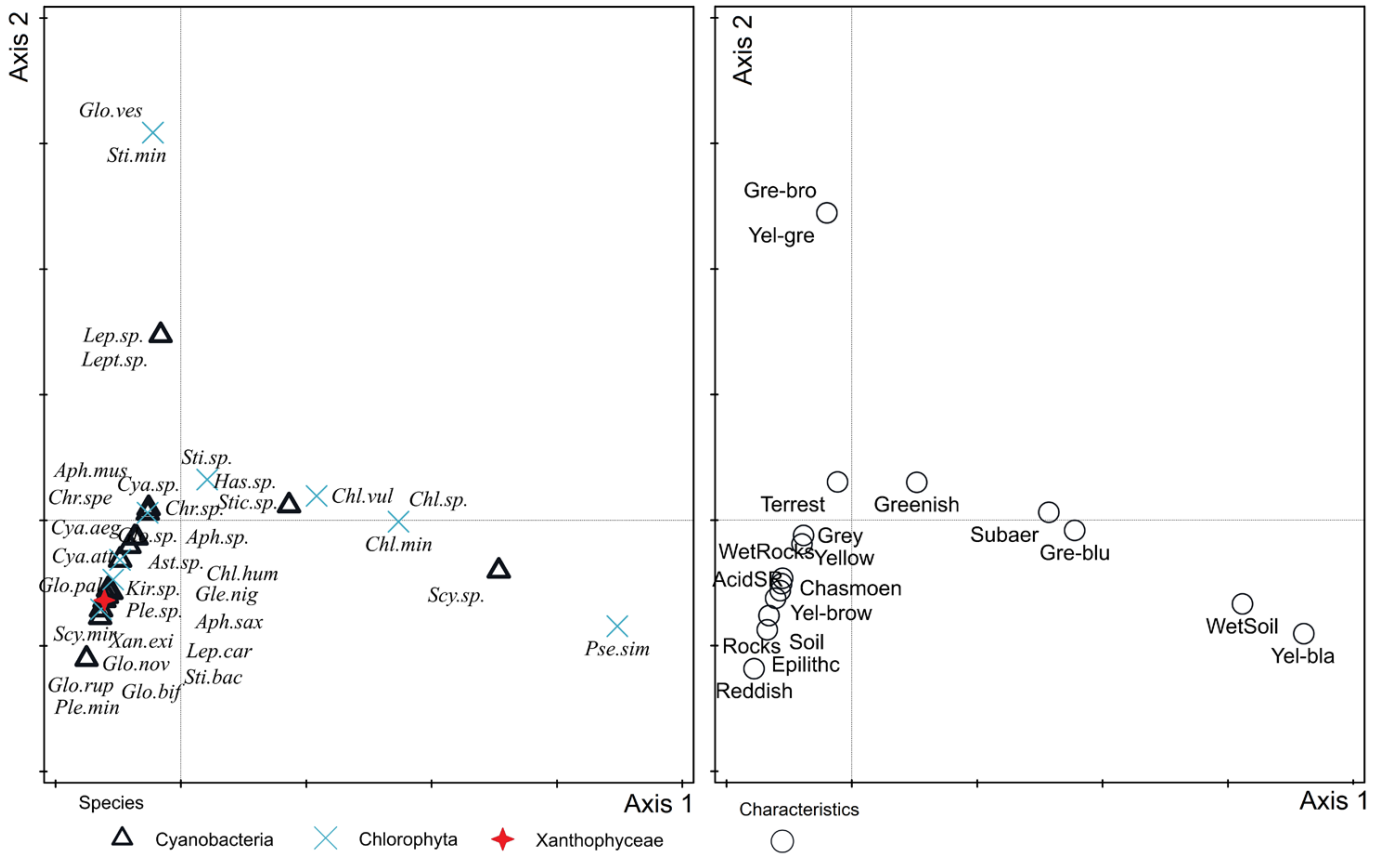

Figure 7. Non-trend analysis of the compatibility (detrended correspondence analysis) of species environments in studied samples. Right diagram – ecological properties of species found in the samples, left diagram – species distribution in terms of these properties. Abbreviations of variables' names: AcidSR (acid soils); Chasmoen (chasmoendolithic); Epilithic (epilithic); Gre-blu (greenish-bluish); Gre-bro (greenish-brownish); Greenish (greenish); Grey (greyish); Reddish (reddish); Rocks (on rocks among other algae); Soil (on moist soil); Subaer (subaerial); Terrest (terrestrial); WetRocks (on wet rocks); WetSoil (on wet soil); Yel-bla (yellowish-blackish); Yel-brow (yellowish-brownish); Yel-gre (yellowish-greyish). Abbreviations of species names are as in Figure 6, and full names are in Table 1.

