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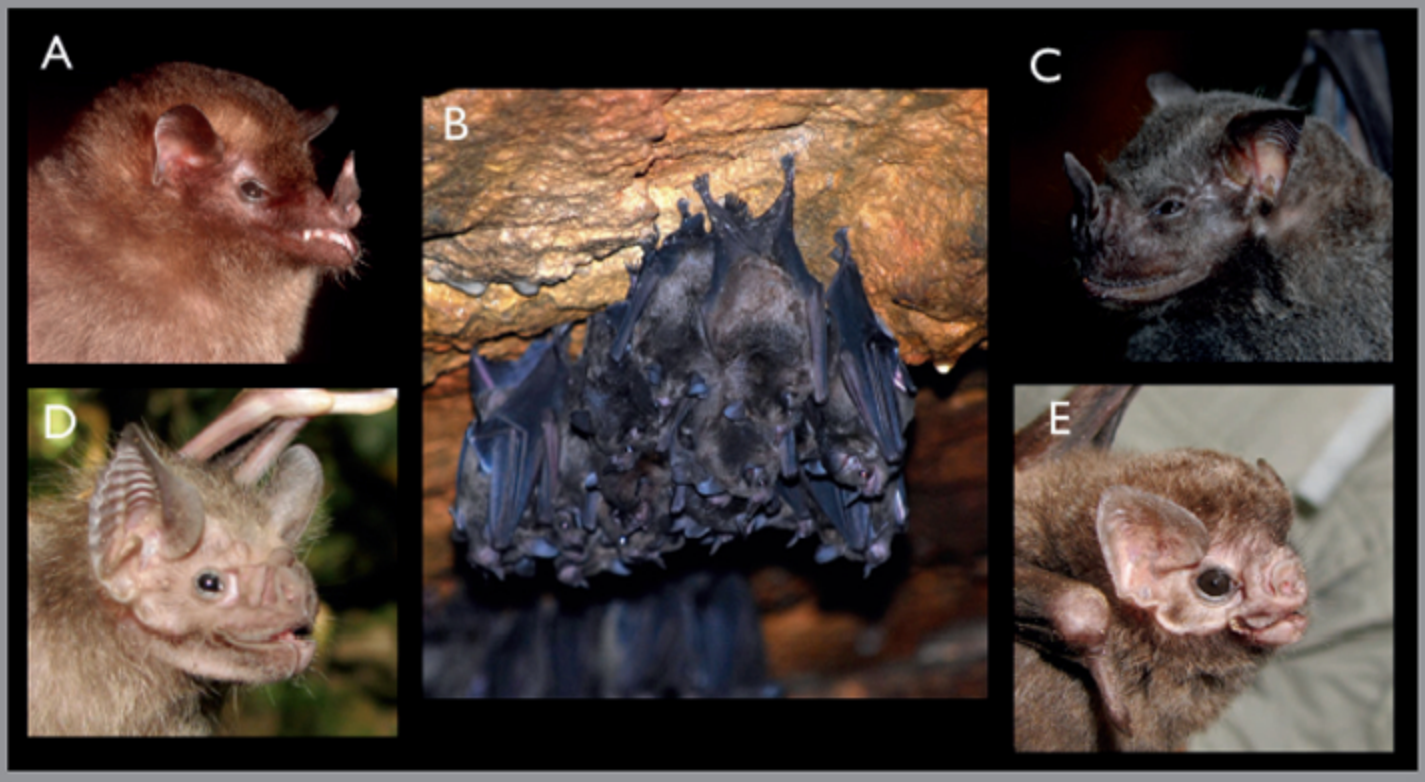

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J. Fernholz and Q.E. Phelps – Influence of PIT tags on growth and survival of banded sculpin (*Cottus carolinae*): implications for endangered grotto sculpin (*Cottus specus*). *Journal of Cave and Karst Studies*, v. 78, no. 3, p. 139–143. DOI: 10.4311/2015LSC0145

INFLUENCE OF PIT TAGS ON GROWTH AND SURVIVAL OF BANDED SCULPIN (*COTTUS CAROLINAE*): IMPLICATIONS FOR ENDANGERED GROTTO SCULPIN (*COTTUS SPECUS*)

JACOB FERNHOLZ[1]* AND QUINTON E. PHELPS[2]

Abstract: To make appropriate restoration decisions, fisheries scientists must be knowledgeable about life history, population dynamics, and ecological role of a species of interest. However, acquisition of such information is considerably more challenging for species with low abundance and that occupy difficult to sample habitats. One such species that inhabits areas that are difficult to sample is the recently listed endangered, cave-dwelling grotto sculpin, *Cottus specus*. To understand more about the grotto sculpin's ecological function and quantify its population demographics, a mark-recapture study is warranted. However, the effects of PIT tagging on grotto sculpin are unknown, so a passive integrated transponder (PIT) tagging study was performed. Banded sculpin, *Cottus carolinae,* were used as a surrogate for grotto sculpin due to genetic and morphological similarities. Banded sculpin were implanted with 8.3 × 1.4 mm and 12.0 × 2.15 mm PIT tags to determine tag retention rates, growth, and mortality. Our results suggest sculpin species of the genus *Cottus* implanted with 8.3 × 1.4 mm tags exhibited higher growth, survival, and tag retention rates than those implanted with 12.0 × 2.15 mm tags. To this end, we recommend 8.3 × 1.4 mm PIT tags as a feasible option for tagging adult sculpin (> 60 mm total length) with minimal impacts on growth and mortality.

INTRODUCTION

Previous researchers have used mark-recapture studies to evaluate fish population dynamics (Hamel et al., 2015; Ruetz III et al., 2015). Data garnered from these studies generally include measures of growth, movement, habitat use, and survival, all of which are imperative for fisheries conservation or restoration (Hamel et al., 2015; Ruetz III et al., 2015). However, obtaining these data requires utilization of batch marking or individual recognition methods, which can be problematic, especially on small fishes (Baras et al., 1999; Brown et al., 1999; Skalski et al., 2009). One promising technique used to mark small fishes is passive integrated transponder (PIT) tags, which alleviate issues associated with size and provide an individual marker for fish (Ruetz III et al., 2006; Skalski et al., 2009; Tatara, 2009; Fuller and McEntire, 2013). The use of PIT tags can provide a more thorough understanding of stream fish ecology (e.g., Bruyndoncx et al., 2002; Knaepkens et al., 2004; Cucherousset et al., 2005; Cunjak et al., 2005) relative to more traditional techniques (Gibbons and Andrews, 2004).

With the ability to uniquely mark small individuals, PIT tags have major advantages over other current marking techniques (Gibbons and Andrews, 2004; Skalski et al., 2009; Fuller and McEntire, 2013). However, apprehension often surrounds tagging small individuals because of high tag-to-body mass ratios (Winter, 1983; Winter, 1996; Baras et al., 1999; Brown et al., 1999; Jepsen et al., 2005; Ruetz III et al., 2006). A critical assumption for tagging studies is that tags do not change behavior, growth, or mortality of marked fish (Nielsen, 1992; Gibbons and Andrews, 2004; Ruetz III et al., 2006). Studies have supported this supposition by demonstrating that PIT tags do not strongly affect growth and mortality of small fishes (Prentice et al., 1990; Quartararo and Bell, 1992; Ombredane et al., 1998; McCormick and Smith, 2004). Despite minimal effects, PIT tagging results can vary depending on tag size, tag-insertion procedure, species tagged, and size of individuals being tagged (Hirt-Chabbert and Young, 2012; Fuller and McEntire, 2013). Due to disagreements among prior researchers about effects on small-bodied fishes and a lack of information regarding effects on non-salmonid species, additional information on the influence of PIT tagging on small-bodied fishes is warranted.

Such small-bodied fish include freshwater sculpin species that are widespread throughout the Northern Hemisphere (Ruetz III et al., 2006). Within this vast range, sculpins play intricate roles in small-stream food webs that are crucial to maintaining ecological integrity (Kohler and McPeek, 1989; Dahl, 1998; Miyasaka and Nakano, 1999; Ruetz et al., 2006; DeBoer et al., 2015). Although considered an important species to small-stream ecosystems, minimal information exists for some sculpin species such as the grotto sculpin. The grotto sculpin, *Cottus specus*, is one sculpin species that is in need of research. Grotto sculpin are an endangered, cave dwelling species only found in Perry County, Missouri. The cave environment that this species occupies, as well as its

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endangered status, inhibits the ability to work directly with the species; and therefore, a surrogate species is necessary. Banded sculpin, *Cottus carolinae*, are closely related genetically and morphologically, with similar reproductive ecology, foraging behavior, and diet (Jason Crites, unpublished data). As such, banded sculpin were used as a surrogate species to provide insight into the feasibility of PIT-tag use to evaluate population metrics for grotto sculpin. The objectives of our study were to evaluate PIT tag retention and estimate survival and growth of tagged banded sculpin using two different sizes of PIT tags, 8.3 × 1.4 mm and 12.0 × 2.15 mm. Results from our study may be used to select a PIT tagging protocol for the endangered grotto sculpin to gain a better understanding of population characteristics.

MATERIALS AND METHODS

Kick-seining was completed to collect 150 banded sculpin from the Castor River near Marquand, Missouri. After collection, they were transported back to a wet lab, acclimated, and placed in a series of tanks. Our arrangement of tanks consisted of sixty-four 37.9 L glass aquaria in rows of eight. Water quality conditions of pH, turbidity, temperature, and dissolved oxygen were monitored and kept constant throughout the experiment. Banded sculpin were randomly sorted into three groups, and individual fish were placed in a randomly selected aquarium. After fish were placed in their aquariums, there was an acclimation period of one week prior to tagging. Fish were fed chironomids ad libitum at the same time of day, every day during the acclimation period and during the duration of the study.