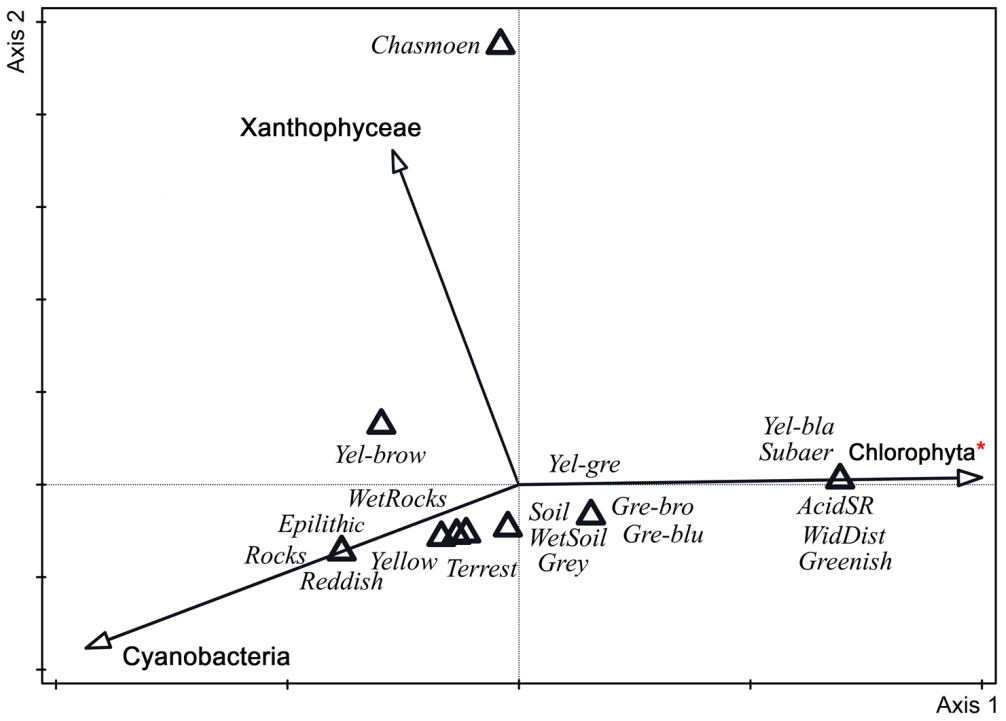

Figure 8. Discriminant analysis (canonical variate analysis) of species environment data in studied samples. The asterisk indicates the variable significant for the data set (Monte Carlo test, $p < 0.05$). Abbreviations of variables' names: AcidSR (acid soils); Epilithic (epilithic); Grey (greyish); Gre-blu (greenish-bluish); Gre-bro (greenish-brownish); Greenish (greenish); Reddish (reddish); Rocks (on rocks); Soil (on moist soil); Subaer (subaerial); Terrest (terrestrial); WetRocks (on wet rocks); WetSoil (on wet soil); WidDist (wide distribution); Yellow (yellowish); Yel-bla (yellowish-blackish); Yel-brow (yellowish-brownish); Yel-gre (yellowish-greyish).

Harding, 2007). Such acidification is the consequence of sulfides in rock strata becoming exposed to water and oxygen, and extremely acidic habitats in adits are associated with mining spoil (Novis and Harding, 2007). Such drainage began during the industrial revolution and now accounts for most of the extremely acidic habitats worldwide (Johnson, 1998). Light quality and its duration are the most obvious factors influencing cyanobacterial diversity in caves and adits (Whitton, 2012), but many studies (e.g., Chapman, 1993; Lundberg and McFarlane, 2011) dealt with one or the other of two well-defined environments. Water availability in adits and caves is important for growth and colonization, but most caves and adits, at least in Europe, North America, and Australia, are deep and damp and their walls are covered with colored cyanobacteria and green algae (see Vinogradova et al., 1998).

Conclusions

Although no visible life of cyanobacteria and algae communities in the old mine adit in Marcinków were present, but applying cultivation, we found 35 species of phototrophic microorganisms mostly known as subaerial forms. Extremely low species diversity of algae in the mine adit might be caused by multifactor stress, combining no light, substrate components, and high humidity. This investigation of the diversity of subaerial cyanobacteria and algae in an old mine adit was the first conducted in Poland.

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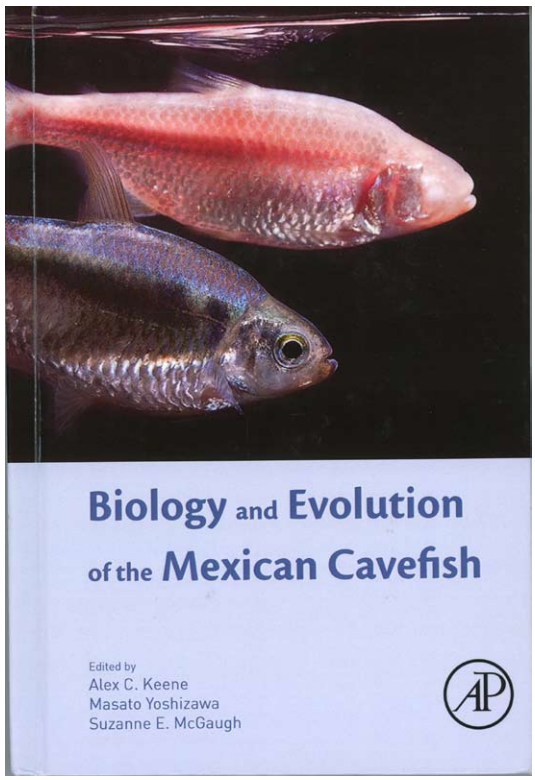
BOOK REVIEW: THE BIOLOGY AND EVOLUTION OF THE MEXICAN CAVEFISH

Alex C. Keene, Masato Yoshizawa, and Suzanne E. McGaugh, editors, 2016, Elsevier/Academic Press, The Netherlands, 403 p., 6.1 X 9 inches, hardbound, $99.95, ISBN 978-0-12-802148.



The Mexican cavefish, *Astyanax mexicanus,* was first discovered and collected by Salvador Coronado in Cueva Chica in Mexico in 1936 and brought to the United States by steamer from Tampico by C. Basil Jordan. It is probably the best known cave animal, especially because it is commonly used in biomedical research as a model of eye development and because it is widely available in aquarium stores. Easy to raise in the lab, it also has the advantage, from an experimental point of view, of hybridizing with its surface ancestors, now placed in the same species as the cave populations.

Its reputation as a model for the evolution of adaptation to cave life is decidedly mixed. At first glance, Mexican cavefish look like their surface relatives except for the lack of eyes and pigment. Relative to other fish in caves, such as the North American amblyopsid fish and the Chinese cyprinid fish, the Mexican cavefish is pedestrian in appearance. First impressions can be deceiving. Sylvie Rétaux and her colleagues, in a chapter in the volume under review, point out that "anyone . . . who has a good sense of observation will at first have a hard time believing that they [surface and cave animals] belong to the same species." The pioneering study of ecology and distribution of the Mexican cave fish (Mitchell et al. 1977) suggested, based on geological, distributional, and hydrological evidence that the populations had been isolated in caves less than 100,000 years, a short time compared to the millions of years of isolation of amblyopsid cavefish. These observations all suggested that the Mexican cavefish was in very early stages of adaptation to cave life. This view was reinforced by the work of Horst Wilkens and his colleagues, beginning in the early 1970s and continuing to the present. Wilkens has championed the view that eye and pigment loss, the most visible of changes in the Mexican cavefish, was entirely due to the accumulation of mutations that reduced eye and pigment, and that these mutations had no advantage or disadvantage with respect to natural selection. The Mexican cavefish became a model for what is often called neutral mutation, but not for adaptation.

All this has changed, beginning in the early 2000s with the work of William Jeffery and his graduate students and post-docs (e.g., Jeffery 2001, Strickler et al. 2001). They showed that eye reduction was associated with changes in the jaw and in taste buds, and that not all proteins involved in eye development actually decreased in function in cavefish but, on the contrary, some increased. Taken together, their studies and many later studies reviewed in this volume make it impossible to sustain the view that the Mexican cavefish is a recent colonist of caves and that the changes are largely non-adaptive. The development of genomics, especially the ability to rapidly sequence genes, together with the ability to do eye transplants from surface to cave individuals, has established the Mexican cavefish as a model, not only of adaptation to caves, but of adaptation in general.

The editors have assembled not only many of the titans in the field, especially William R. Elliott, William R. Jeffery, Sylvie Rétaux, and Clifford J. Tabin, but also a new generation of biologists at the forefront of the emerging studies of the Mexican cavefish. The book is organized into five parts, with twenty chapters:

I. Ecology and Evolution
II. Genetic Diversity and Quantitative Genetics
III. Morphology and Development
IV. Behavior
V. Future Applications

There are opening and closing chapters by Tabin and Jeffery. Tabin, in whose Harvard laboratory many of the authors trained and who discovered the most fundamental genes of animal development, outlines the advantages of the Mexican cave fish as a model system, including its morphological variability and the ease of maintaining reproductive populations in the laboratory. Jeffery, whose pioneering work on the pathways of eye degeneration served as an impe-

tus for many of the studies, closes the book with a review of the research groups involved with the Mexican cavefish, beginning in the 1940s and 1950s with José Álvarez, Charles Breder, and Perihan Şadoðlu.

Part I, on ecology and evolution, is especially welcome because relatively little has been written previously about what the ecologist G. Evelyn Hutchinson called the "ecological theater" of Mexican cavefish, compared to the "evolutionary play." There are two chapters by Elliott, who provides detailed descriptions of both the 29 caves with cavefish and the community of organisms that occupies the caves. One message from these chapters is that there is a wide variety of subterranean habitats occupied by the cavefish. One interesting point he makes is that the aquarium fish from Cueva Chica are atypical, both because they are from a hybrid population and because they have been subjected to many generations of artificial selection in aquaria. Espinasa and Epinasa provide a fascinating chapter on the hydrologic context and point out that many of the caves with cavefish, such as Cueva Chica and Cueva de El Pachón, are much younger than the likely age of the cavefish populations, so that it is a mistake to think current conditions are those under which the population evolved. Ornelas-García and Padraza-Lara review the taxonomic history, back to the time when each cave population was thought to be a separate species in the troglomorphic genus *Anoptichthys.* They hold that too much emphasis has been placed on hybridization in taxonomic considerations, that genetic evidence suggests that there are four or five separate cave invasions and species, and that these species may even have had different surface ancestral species in the genus *Astyanax.* While they do not formally describe these species, they set the stage for a reconsideration of the taxonomy of what we currently call *Astyanax mexicanus.*

Part II focuses on population genetics. Borowsky focuses on the decades-long debate about the relative importance of selection versus genetic drift (neutral mutation) in eye and pigment loss. He takes a statistical approach, rather than focusing on an individual pathway, and argues that eyes are lost through the direct (energy economy) and indirect effects (pleiotropy) of selection and that pigment is lost through drift. As more evidence becomes available, it remains to be seen whether his predictions will hold. O'Quin and McGaugh examine the genetic basis of troglomorphy (those traits convergent in cave populations) using quantitative trait loci that link genotype to phenotype. The technique, still in its beginning phases with respect to Mexican cavefish, offers considerable promise in the analysis of adaptation, especially the linkages between traits such as eye loss and taste bud number. They point out that except for albinism and the "brown" phenotype, all traits examined, morphological and behavioral, are polygenic. Rohner looks at natural selection even more directly. He examines the hypothesis, originally due to C.H. Waddington, that much genetic variation is canalized (i.e., silenced with respect to phenotype) and can be released by environment perturbation. He shows that such variation exists in surface populations and is released in cave populations, and it may be the important driver of adaptation. His suggestion that the environmental perturbation responsible is a drop in conductivity in cave streams cannot be generally true, since conductivity is typically higher in cave streams because water in contact with limestone rock increases in concentration of calcium ions. Nevertheless, this is really a quibble, because there are many candidates for the environmental perturbation of cave water, starting with darkness itself.