The three groups of 50 fish each were either tagged with 8.3 × 1.4 mm tags (small tags), tagged with 12.0 × 2.15 mm tags (large tags), or untagged as a control group. Fish in each group were divided into size classes of less than 49 mm in length (juveniles), 50–59 mm (subadults), and 60 or more mm (sexually mature) for the assessment of growth, sizes selected based on previous observations of life stages in grotto sculpin by Adams et al. (2008).

After acclimation, banded sculpin were measured for total length to the nearest millimeter and after tagging, if any, weighed to the nearest 0.001 g (Ruetz III et al., 2006). The 12.0 × 2.15 mm tags were inserted using a 12-gauge needle, while 8.3 × 1.4 mm tags were inserted with a 14-gauge needle. Insertion location of tags was similar to that in the study completed by Ruetz III et al. (2006). Tags were inserted into the body cavity just off the mid-ventral line, anterior of the vent. Needles were inserted at a 45-degree angle and were positioned parallel to the long axis of the body. Once the needle penetrated the musculature, the tag was pushed into the body cavity with the injector (Nielsen, 1992; Ruetz III et al., 2006). Fish were weighed and measured for total length every 7 for 28 days after the tag date (Ruetz III et al., 2006).

Survival percentage of fish group and size class was observed. Average change in weight for fish that survived and retained their tags throughout the study period was used to assess growth. Casualties were not used in change-in-weight calculations because using fish that expired or dropped their tag during the study could skew growth data. Change in weight was calculated by subtracting the original post-tagging weight of an individual from the weight of the individual at the end of the experiment. Calculated weight change was log transformed, and a one-way ANOVA was used to compare for differences among tag and control groups. All post-hoc comparisons of average change in weight between tag groups were Bonferroni corrected.

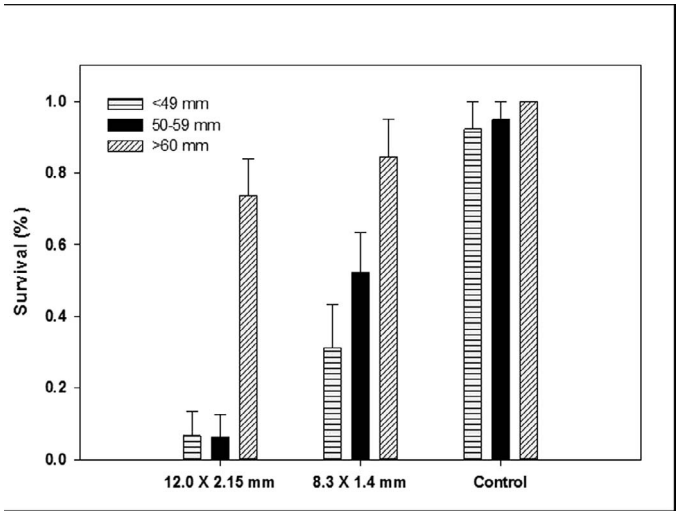

Figure 1. Survival percentage of banded sculpin after 28 days in different size classes within each group (8.3 × 1.4 mm PIT tag, 12.0 × 2.15 mm PIT tag, and control). Lines indicate standard error.

A binomial logistic regression analysis was also completed to determine size of individuals that could be successfully tagged. A success was viewed as an individual that survived and retained a tag for the extent of the 28-day study period. A failure was viewed as an individual either dropping its tag or dying at any point in time prior to the completion of the study. These two possibilities were used in conjunction with the initial length of fish within the binomial logistic-regression model to calculate the probability of a successful tagging event at a given length.

RESULTS

Survival percentages for each size class are shown in Figure 1. Among juvenile fish, the lowest survival percentage was in fish tagged with large tags (6.7 %), followed by fish tagged with small tags (31.3 %). The control group exhibited the highest survival for juvenile fish (92.3 %). The control group also had the highest survival percentage for sub-adult fish (95.0%), followed by fish tagged with small tags (52.4 %). The group that had the lowest survival percentage for sub-adult fish was the large-tag group (6.3 %). Finally, for adults

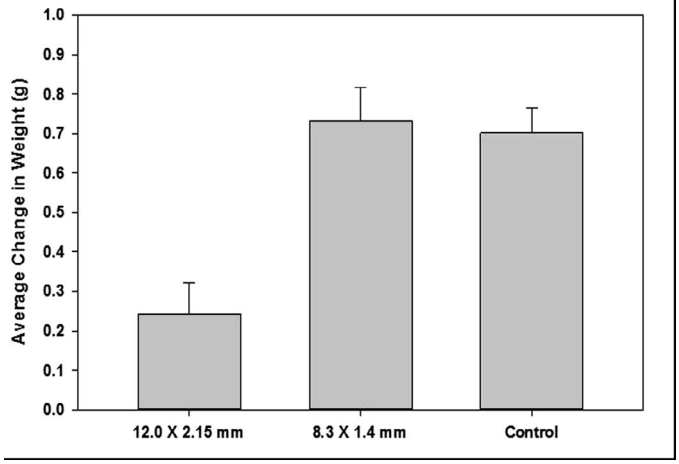

Figure 2. Growth indicated by average change in weight (g) by group (8.3 × 1.4 mm, 12.0 × 2.15 mm, and control). Standard error of the averages indicated.

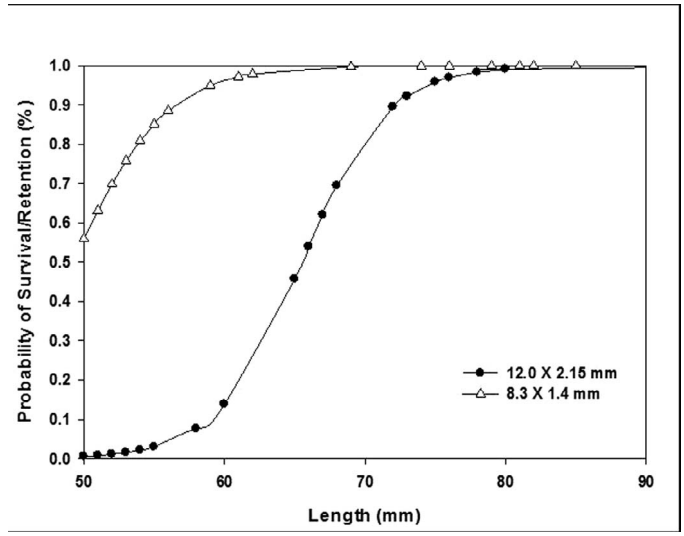

Figure 3. Probability of survival and tag retention calculated with a binomial logistic-regression model for fish tagged with PIT tags (8.3 × 1.4 mm and 12.0 × 2.15 mm tags).

the control group exhibited the highest survival percentage (100%) followed by fish tagged with small tags (84.6 %), and again fish tagged with large tags had the lowest survival percentage (73.7 %).

There was no significant difference in growth between the control (0.7011 ± 0 .0611 g) and small tag groups (0.7334 ± 0.0777 g) ($F = 9.22$; df = 2, 65; $p = 0.0003$). However, the large tag group (0.2432 ± 0.0987 g) was significantly different from both the control and small tag groups. Generally, growth rates of fish within the control group and fish tagged with small tags were higher than growth rates of fish tagged with large tags (Figure 2).

Using the binomial logistic-regression model, the size of fish that can be effectively tagged with each tag size was assessed. The effective level, in this case probability of retention and survival, was set at 95 %. These levels were met for small tags when fish were 60 mm in length or greater (Figure 3). For large tags, a 95 % success rate was attained with fish that were at least 75 mm in length (Figure 3).

Discussion

Our study allowed us to accomplish the objectives of evaluating PIT tag retention and estimating survival and growth of tagged individuals when utilizing two different sizes of PIT tags (8.3 × 1.4 mm and 12.0 × 2.15 mm). Based on results garnered, future studies can utilize the tagging methods outlined in our study on the banded sculpin and other closely related sculpin species, such as the endangered grotto sculpin, that are longer than 60 mm in total length. However, fish less than 60 mm in length should not be tagged, and future research should focus on determining tagging protocol to effectively tag smaller fish.

We assume our findings will translate to field studies, because previous research indicates PIT tagging results are similar between controlled and environmental settings (Tatara, 2009). Therefore, because our tagging techniques were successful in utilizing small tags on fish longer than 60 mm in length, our tagging protocol can be utilized in field studies in relation to both banded and grotto sculpin. By utilizing PIT tagging techniques beneficial ecological information such as spawning habitat and intra-/inter-species interactions can be obtained and used in conjunction with population indices to help direct conservation and restoration efforts for the grotto sculpin. With freshwater sculpin species being widespread throughout the Northern Hemisphere, the potential for obtaining valuable intra- and inter-species ecological interactions in sculpin-occupied streams is also widespread.

We are able to support the premise that utilizing PIT tags can be effectively used to mark small bodied fishes (Gibbons and Andrews, 2004; Skalski et al., 2009; Fuller and McEntire, 2013). Supporting evidence included effectively tagging fish 60 mm and larger when utilizing 8.3 × 1.4 mm tags. The apprehension surrounding tagging small individuals can be alleviated based on our results, especially when using small tags on banded and grotto sculpin longer than 60 mm in total length. Our findings also support the assumption found in other studies that PIT tags do not strongly affect the growth and mortality of small fishes (Prentice et al., 1990; Quartararo and Bell, 1992; Ombredane et al., 1998; McCormick and Smith, 2004). We were able to support this assumption with small tags exhibiting no significant difference in growth in comparison to the control group. We were also able to support the findings of other studies by demonstrating that results vary depending on tag size, tag insertion procedure, species being tagged, and size of the individuals being tagged (Hirt-

Chabbert and Young, 2012; Fuller and McEntire, 2013). Results garnered from our study provide evidence for the effects of PIT tagging small non-salmonid species. More specifically, our study provides insight into PIT tagging two sculpin species, but there is still a need for more research concerning the effects of PIT tagging in other small-bodied fishes.