In Part III, on morphology and development, individual traits are considered. In general, the debate on selection and neutrality recedes a bit, and the focus becomes whether the hypothesis that a particular trait is adaptive. With the exception of eyes, pigment is the best-studied trait in Mexican cavefish, and while there is an emerging consensus about the involvement of natural selection in eye loss, there is no consensus about pigment. Jeffery and his colleagues provide an exceptionally clear description of pigment development in cavefish. They convincingly argue that while the number of pigment producing cells (melanophores) may be controlled by drift, melanin production is affected by natural selection, since the blockage of the conversion of L-tyrosine to L-DOPA allows L-tyrosine to increase activity levels in cavefish, an adaptation to low food. While they may ultimately be wrong, they very clearly lay out the evidence and the steps involved. Yamamoto lays out in detail the development of the eye and its degeneration in adult cavefish, pointing out the critical role of an increase in the activity level of the sonic hedgehog (*shh)* gene in cavefish. The increase in *shh* and other genes was one of the key findings that led biologists to look to natural selection as a cause of eye degeneration. Curiously, Yamamoto takes an agnostic view with respect to the selection-versus-drift controversy at the end of his chapter. Gross and Powers examine and document the changes in the craniofacial complex in cavefish, a topic deserving further attention. They document the differences between surface and cavefish and between different cavefish populations. Some of the changes are the consequence of eye loss and reduction in the size of the orbit, and many changes are difficult to characterize as adaptive or not. Atukorala and Franz-Odendaal look closely at the lower jaw and taste buds on the lower jaw. Their chapter is very much in the context of hypothesizing how the changes in jaw and taste buds are an adaptation and how they are linked to eye reduction. They make the point that taste buds and eyes are linked by the *shh* gene pathways. The final chapter in this section is by Rétaux and colleagues. They look at brain neuroanatomy and neurochemistry, documenting changes in the brain associated with loss of vision, such as an increase in olfactory components. It is a clear demonstration that there is more to Mexican cavefish than loss of eyes and pigment.

Part IV concerns behavior. Yoshizawa reviews sensory adaptation, emphasizing vibration-attraction behavior (VAB).

Occasionally found in surface fish and ubiquitous in cavefish, the lateral line system allows the fish to detect vibrations in the water. He carefully builds the case that VAB is an adaptation and connects behavior and morphology by showing that the quantitative trait loci for VAB and eye size overlap and that the position of taste buds in the lower jaw is connected to the 45° feeding angle. As Yoshisawa and collaborators have shown, the genetic bases of these two traits are entirely different, and thus are convergent in different cavefish populations. Perhaps more than any other system, the work on VAB, taste buds, and feeding angle is the closest to a complete adaptationist hypothesis. Volkoff also looks at feeding behavior, but focuses on the peptide regulation of feeding. She points to potentially interesting peptides like orexin, but comparisons between surface and cavefish have not been done. What she does is point out an interesting research agenda for future researchers. The same can be said for Duboue and Keene's study of sleep and Beale and Whitmore's study of circadian clocks. They point out the utility of these systems not only as models of adaptation, but also as models of study for the phenomena of sleep and rhythmicity itself. Hinaux and colleagues review social behavior and aggressiveness in cavefish. As they point out, schooling behavior is unimportant in a cave environment that lacks predators and may even be maladaptive in that it reduces foraging success. They are firmly in the adaptationist camp and propose the loss of territorial behavior and aggressiveness may be adaptive. In the final chapter of this section, Santacruz and colleagues pose the basic question of how the brain makes sense of space, especially in blind cavefish. This chapter also poses a research agenda, and proposes an expansion of the work that has been done on spatial orientation in Mexican cavefish.

Part V, on future applications, consists of a single chapter by Tabor and Burgess, who urge the Mexican cavefish research community to take up the techniques and procedures of transgenetics to understand more fully the biology of Mexican cavefish.

Anyone with an appreciation of cave life will find this book interesting. The earlier chapters are perhaps of more general interest and are the least technical in nature. However, all readers should find the final chapter by Jeffery on the social history of Mexican cavefish research interesting. As is the case for nearly all edited volumes, there is no one clear voice. For example, a number of authors mention the pigmentation system in Mexican cavefish and the gene responsible for albinism—*oca2*—but they do not all come to the same conclusion as to whether it has been subject to natural selection. Overall, the decades-long debate on selection versus drift is receding, and a number of the chapters ignore the role of genetic drift or only give it lip service. This is due in part to the success investigators have had in finding evidence that is hard to explain by genetic drift, such as the increased expression of the *shh* gene in cavefish, and in part, because it is easier to design an experiment to test the hypothesis of natural selection than it is to test the hypothesis of neutral mutation and genetic drift. Nonetheless, the issue is certainly not resolved, and what the book represents is a snapshot of the state of the field, clearly a very active and exciting one. While the authors represent the range of researchers working on Mexican cavefish, I find it a pity that two authors were not included. One is the great champion of neutral mutation and genetic drift Horst Wilkens, who is still active after five decades of research in the field. The other is a young researcher who is a leader in integrating behavior, morphology, and their genetic mapping, Johanna Kowalko. Her work is very much in evidence in a number of chapters.

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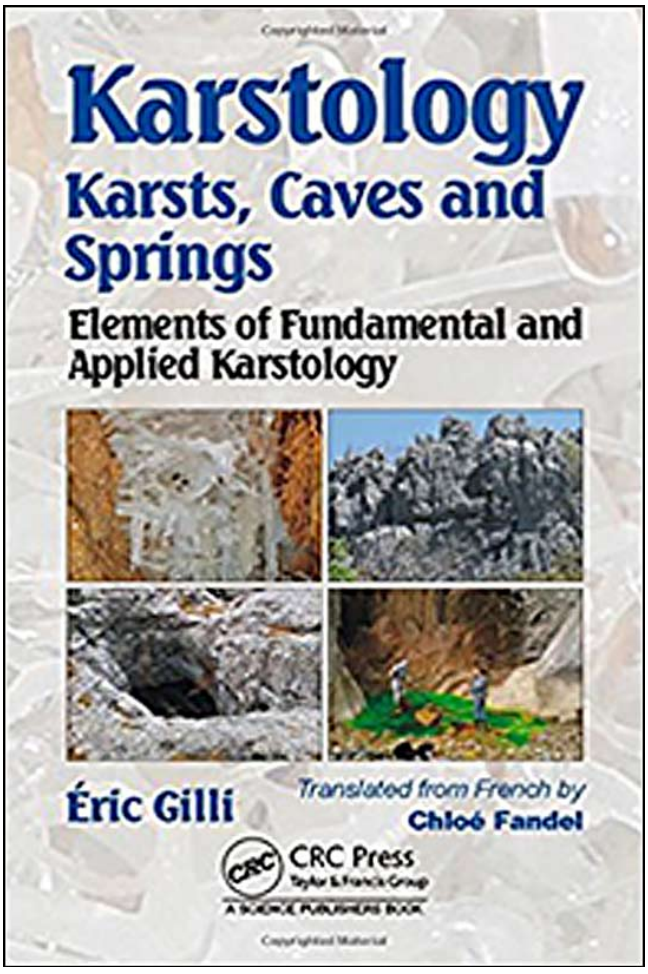
BOOK REVIEW: KARSTOLOGY: KARSTS, CAVES AND SPRINGS: ELEMENTS OF FUNDAMENTAL AND APPLIED KARSTOLOGY

Eric Gilli; CRC Press, Taylor & Francis Group, Abingdon, UK 2015, 256 p., hardcover, 6.2 × 9.2 inches, hardbound $63.99, eBook $44.79. hardbound ISBN 9781482243154, e-book ISBN 9781482243161



This book by Prof. Eric Gilli of the University of Vincennes, Paris, was originally published in French as *Karstologie—Karsts, Grottes et Sources* (2011), Dunod Editeur, Paris. The reviewed English version (2015) was translated by Chloé Fandel, Department of Water Resources, University of Arizona.

Karstology is a field that combines geomorphology, geology, hydrogeology, engineering, paleontology, archeology, and climatology. However in France, it is traditionally considered a subtopic of geography. This book contains 22 chapters, as well as an introduction and bibliography. It gives a good representation of the field, with many color images, and explores a wide range of topics from the viewpoints of surface and underground morphology, as well as time. It covers many karst areas, although many world-famous examples are not included.

Chapters 1 and 2 introduce definitions, principles, and the history of karst science. Many authors consider Cvijić to be the founder of modern geomorphic and hydrologic karst studies in the late eighteenth and early nineteenth centuries (Ford, 2007). But Gilli regards Hacquet in the late eighteenth century to be a more valid candidate on the basis of his several books on karst written in 1778–79.

Chapter 3 concerns carbonate rocks. The coverage is not as comprehensive as that of Ford and Williams (2007). From the book title, one might expect greater attention to this topic. Chapter 4 covers the process of dissolution and other relevant factors, and Chapter 5 introduces karst surface forms such as karren, dolines, and poljes. Gilli divides the factors affecting karst landforms into five categories: structural, topographic, pedological (soil types), meteorological, and biological. The more common classification of these features as given by Bögli (1980) and Ford and Williams (2007) receives limited coverage.

In Chapter 6, karst landscapes in various climates are considered. Caves and other underground karst features are divided between two chapters, 7 and 10. In addition, Chapters 11, 12, and 14 about aquifers could have been combined into a single chapter. In view of the growing interest in coastal karst, Gilli has covered this topic in a separate Chapter 14 on coastal and submarine karst aquifers.

Chapter 13 on water use, management, and risks in karst areas, is very important. Given the fragility of karst areas, improper use of land and water resources has caused many problems in karst regions. In Chapter 15, land management in karst is the main issue. Many examples are given of land subsidence and dam failure in various karst areas of the world, and methods of detecting them are described, such as the use of radar tracking.

This book gives greater attention to tourism in karst and caves than do other general books on karst. In recent years, tourism in karst areas has been an increasing consideration, and its importance is emphasized in Chapter 16. This chapter is limited only to caves, although surface karst forms are also important for tourism. Many tourist caves in the world are described, but there are no photos of dramatic surface karst such as tower karst in southeastern Asia. Damage to caves by tourists and amateur cavers is mentioned only briefly.