Although batch marking or individual recognition techniques can be problematic on small fishes, these techniques can be used to gain data required to make good management decisions, especially in regards to conservation and restoration of a species (Ruetz III et al., 2006; Skalski et al., 2009; Tatara, 2009; Fuller and McEntire, 2013). However, PIT tagging small fish is supported by the results found within our study with banded sculpin and likely can be applied to a closely related sculpin species such as the endangered grotto sculpin. Therefore, our tagging procedures likely can be utilized in future mark-recapture studies concerning both banded and grotto sculpin. Such mark-recapture studies can provide a non-lethal way to monitor and assess population characteristics, thus being an effective tool for monitoring endangered species. Specifically, the information that can be provided by mark-recapture studies includes insight into growth, recruitment, and mortality, providing insight into the ecological function of a species which is essential for fisheries conservation and restoration decisions (Gibbons and Andrews, 2004; Ruetz III et al., 2006; Skalski et al., 2009; Hamel et al., 2015; Ruetz III et al., 2015). Mark-recapture studies are an effective tool when evaluating population dynamics and ecological role and can allow researchers the ability to make better restoration decisions concerning grotto sculpin and the cave ecosystems they inhabit.

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S. Banerjee and S.R. Joshi – Culturable bacteria associated with the caves of Meghalaya in India contribute to speleogenesis. *Journal of Cave and Karst Studies*, v. 78, no. 3, p. 144–157. DOI: 10.4311/2015MB0131

CULTURABLE BACTERIA ASSOCIATED WITH THE CAVES OF MEGHALAYA IN INDIA CONTRIBUTE TO SPELEOGENESIS

Subhro Banerjee and SantaRam Joshi*

Abstract: The caves of Meghalaya in India are some of the longest caves in the subcontinent that have so far received negligible attention of geomicrobiologists. The present work was undertaken to discover and explore bacterial biofilms of various textures and colors from five caves of Meghalaya in North-East India. There are no previous specific scientific investigations from three of the studied caves. Thirty-two different culturable bacterial species belonging to sixteen different genera were characterized. Based on molecular identification, the isolates were related to nearest taxa, with the majority belonging to *Bacillus* and *Pseudomonas*. The study also indicated the capabilities of the isolated microbial strains to precipitate calcite, providing evidence for biotic processes involved in the formation of natural speleothems. SEM studies revealed an array of crystal polymorphs generated *in vitro* by the isolated bacteria similar to the microscopic observations of speleothems. The EDX spectrum showed that the precipitated crystals were predominately composed of calcium carbonate. The results endorse the hypothesis that the isolated chemoheterotrophic bacterial species contribute to the process of cave-speleothem formation in a hypogean environment.

Introduction

Microorganisms are active and passive promoters of redox reactions, influencing geological processes in caves that contribute to cave ecology (Northup and Lavoie, 2001). Caves with dim natural light and artificially lighted hypogean environments have been found to host diverse microorganisms that group themselves into biofilms associated with rock surfaces. These biofilms are complex aggregates of microorganisms embedded in a self-produced matrix (Banerjee et al., 2012) that provides protection for growth, enabling microorganisms to survive adverse cave environments.

Ideal combination of high grade limestone, the world's highest precipitation, low elevation, and a hot and humid climate have resulted in the formation of Meghalaya's subterranean caves and caverns. The three hills, Khasi, Jaintia, and Garo, contain limestone of variable quantity and quality (Daly, 2009). Knowing the composition of bacterial communities forming the biofilms represents a first approach to understanding the development of bacteria on speleothems, as different bacterial communities can lead to distinct effects on their environment, such as precipitation or dissolution of carbonates in caves (Legatzki et al., 2012; Banerjee and Joshi, 2013; Jones and Bennett, 2014).

Biomineralization is the natural process by which living organisms form minerals from bioorganic molecules and inorganic solids (Bäuerlein, 2004). The living organism provides a chemical environment that controls the nucleation and growth of the mineral phases. Biominerals meet the criteria for being true minerals, but they are often distinguishable from their inorganically produced counterparts by their peculiar shape, size, crystallinity, or isotopic and trace-element compositions (Weiner and Dove, 2003). Bacteria and fungi can induce the precipitation of calcium carbonate extracellularly through a number of processes, including photosynthesis, ammonification, denitrification, sulphate reduction, and anaerobic sulphide oxidation (Riding, 2000). The introduction of new molecular techniques, along with mineralogy techniques such as energy dispersive spectroscopy, enabled the investigation of the complex reactions of microorganisms with minerals (Baskar et al., 2006). Microbes can cause dissolution and precipitation reactions in caves for carbonates, moonmilk, silicates, clays, iron, manganese, sulfur, and saltpeter. They may produce active biogenic influence in the cave formations (Baskar et al., 2009; Li et al., 2014; Daskalakis et al., 2015).

The present study is an attempt to add further information about the complex biofilm associations in the surveyed caves and to provide information about the interaction of bacteria with the geologic substrate. The caves in Meghalaya (locally called *krem*) are not easily accessible, since they are situated in hilly, uninhabited, and remote areas. The present geomicrobiological study is based on five caves, four of which are located in the East Khasi Hills district and one in East Jaintia Hills district of Meghalaya. The caves located in East Khasi Hills district are Mawsmai (N 25°14.68′; E 91°43.48′), Mawmluh (Mawkhyrdop) (N 25°15.548′; E 91°42.749′), Mawjymbuin (N 25°18.294′; E 91°35.109′) and Dam (N 25°18.491′; E 91°35.496′) caves; the cave located in East Jaintia Hills is Labit (N 25°21.16′; E 92°31.0.23′) cave. The isolated biofilm bacteria were studied with scanning electron microscopy coupled with energy dispersive X-ray analysis for

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in vitro generation of calcium crystals, since calcium precipitation is the predominant form of mineralization involved in speleothem formation, and to analyze whether the chemoheterotrophic bacteria play any role in formation of stalactites, stalagmites, and various cave-wall deposits.

Materials and Methods

Krem Mawsmai is located south of lower Cherrapunji below a zone thickly forested with pine and broad-leafed trees, the Mawsmai (or locally Mawlongsyiem) sacred forest, which receives very high rainfall. The dense canopy cover of the groves provides an ideal microclimate for the survival of certain species due to the high nutrient release in the soil. The 160 m long cave is totally aphotic and has plenty of stalactites and stalagmites. The cave was moist, and water dripping from the roof was observed during sampling. *Krem Mawmluh,* also known as Mawkhyrdop cave, is located at a distance of approximately 1 km west of Sohra (Cherrapunji), adjacent to the cement factory Mawmluh-Cherra Ltd. and at a distance of 10 km from Mawsmai cave. The cave is about 7.1 km long and is the longest cave system in the Khasi Hills. The entrance lies at the bottom of the western flank of the Lum Lawbah, which flows throughout the year and most prominently during monsoons, and is contaminated by effluents from the cement factory. As the sampling time was during the monsoons in the month of July, we could collect biofilm samples only from the cave entrance, rather than venturing inside. *Krem Mawjymbuin* is situated in Mawsynram in eastern Khasi Hills district of Meghalaya at an elevation of 209 m. Years of weathering and dripping of mineralized solutions have formed magnificent stalagmites of calcum salts. Inside the cave is a pair of notable speleothems that are shaped into a Shivalinga, Hindu mythological god. *Krem Dam,* measuring 1297 m in length, lies at the foot of a large blind valley approximately 1 km to the east of Mawsynram village and is the biggest cave in the entire subcontinent of India that is totally in sandstone. The titanic entranceway approximately 30 m across is the chief attraction of the cave. The cave consists of a very large river passage ending in a roof collapse where daylight can be seen, but no significant calcite formations are found in this cave. *KremLabit* is located in NongkhliehElaka (the Shnong Rim area) of east Jaintia Hills district and is a part of the longest cave system in India, *Krem Laitprah*/*Um Im-Labit*. The current length of the cave system is approximately 31 km, which is likely to be increased as nearby caves continue to be explored.

Biofilm samples were gently and aseptically scrapped off from areas of the caves minimally disturbed by anthropogenic activities. They were then placed in sterile sample containers and kept in icebox. The samples were immediately brought to the laboratory, kept at 4 °C, and analyzed within 24 hours.