In Chapter 17, mineral resources in karst are described. Traditional resources such as bauxite and guano are covered, but the greatest attention is given to hydrocarbon resources in karst. A minor omission is travertine, which is widely used as building stone.

Chapters 18 and 19 concern thermal springs and paleokarst respectively. They are only 2 and 21 pages long, and

their topics could probably have been included in other chapters. Discussions of paleokarst and paleo-caves are also in Chapters 7, 8, and 10. Methods for determining relative and absolute ages are described, such as paleomagnetic and isotopic techniques. The consequences of climate change in karst areas are described, with the example of growth and destruction of the Maya empire.

Chapter 20 includes a discussion of two karst systems in France, with special attention to geodesy and rock deformation. Chapter 21 gives a brief coverage of the paleontology, archeology, and biology of karst environments, especially caves. The topics of "cave men" and extremophiles are included in this chapter, although the world-famous archaeological cave site, Shanadar in Iraq, is not included.

Chapter 22 ends the book with a discussion of the importance of karst studies and modern perspectives of karstologists on topics such as microorganisms in speleology, karst systems, and speleogenetic modeling.

Unlike the Ford and Williams book, which gives more attention to surface landforms, Gilli emphasizes karst groundwater and caves. His book covers mainly karst in limestone, while omitting other types of karst, such as evaporite karst and pseudokarst. The book would also have profited from additional information about natural and anthropogenic hazards in karst areas. Given the importance of this subject, I think it deserves a separate chapter. However, a very useful topic included in this book is a list of important questions in karstology that are still open.

From the book's title, one would expect it to include examples and illustrations of the various karst regions throughout the world, but there is a strong emphasis on France and other European countries. However, the main goal of this book is to emphasize the fundamentals of karstology. References could have been given to literature that provides more detailed information on other related fields and geographic areas. Unlike many recent books, this one does not list the names of people and places in the index, so it is difficult to find them in the book.

Each chapter in this book should be of interest to students and professionals. They will be valuable to geographers, geomorphologists, geologists, hydrogeologists, speleologists, and land-use managers, as well as students at many levels from bachelors to PhD and post-graduate. It follows the strict conventions of academic and scientific writing, but is also clearly written, and the topics are easy to apply. Although the Ford and Williams book (2007) will probably remain the professional standard for many decades, this new book by Gilli can serve as a helpful guide for karst researchers at many different levels now and in the future.

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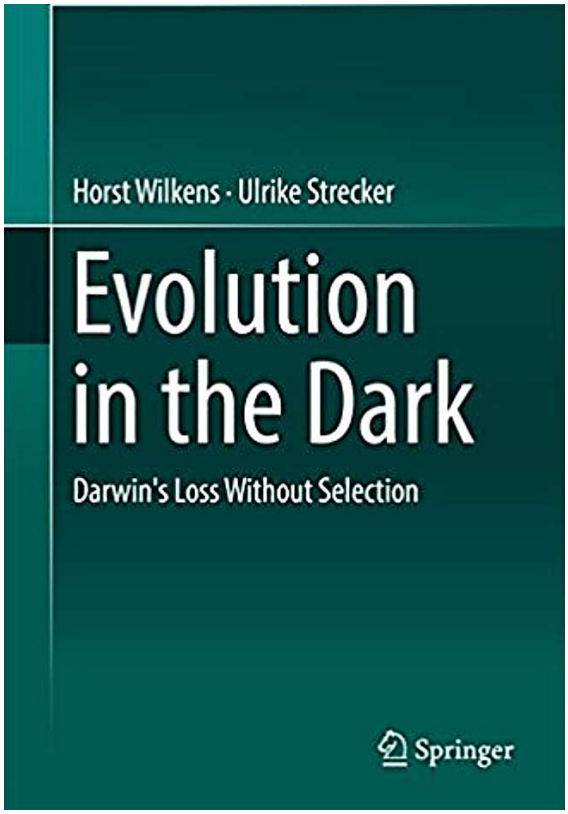
BOOK REVIEW: EVOLUTION IN THE DARK: DARWIN'S LOSS WITHOUT SELECTION

Horst Wilkens and Ulrike Strecker 2017. Springer, Berlin. 217 p., 25 × 33 cm, hardbound, $159, ISBN 9783662545102,



I give this new book my strongest possible endorsement. It is a tour de force and the best overview of evolution in cave organisms since David Culver's 1982 book. It is even better-written and argued than many of Wilkens's earlier publications of his pioneering 47 years of research. The sequence of chapters reads like a detective story with background, patterns, hypotheses, and tests.

Each of seven chapters has a great abstract, self-contained sub-sections, and hundreds of references. For every other page there is an average of one graph, diagram, table, photograph, map, or drawing, each with a self-contained legend. Many photos and photomicrographs are in color. Theorists may be disappointed not to find any equations, but Wilkens has covered both selection and neutrality theories in a critical review with David Culver (2000, p. 381–398, *Ecosystems of the World: Subterranean Ecosystems*).

Despite many disagreements, Wilkens and all others agree that *Astyanax mexicanus* is an especially good model for study of evolution in caves. Everyone cites its short generation time, hundreds of offspring at each spawning, ease of husbandry in the lab, and that surface and cave populations are inter-fertile. Colonizations have been sudden, and many are recent and so different that populations have not diverged enough to become reproductively isolated. This is different from most cavefish, which have been isolated so long that their putative ancestors do not exist.