Isolations of bacteria from the speleothems of caves were made to identify the culturable, aerobic, heterotrophic fraction of the total microbial community. Duplicate samples of biofilm (1g) were aseptically transferred into 9 ml sterile Ringer salt solution (SRL, India), 1/4 strength, and vortexed briefly for 2 minutes. Serial 10-fold dilutions ranging from 10^{-1} to 10^{-5} were plated in triplicates onto Nutrient Agar and R2A Agar. The plates were incubated at 25 °C to mimic cave temperatures in an inverted position for 3 days, and the colony-forming units were recorded. Nutrient Agar was used

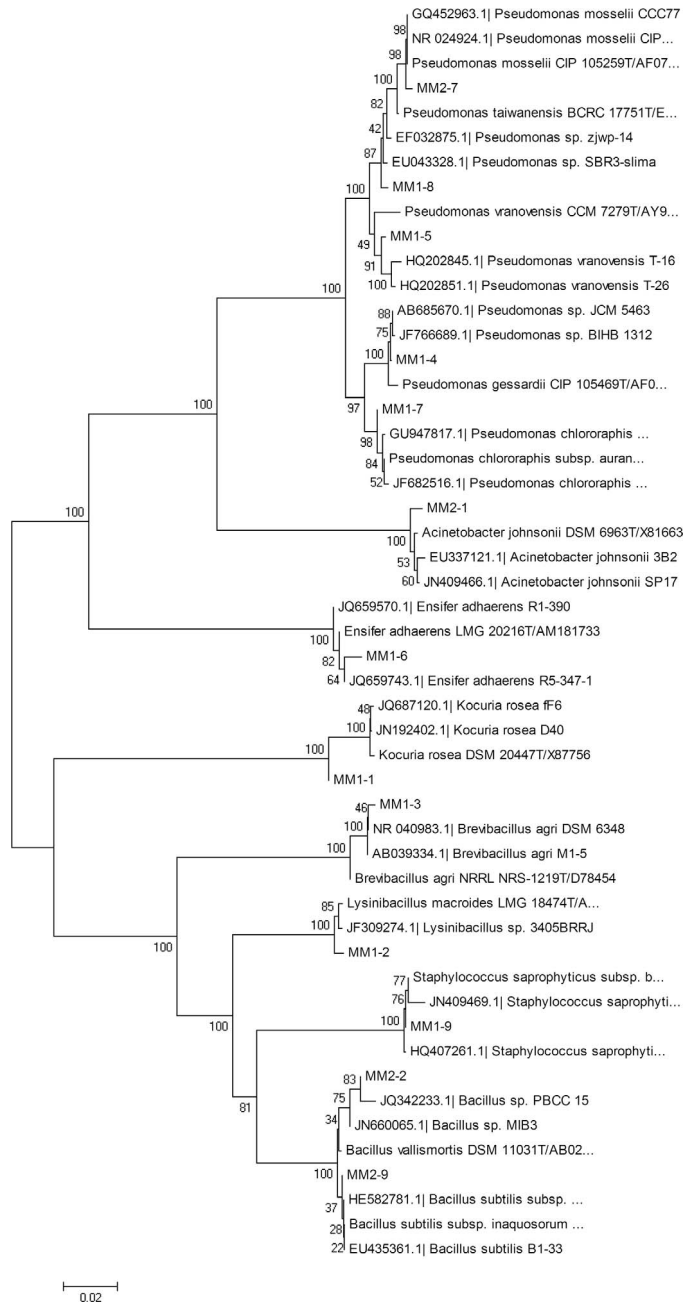

Figure 1. Neighbor-joining tree based on 16S rRNA gene sequences depicting the phylogenetic relationships between the isolates obtained from *Krem Mawsmai* listed in Table 3. The scale bar corresponds to the expected number of changes per nucleotide position.

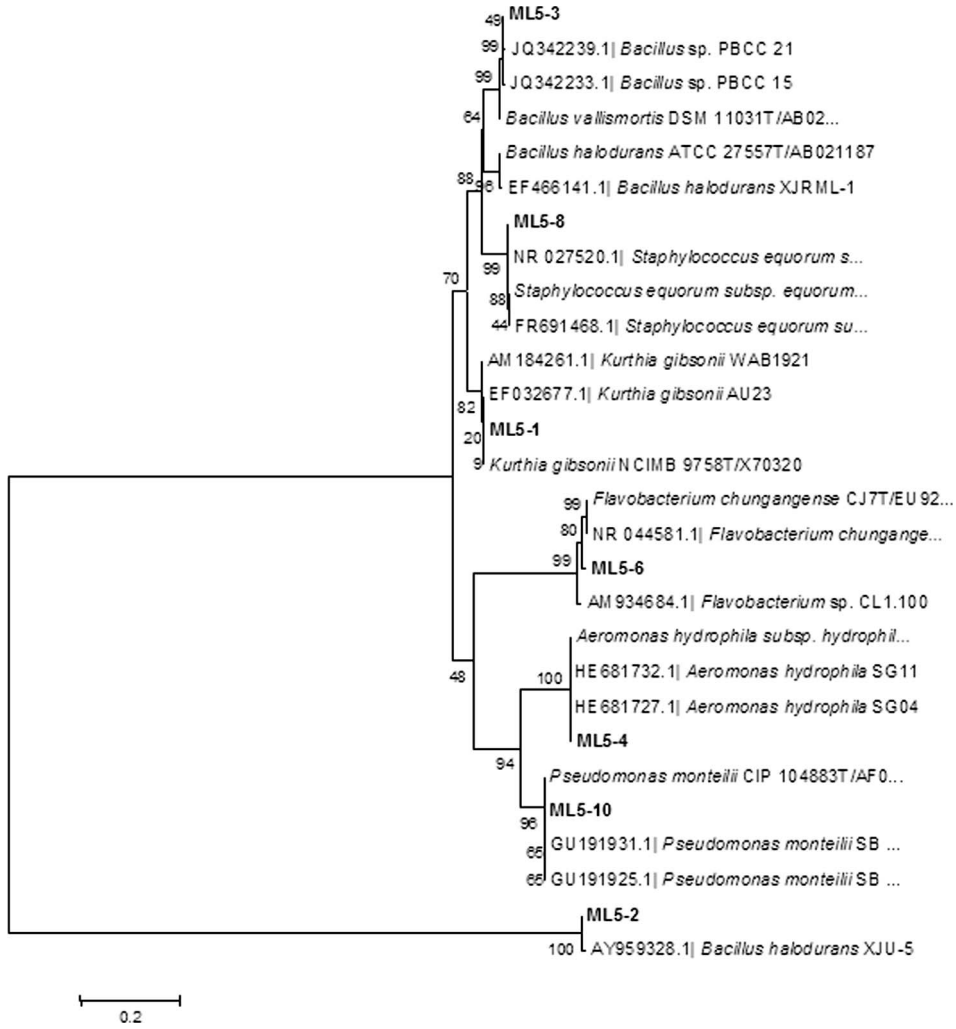

Figure 2. Neighbor-joining tree based on 16S rRNA gene sequences depicting the phylogenetic relationships between the isolates obtained from *Krem Mawmluh* listed in Table 3. The scale bar corresponds to the expected number of changes per nucleotide position.

as the standard nutrient-rich media, and R2A Agar was used as the minimal media to enumerate the aerobic heterotrophic count (Baskar et al., 2006). Controls consisting of autoclaved distilled water and 0.9% saline solution were also incubated. Individual colonies were selected based on color and, colony morphotypes and purified by repeated streaking.

The cell shape, size, arrangement, and Gram-staining parameters were observed for all the isolates under a bright-field microscope (Leica DM 5500, Germany). Preliminary identification and characterization were done by morphological and biochemical analysis following *Bergey's Manual of Determinative Bacteriology* (Holt et al., 2000) using standard protocols.

Genomic DNA isolation was done by Bacterial Genomic DNA Purification Spin Kit (HiMedia, India), followed by amplification of bacterial 16S rRNA gene using the universal bacterial 16S rRNA primers, 27F [5′-AGA GTT TGA TCC TGG CTC AG-3′] and 1541R [5′-AAGGAG GTG ATC CAG CCG CA-3′] (Cao et al., 2003). PCR was performed at a final reaction volume of 50 µL containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM each of deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 0.2 µM primers, 0.2 µL of 3 U/µLTaq DNA polymerase (Bangalore Genei, India), and 3 µL of the extracted DNA as template (approximately 150 ng). The amplification was programmed as initial denaturation for 5 min at 94 °C, followed by 35 cycles consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 2 min, and then cycling was completed by a final elongation step for 5 min at 72 °C in a Gene AMP PCR system 9700 (Applied Biosystems, California, USA). A control tube containing sterile water instead of DNA solution was used as a negative control. Results were then purified using QIAquick Gel Extraction Kit (QIAGEN, Germany). Bi-directional sequencing reactions of

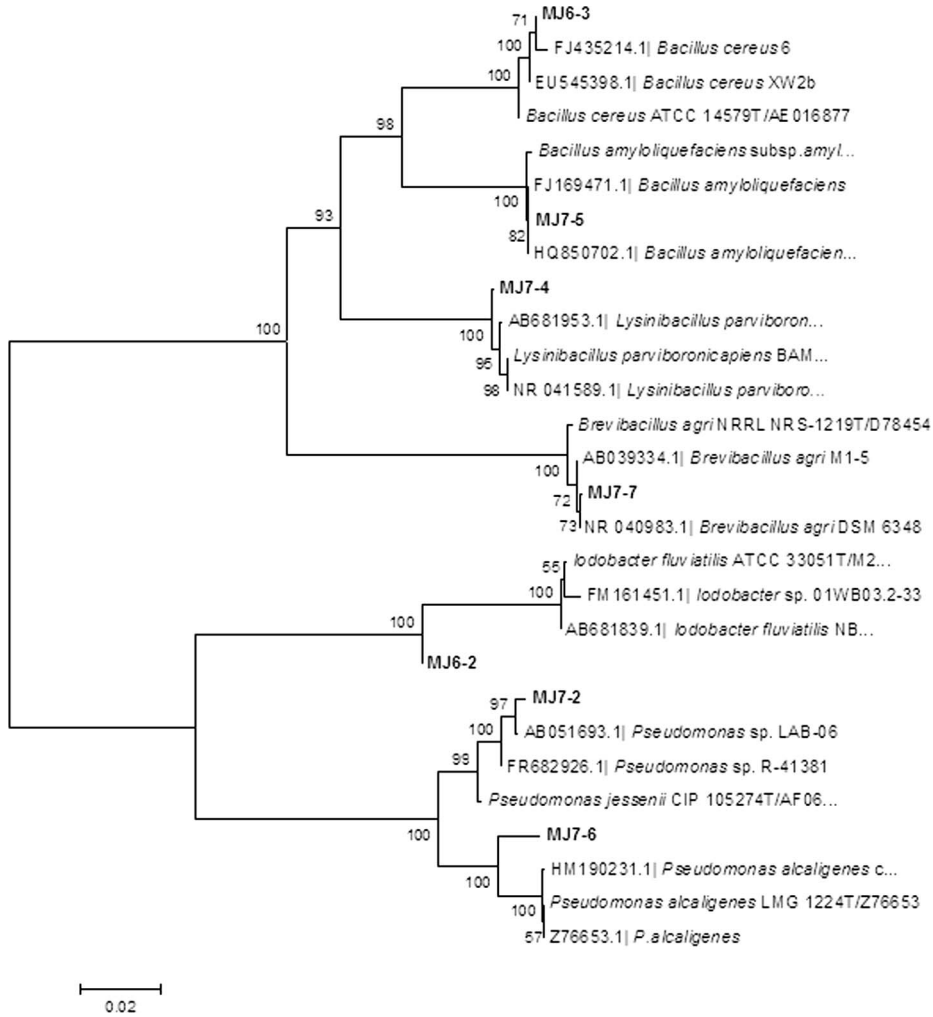

Figure 3. Neighbor-joining tree based on 16S rRNA gene sequences depicting the phylogenetic relationships between the isolates obtained from *Krem Mawjymbuin* listed in Table 3. The scale bar corresponds to the expected number of changes per nucleotide position.

the 16S rDNA fragments were performed using the above forward and reverse primers by genetic analyser ABI 3130XL (Applied Biosystems, USA) with the Big Dye (3.1) terminator protocol. The sequencing reaction was performed with 20 μL reaction mixture containing approximately 50 ng of template DNA, 1 pmol of primer, and 8 μL of ABI BigDye (Applied Biosystems, USA).

The Basic Local Alignment Search Tool (BLAST) was used initially to determine phylogenetic neighbors from the nucleotide database of the National Centre for Biotechnology Information (NCBI) (Altschul et al., 1997). For the isolates selected for the present investigation, the phylogenetic neighbors were obtained using the BLAST program against the database of type strains with manually curated and validly published prokaryotic names on the EzTaxon-e server at http://eztaxon-e.ezbiocloud.net/ (Kim et al., 2012). Molecular Evolutionary Genetics Analysis software (MEGA version 4) was used for phylogenetic analyses (Tamura et al., 2007). The sequences of identified phylogenetic neighbors were aligned with the sequences of representative strains by using the CLUSTAL W routine built into MEGA 4. The neighbor-joining method was employed to construct the phylogenetic tree with 1000 bootstrap replications to assess nodal support in the tree (Felsenstein, 1985). The nucleotide sequences were then deposited in NCBI database and accession numbers obtained.

The bacterial isolates were spot-inoculated on B4 agar (2.5 g L^{-1} calcium acetate, 4 g L^{-1} yeast extract, 10 g L^{-1} glucose and 18 g L^{-1} agar) for detection of calcite (calcium carbonate) precipitation (Boquet et al., 1973). Controls consisted of uninoculated culture medium and medium inoculated with autoclaved, dead bacterial cells. The plates were incubated aerobically at 25 °C to mimic cave temperatures in an inverted position and each isolate was periodically examined up to 25 days for the presence of crystals (Banerjee and Joshi, 2014).

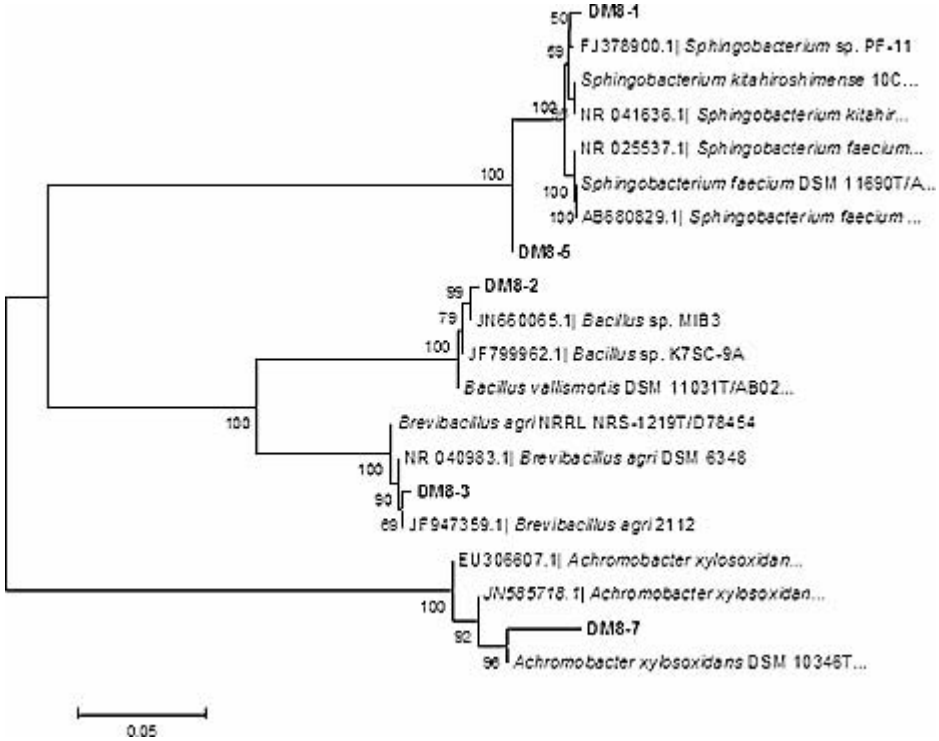

Figure 4. Neighbor-joining tree based on 16S rRNA gene sequences depicting the phylogenetic relationships between the isolates obtained from *Krem Dam* listed in Table 3. The scale bar corresponds to the expected number of changes per nucleotide position.

The crystal-precipitating cultures were aseptically excised from the media. Morphology and size characteristics of both the crystals and the microorganisms were studied by scanning electron microscopy (JSM 6360 [JEOL], resolution 3 nm, magnification 8× through 300,000×, accelerating voltage, 1–30 kV). SEM samples were prepared as follows: agar blocks of cultures grown on B4 medium were fixed onto aluminum stubs with two-way adherent tabs with conductive paint and allowed to dry. They were then gold-coated by sputtering for approximately 2 min to 3 min, dried at 37 °C, and gold shadowed (Baskar et al., 2009). The SEM was equipped with an energy dispersive X-ray (EDX) analyzer (INCA Penta FET X3 Model 7582, Oxford Instruments, England) with a resolution at 5.9KeV of 133 eV. That instrument was used to examine microbial colonies for their cells, filaments, or biofilms, and the microstructures and mineral compositions of associated crystals.

SEM studies were also performed on a few representative speleothems from *Krem Mawsmai* and *Krem Mawmluh*. EDX was used for quantitative estimation of the chemical composition of the minerals involved in the cave deposits following the same procedures as previously described for examination of crystals. The data generated by EDX analysis consisted of spectra showing peaks corresponding to the elements making up the true composition of the sample being analyzed.

RESULTS

Bacteria with diverse morphologies and cultural characteristics were observed on dilution plates from each cave. Gram staining showed the presence of both Gram-positive and Gram-negative bacteria. Pure cultures of the bacterial strains showed marked variation in colony morphology and pigmentation. The isolated bacteria were identified mostly to genus level and some to species level with the aid of biochemical tests.

The isolates identified to genus level through biochemical tests were further characterized to species level using molecular techniques. The PCR products of 16S rRNA were sequenced. The sequences of these isolates were aligned with the database of EzTaxon-e. Five different phylogenetic trees, one for each cave studied, were constructed using Neighbor-Joining method in Mega 4.1 software (Figs. 1–5). Table 1 lists the bacterial isolates identified from the studied caves. Table 2 summarizes the bacteria isolated and identified by both biochemical and molecular approaches, grouped by higher divisions.

The 16S rRNA partial sequences were deposited to the National Centre for Biotechnology Information GenBank under the GenBank IDs JX040437–JX040447, JX144942–JX144954, JX144956–JX144960, JX298811, JX298812, and KF515731–KF515736 (Table 3).

Table 1. Bacterial isolates identified from the studied caves.

Isolates	Caves				
	Mawsmai	Mawmluh	Mawjymbuin	Dam	Labit
Bacillus subtilis	+	−	−	−	−
Bacillus cereus	−	−	+	−	+
Bacillus vallismortis	+	−	−	−	−
Bacillus halodurans	−	+	−	−	−
Bacillus amyloliquefaciens	−	−	+	−	−
Bacillus thuringiensis	−	−	−	−	+
Bacillus circulans	−	−	−	−	+
Bacillus isronensis	−	−	−	−	+
Bacillus sp.	−	−	−	+	−
Pseudomonas gessardii	+	−	−	−	−
Pseudomonas vranovensis	+	−	−	−	−
Pseudomonas chlororaphis	+	−	−	−	−
Pseudomonas taiwanensis	+	−	−	−	−
Pseudomonas mosselii	+	−	−	−	−
Pseudomonas monteilii	−	+	−	−	−
Pseudomonas alcaligenes	−	−	+	−	−
Pseudomonas sp.	−	−	+	−	−
Kocuria rosea	+	−	−	−	−
Lysinibacillus macroides	+	−	−	−	−
Lysinibacillus parviboronicapiens	−	−	+	−	−
Brevibacterium frigoritolerans	−	−	−	−	+
Brevibacillus agri	+	−	+	+	−
Paenibacillus massiliensis	−	−	−	−	+
Ensifer adhaerens	+	−	−	−	−
Achromobacter xylosoxidans	−	−	−	+	−
Staphylococcus saprophyticus	+	−	−	−	−
Staphylococcus equorum	−	+	−	−	−
Sphingobacterium faecium	−	−	−	+	−
Sphingobacterium kitahiroshimense	−	−	−	+	−
Acinetobacter johnsonii	+	−	−	−	−
Iodobacter fluviatilis	−	−	+	−	−
Kurthia gibsonii	−	+	−	−	−
Aeromonas hydrophila	−	+	−	−	−
Flavobacterium chungangense	−	+	−	−	−

The overall structure of the selected bacteria and various crystal morphologies associated with them were clearly evident when examined under the electron microscope. SEM photomicrographs showed the presence of calcite crystals of various sizes, and most strikingly, a significant number of nanoscale to microscopic-size calcite crystals (Fig. 6). In addition, biofilms that were interwoven throughout the fiber network and bacterial filaments were observed under the SEM. Furthermore, EDX analyses confirmed that the crystals were composed predominately of calcium, carbon, and oxygen, suggesting precipitation solely of calcite and not of any other compound (Fig. 7).