Wilkens explains how the surface-living *Astyanax* Mexican tetras are different from the ancestors of most cavefish, such as catfish, because they are only minimally pre-adapted to life in caves. As day-active schooling fish they strongly rely on vision. They do not have great elaboration of sense organs that are important for finding food in caves, like lateral line neuromasts, taste buds, and olfactory rosettes. But we know now that the surface fish do have taste buds and lateral-line sense organs and that they have standing genetic variation for these traits. Those surface fish individuals with the greatest lateral line, taste, and smell senses may have been the only ones to survive when first isolated in caves. They colonized successfully, whereas those colonizing later die of starvation.

Astyanax provides a huge advantage for reconstruction of the evolution of cave adaptation because it has had many independent colonizations of caves that range from old to recent. All populations are inter-fertile, and this has allowed a full range of classic genetic and molecular genetic studies in the laboratory. It has also allowed paleo-biogeographic reconstruction of past and ongoing colonizations. The dynamic evolution of caves and karst along with Pleistocene climatic change has resulted in continued extinctions and recolonizations, some with introgressive hybridization between surface and cave in at least the distant past.

Wilkens and all others also agree that there has been direct natural selection to elaborate sense organs and metabolic economy. But they disagree about mechanisms of regression or reducing of eyes and pigment. Wilkens shows that there have been many mutations in eye and pigment genes and that these have no disadvantage in caves. These mutants are not eliminated by purifying selection, and eyes become more regressed with time through still more mutations. Others argue that there has been both direct and indirect selection, by pleiotropy, against eyes and pigment.

Horst Wilkens, Richard Borowsky, and William Jeffery are the three titans of Mexican cavefish study. Each is interested in both regressive changes, especially of eyes and pigment, and adaptations of behavior and physiology to survive in caves. But their conclusions differ in many ways. Beginning in the late 1960s, Horst Wilkens and a few students and close colleagues did by far the earliest research on the biology, genetics, and history of *Astyanax* surface and cave populations. He is the only one who has consistently studied many surface populations and almost all the cave popula-

tions. He always emphasizes classic genetic crosses to study all aspects of cave and surface fish biology. Borowsky's expertise is with sophisticated ways of quantitative trait locus (QTL) mapping traits on chromosomes and searching for candidate genes. Jeffery emphasizes experimental developmental approaches. Wilkens and his colleagues and students have studied many other cavefish and crustacean species. Other groups, except for Borowsky's studies of Thai Balitorid fish and some recent work on cave isopods, only study the Mexican cavefish. The Wilkens group has used many approaches and studied both regressed and elaborated traits, but the other titans have narrower research niches. Wilkens and his colleagues give detailed attention both to adaptive elaborated traits and neutral regressed traits, and many of that group have pioneered the study of different traits. Some of Borowsky's colleagues have also pioneered new areas of study, particularly of sleep and hyperphagia in cavefish and possible standing genetic variation in surface fish. But most of Jeffrey's colleagues and students continue to push the same hypothesis of antagonistic pleiotropy between eye or pigment reduction and sense-organ elaboration, often uncritically, as Wilkens and Strecker document.

The other research groups give almost no attention to alternative hypotheses for regressed eyes and pigment in cavefish. They only cite a few of Wilkens' papers and never the critical review of theories with Culver, and rarely the historic luminaries who have contributed to recognizing the general importance and understanding of regressive evolution. Historically, Kosswig, Wilkens's hero and PhD advisor, expounded his neutral mutation theory. A modern version by Kimura and Nei is the neutral theory of molecular evolution. Central to both neutral theories, mutations on any trait are overwhelmingly neutral or negative. This was first recognized by Nobel Laureate Muller, but suggested by Darwin in a prescient paragraph quoted by Wilkens and Strecker (page 202). According to much recent research, this prevalence of deleterious mutations may result in what are called "pseudogenes" that have lost function owing to deletions and insertion mutations. Less extreme, but prevalent when looked for, is that function is compromised when non-synonymous substitution mutants hugely outnumber synonymous substitutions. Some non-synonymous mutations result in radical amino acid changes in protein. Without purifying, selection eye mutants are neutral and accumulate in cave populations.

Wilkens and Strecker are the only researchers who discuss the generality and mechanisms of regressive evolution, and they strongly support it for cavefish. As recognized by Darwin and emphasized by Kosswig, regressive evolution is almost universal in species and too little studied. Most regressive evolution is gradual over long periods of evolutionary time, so often we can only study fossils to see the ancestral conditions. Examples discussed by Wilkens and Strecker are teeth in humans, pelvic fins in whales, wings in Ratite birds, and eyes in naked mole rats and cavefish.

Research groups other than Wilkens's differ in the degree to which they espouse the importance of indirect selection against cavefish eyes and pigment via pleiotropy. To William Jeffery and also students and colleagues, antagonistic pleiotropy continues to be central (e.g., a 2005 review, *J. Heredity* 96). As functionless eyes are reduced, adaptive systems are enhanced. Two examples are eyes and taste buds and eyes and lateral line senses. Richard Borowsky is more even-minded. He even critiques the Jeffery et al. eye and lateral-line antagonistic pleiotropy. But he misses some of the same points he criticizes in his 2016 chapter in *Biology and Evolution of Mexican Cavefish*, "Regressive evolution: testing hypotheses of selection and drift." In fact, he tests three predictions that he believes falsify drift, but none that might falsify selection. One of his predictions is weakly correlative and two others are either open to other interpretations (Wilkens) or shown to be based on incorrect theory (Wilkens's citation of Lande). Sylvie Retaux (with Casane) claims that there is support for both selection and drift in a 2013 review paper (*EvoDevo* 4(1) entitled "Evolution of eye development in the darkness of ccaves: adaptation, drift, or both?") They uncritically evaluate antagonistic pleiotropy and cite many lines of evidence from cave and other animals that clearly support the accumulation of neutral mutations that result in different degrees of loss of function.