Electron-microscopy studies of the speleothem samples were also done to investigate if the crystals generated by the isolated bacteria *in vitro* and the microorganisms themselves form a part and parcel of the speleothem itself, thereby to confirm the biogenic role, if any, in cave formations. All the observed structures, coupled with the highly weathered and disintegrated crystals of calcite, organic inclusions, and lithified structures indicated that they may have been formed by the metabolic activities of the associated bacterial communities (Fig. 8). The high concentration of carbon and oxygen as evidenced by EDX in the original cave wall and cultured samples may possibly reflect the visible microbial colonization of the cave walls. EDX of the speleothems revealed that apart from calcium, oxygen, and carbon expected for calcite, traces of deposits of magnesium, silica, iron, scandium, and tellurium were are also present (Figs. 9, 10).

Table 2. Phyla and classes of genera identified by biochemical and 16S rRNA gene sequencing.

Phylum	Class	Genus
Firmicutes	Bacilli	*Bacillus*
		Brevibacillus
		Lysinibacillus
		Paenibacillus
		Staphylococcus
		Kurthia
Bacteroidetes	Sphingobacteria	*Sphingobacterium*
	Flavobacteria	*Flavobacterium*
Actinobacteria	Actinobacteria	*Kocuria*
		Brevibacterium
Proteobacteria	Alpha	*Ensifer*
	Beta	*Achromobacter*
		Iodobacter
	Gamma	*Aeromonas*
		Acinetobacter
		Pseudomonas

DISCUSSION

Despite numerous studies performed to determine the mechanisms of biomineralization, the precise biomineral formation mechanisms remain uncertain. The geomicrobiology of subsurface rock environments of Meghalaya in North-East India has lacked investigation and understanding, even though the state hosts some of the longest caves in the Indian sub-continent and could provide useful information on general and applied microbiology as well as geosciences. Analysis of microbial communities, their role in biomineralization processes and their geomicrobiological interactions can help to understand the steps of colonization, subsurface microbial diversity, biomineral diversity, and various biomineralization mechanisms. Research on the role of microbial species in the development of secondary carbonate deposits in caves is still going on. Irrespective of the pathway, bacterial metabolic activity in these environments appears to lead to the precipitation of various polymorphs of $CaCO_3$, suggesting that bacterial metabolism plays a dominant role in calcification processes (Banks et al., 2010). Bacteria may also act as highly reactive geochemical interfaces, and their extracellular polymers are especially effective at binding ions from solution and serving as nucleation surfaces for mineral formation (Merz, 1992). These metabolic activities of microbes can, therefore, induce localized conditions that are favorable for mineral precipitation

The study revealed different indigenous chemoheterotrophic bacterial strains from five caves of Meghalaya, with the predominant bacterial genera belonging to *Bacillus* and *Pseudomonas*, which is in accordance with other studies where *Bacillus* is the predominant genera involved in calcium-carbonate precipitation (Baskar et al., 2009). In the present

Table 3. Closest match of the bacterial isolates based on 16S rRNA gene phylogeny analysis.

Isolates	GenBank Accession Number	Closest Related Microorganism
DM8-1	JX040437	*Sphingobacterium kitahiroshimense*
DM8-2	JX040438	*Bacillus* sp.
DM8-3	JX040439	*Brevibacillus agri*
DM8-5	JX040440	*Sphingobacterium faecium*
DM8-7	JX040441	*Achromobacter xylosoxidans*
MJ6-2	JX040442	*Iodobacter fluviatilis*
MJ6-3	JX040443	*Bacillus cereus*
MJ7-2	JX040444	*Pseudomonas* sp.
MJ7-7	JX040445	*Brevibacillus agri*
MJ7-4	JX040446	*Lysinibacillus parviboronicapiens*
MJ7-5	JX040447	*Bacillus amyloliquefaciens* subsp. *amyloliquefaciens*
MM1-1	JX144942	*Kocuria rosea*
MM1-2	JX144943	*Lysinibacillus macroides*
MM1-3	JX144944	*Brevibacillus agri*
MM1-4	JX144945	*Pseudomonas gessardii*
MM1-5	JX144946	*Pseudomonas vranovensis*
DM8-1	JX040437	*Sphingobacterium kitahiroshimense*
MM1-7	JX144947	*Pseudomonas chlororaphis* subsp. *aurantiaca*
MM1-8	JX144948	*Pseudomonas taiwanensis*
MM1-9	JX144949	*Staphylococcus saprophyticus*subsp. *bovis*
MM2-1	JX144950	*Acinetobacter johnsonii*
MM2-2	JX144951	*Bacillus vallismortis*
MM2-7	JX144952	*Pseudomonas mosselii*
MM2-9	JX144953	*Bacillus subtilis* subsp. *inaquosorum*
ML5-1	JX144954	*Kurthia gibsonii*
ML5-4	JX144956	*Aeromonas hydrophila* subsp. *hydrophila*
ML5-6	JX144957	*Flavobacterium chungangense*
ML5-8	JX144958	*Staphylococcus equorum*subsp. *equorum*
ML5-10	JX144959	*Pseudomonas monteilii*
MJ7-6	JX144960	*Pseudomonas alcaligenes*
MM1-6	JX298811	*Ensifer adhaerens*
ML5-2	JX298812	*Bacillus halodurans*
LB1	KF515731	*Bacillus thuringiensis*
LB2	KF515732	*Bacillus circulans*
LB3	KF515733	*Paenibacillus massiliensis*
LB4	KF515734	*Brevibacterium frigoritolerans*
LB5	KF515735	*Bacillus isronensis*
LB6	KF515736	*Bacillus cereus*

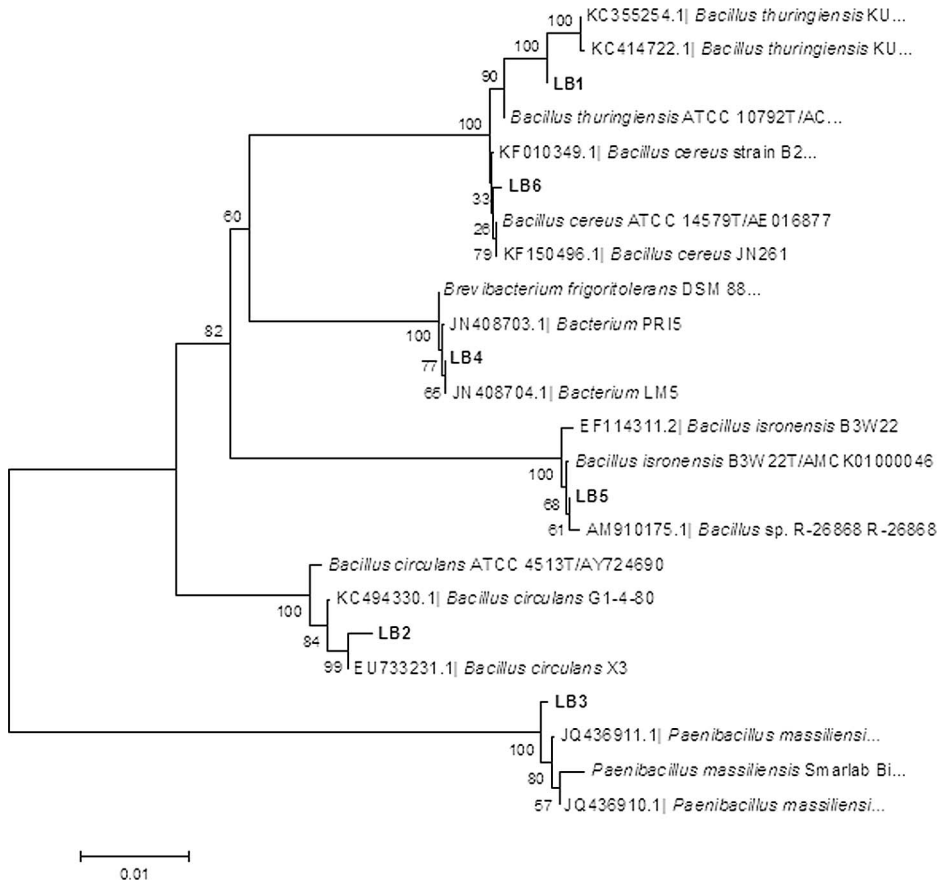

Figure 5. Neighbor-joining tree based on 16S rRNA gene sequences depicting the phylogenetic relationships between the isolates obtained from *Krem Labit* listed in Table 3. The scale bar corresponds to the expected number of changes per nucleotide position.

study, it was determined that a few genera are prevalent in one cave but absent in the other selected sampling sites. The prevalence of *Sphingobacterium* in *Krem Dam* only can shed some light on the uniqueness of this organism to adjust itself in this yet untouched cave. On the other hand, diverse species of *Pseudomonas* encountered in *Krem Mawjymbuin* can be related to their versatile nature and ability to form biofilm. From the various phyla obtained in the present study, it was evident that bacteria belonging to phylum Firmicutes predominated in all the caves studied except in *Krem Mawsmai*, where the predominant bacteria belonged to Gammaproteobacteria. Studies have shown that Gammaproteobacteria were found to be important biofilm-forming groups in Lower Kane Cave, Wyoming, and Movile Cave, Romania (Engel et al., 2001, 2003). Such results led the investigators to conclude that the surface of the Gammaproteobacteria species found within the bacterial filaments of the cave played a crucial role in calcite deposition (Holmes et al., 2001). Moreover, this group of bacteria are commonly present as yellow biofilms in oxygen-deprived microenvironments where the fermentation of organic matter by Gammaproteobacteria is known to induce acidification of the medium (Madigan et al., 2003). It was also striking to note that bacteria belonging to Alphaproteobacteria were encountered only in *Krem Mawsmai*, which could be attributed to the nature and anthropogenic influences prevalent in the cave. Moreover, the present findings show that a particular assemblage of bacteria may predominate in a particular cave but be completely absent in other caves, and this observation needs to be investigated in detail for understanding the geomicrobiologal contributions to formation of cave speleothems.

Complex microbial communities producing colored colonizations on Altamira Cave walls in Spain have been reported, with white, yellow, and gray biofilms having been analyzed and distinguished based on the bacterial communities forming these colonizations (Portillo et al., 2009). In addition, a morphological study has been reported on the characteristics of these differently colored biofilms that suggests that white and gray biofilms were associated with mineral deposits, while yellow colonies did not present associated mineral formations (Cuezva et al., 2009). The different coloration of these biofilms is the result of their distinctive composition of bacterial phylotypes

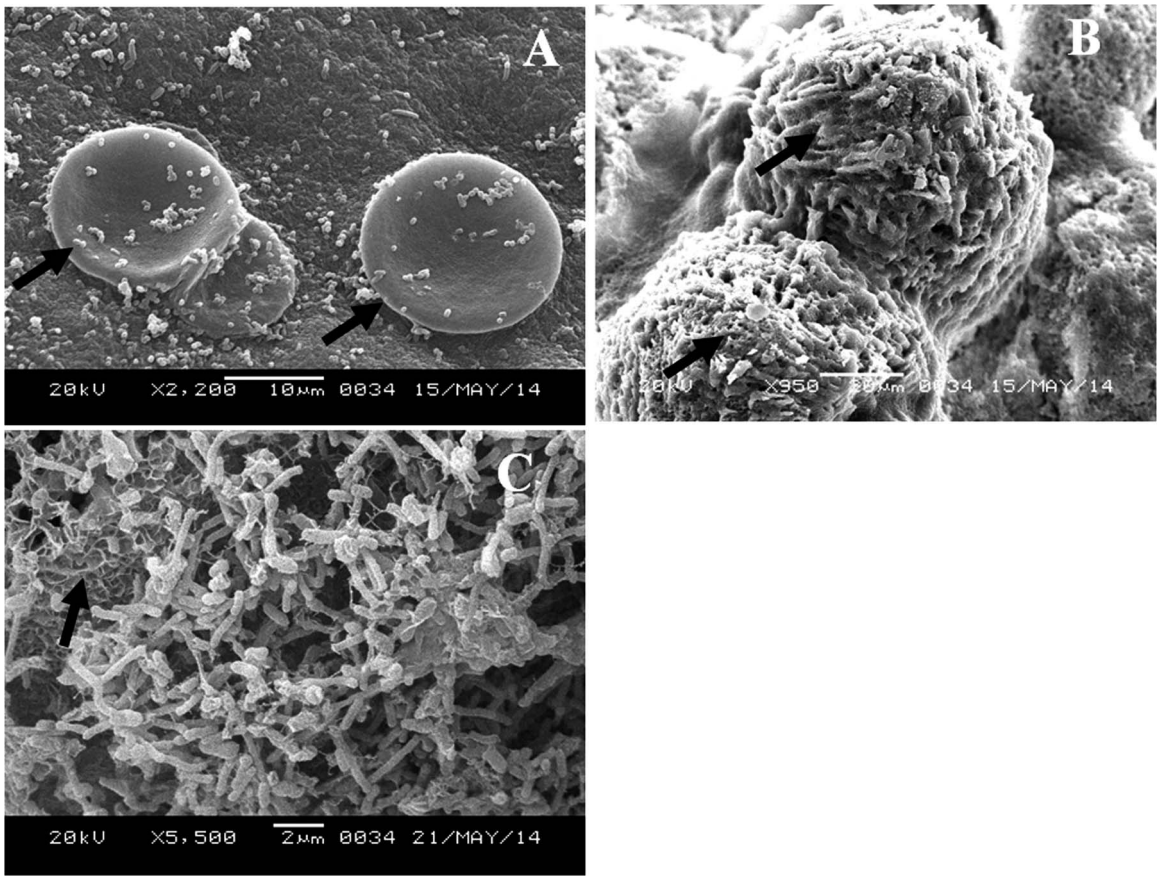

Figure 6. SEM photomicrographs showing varied morphologies of *in vitro* crystal formation by selected bacterial genera. (A) Numerous bacterial cells of *Kurthia gibsonii* isolated from a stalagmite in *Krem Mawmluh* across the disc shaped crystals generated by them. (B) The close association between microbial growth and the precipitation of minerals, including cell-shaped pits and septa on the porous hemispherical crystal as indicated by arrows generated by *Pseudomonas gessardi* isolated from a stalagmite in *Krem Mawsmai*. (C) A mesh of bacterial ligaments and filaments in association with micro- and nano-sized crystals in case of *Brevibacterium frigoritolerans* isolated from a cave-wall deposit of *Krem Labit*.

(Portillo et al., 2009). The consequences of bacterial metabolism represent an important aspect to be monitored during the assessments of conservation strategies and the evaluation of potential for the transformation of cave environments.

Electron-microscopy studies of the speleothem samples were done to investigate if crystals similar to those generated *in vitro* by isolated bacteria and the microorganisms themselves form a part of the speleothems, thereby to confirm the biogenic role, if any, in the formation of the studied caves. The strains were tested for their ability to precipitate calcium carbonate. The presence of crystals and their microscopic sizes were related to bacterially mediated precipitation.

Conclusion

Based on our microbiological observations and SEM-EDX studies, the isolated biofilm bacteria from the studied caves are able to precipitate minerals, thus reaffirming the role of these microorganisms in biospeleogenesis. The results obtained in this study indicate a geomicrobiological contribution in the precipitation of calcium carbonate. Similarly, this interpretation could be extended to some other elements that would be specific to a hypogean environment. Further work should be focused toward deciphering the underlying molecular mechanisms behind these complex mineralogical features and microbial assemblages.

Acknowledgements

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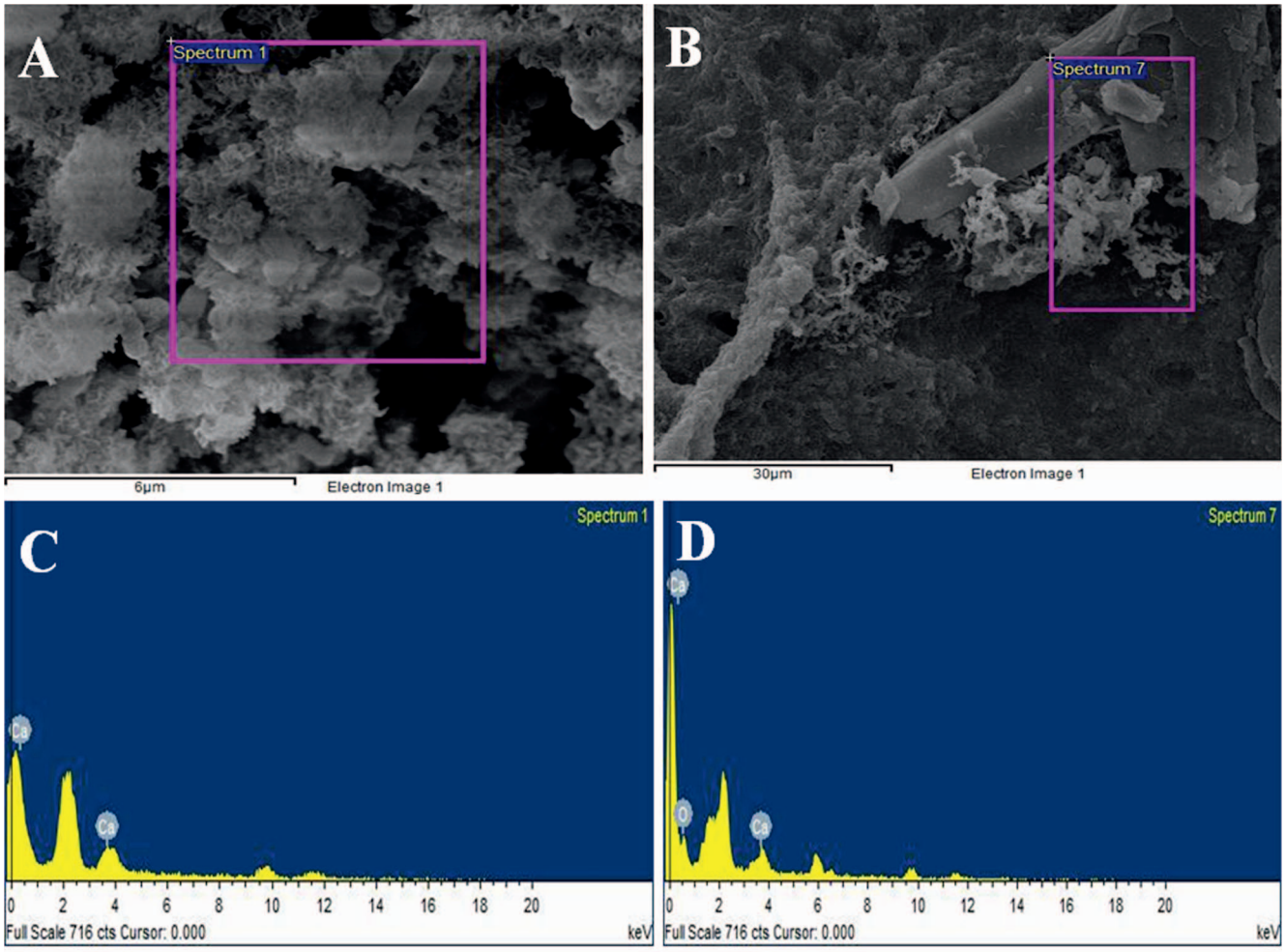