The strongest evidence Wilkens and Strecker adduce for accumulation of neutral mutations comes from various classic genetic crosses between surface and cave populations and quantitative trait locus mapping by Wilkens and by Borowsky. Wilkens and Strecker show that all studied traits are polygenic. For example, QTL mapping suggests at least 12 eye genes and 16 pigment genes. And F2 crosses (i.e., of the second filial generation) show that they are inherited independently. Wilkens and Strecker are the only workers to show that both eyes and pigment have two modules that control different aspects, i.e., lens and retina for eyes, and the number of melanophore cells and light-induced color change for pigment. In all cases, F2 crosses show all combinations of reducing and elaborating traits are not genetically linked, resulting in what Wilkens calls mosaic evolution. Mosaic evolution seems to be universal with polygenic traits, in which, as recognized only by Wilkens, there is epistasis with threshold effects. That can result in what appear to be sudden changes in traits, apparently including rapid declines in eye size.

Below I have summarized each of the book's chapters with a few notes that indicate where the authors' studies are pioneering:

1. Evolution in the dark: Introduction. 2 p. Constructive/elaborated traits are Darwin's gain and less-studied regressive/rudimented traits are Darwin's loss without selection.

2. The role of rudimentation in evolution. 8 p. Rudimentation is universal in all kinds of life, but too rarely studied. Some examples are loss of functional wings in Ratite birds, loss of hind limbs in whales, and extreme reduction in eyes in fossorial mammals and cave animals. Most cave animals' ancestors were pre-adapted to life in the permanent darkness of caves because they were nocturnal, with sensory systems that function without light.

3. Diversity and the phylogenetic age of cave species. 34 p. The authors concentrate on an aquatic hot spot around the Gulf of Mexico, where they have studied fish and crustacean cave species of very different biogeographic origins. These species became isolated in caves in different ways during past climatic change. With different time durations in caves, they have different degrees of evolutionary regression of eyes and pigment.

4. Surface and cave populations of Mexican *Astyanax*. 16 p. The authors distinguish "phylogenetically young" with variable eye and pigment reduction (VEP) and "phylogenetically old" with much less phenotypic and genetic variation and strong eye and pigment reduction (SEP). The SEP populations have both the most regressed and most elaborated traits. The most elaborated adaptations to restricted food supplies are metabolic economies, increased activity, decreased sleep, and increased appetite and fat storage when food is available. These go along with elaborated sensory systems, especially taste and lateral line, which are needed when sight is impossible in the dark with regressed eyes. The authors also discuss all other regressed traits related to blindness, including losses of aggressive interactions, phototactic behaviors, and schooling. Molecular markers used by Strecker (mitochondrial DNA and microsatellites) show multiple colonizations and extinctions. Cave colonization in VEP and SEP populations have taken place in parallel and resulted in multiple convergent evolutions.

5. Complexity of interrelationship between *Astyanax* cave and surface fish. 26 p. The authors compare divergence in nuclear DNA and mitochondrial DNA. The haplotype distributions show that four different VEP and three SEP populations have had a complex history of colonizations, bottlenecks, extinctions, and introgressive hybridization as the cave systems have come and gone, both connected and disconnected. The authors are the first to show that hybridization, which may add new variants to cave populations and so fuel new evolution, occurs only between SEP and VEP populations and between SEP and SEP populations. The authors suggest that all of the cave populations fit the criteria of being biological species.

6. Regressive and constructive traits in surface and cave fish. 112 p. The authors point out that with recent and ongoing colonization, the cave and surface populations are inter-fertile, and that this has allowed multiple kinds of studies of constructive/elaborated traits and regressed/rudimented traits. Of the 10 elaborated traits (27 p.) all but two—sleep and hyperphagia—have been studied only by Wilkens with his group of students and colleagues. Aside from pigment (7 p.) and eyes (25 p.), both studied by many researchers, there are seven regressive traits studied only by Wilkens and his group and only one studied by others (20 p.). The last ten pages of the chapter give a careful analysis of the genetics of complex regressive and constructive traits.

7. Mechanisms of regressive evolution. 26 p. Wilkens and Strecker evaluate hypotheses to support Darwin's concept of gain by selection, and to support Darwin's loss by accumulation of neutral mutations. They use both cave organisms and other animals like whales, flightless birds, and fossorial mammals. They give evidence that falsifies each hypothesis of selection against eyes or pigment in caves. Direct selection for regression based on energy conservation is not supported, since regression of eyes and pigment is universal in caves even with abundant food supplies. Each case of purported indirect selection based on antagonistic pleiotropy is convincingly flawed. Next, they review all the historic and current evidence that regression is based on accumulation of deleterious mutations in the absence of stabilizing selection in caves. This is Kosswig's neutral mutation theory and Nei and Kimura's neutral theory of molecular evolution. They show that there is always variability of regressive traits, always polygenic inheritance, and always a huge preponderance of deleterious mutations. For constructive/elaborated traits, deleterious mutants are eliminated by purifying selection and so these traits show low variability.

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