E

Spectrum 1			Spectrum 7		
Element	Weight %	Atomic %	Element	Weight %	Atomic %
Ca K	100	100	O K	86.69	94.22
Totals	100		Ca K	13.31	5.78
			Totals	100	

Figure 7. SEM-EDX of a few crystal polymorphs formed *in vitro* by *Kurthia* and *Pseudomonas*. (A) Fluffy irregular crystals and (B) rhombohedral crystals precipitated by bacteria. (C) EDX showing composition of Spectrum 1 in (A). (D) EDX showing composition of Spectrum 7 in (B). (E) Semiquantitative analysis of the EDX spectra of precipitated crystals of calcite by bacteria. The symbol "K" signifies electron shell of the elements.

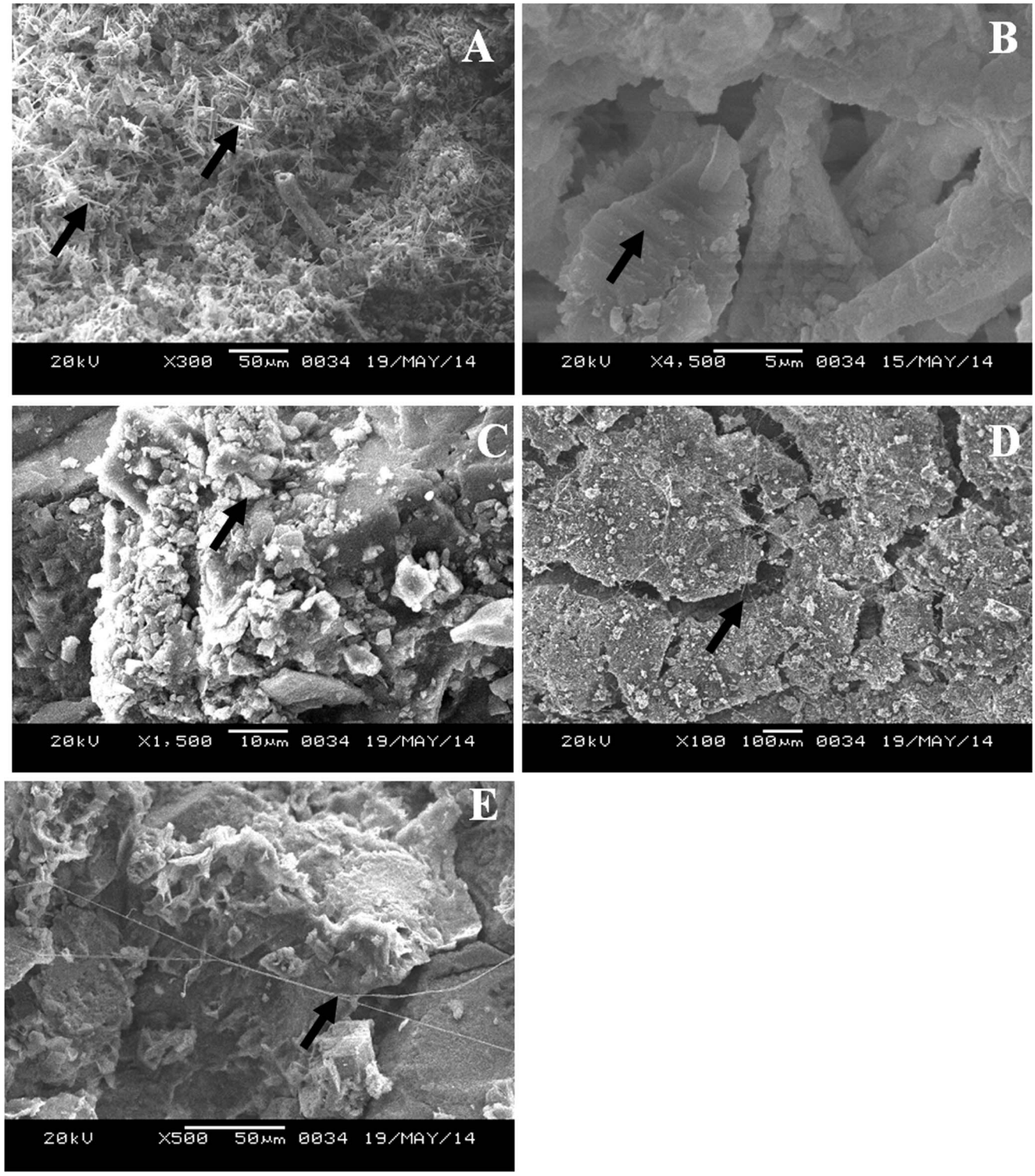
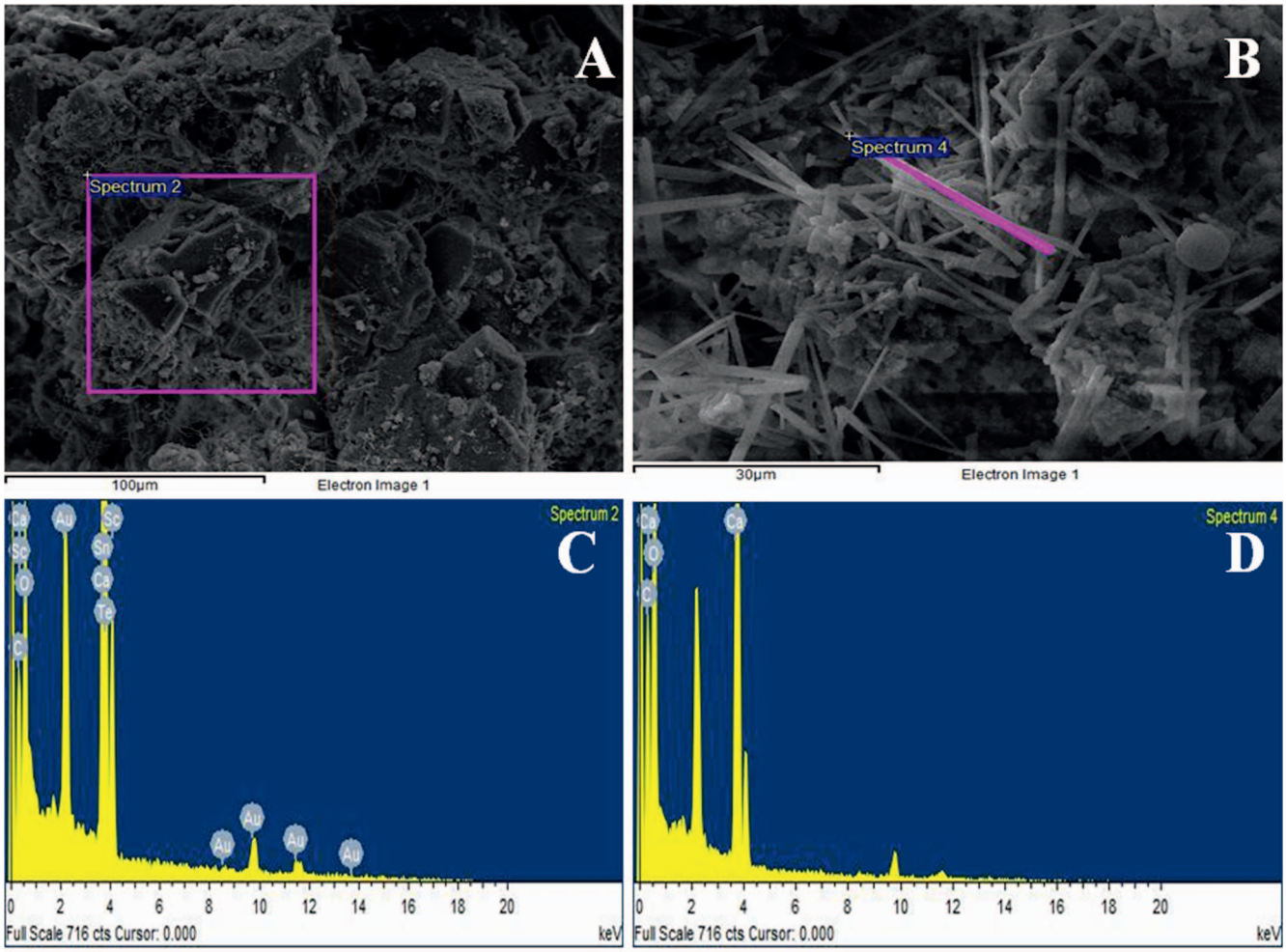


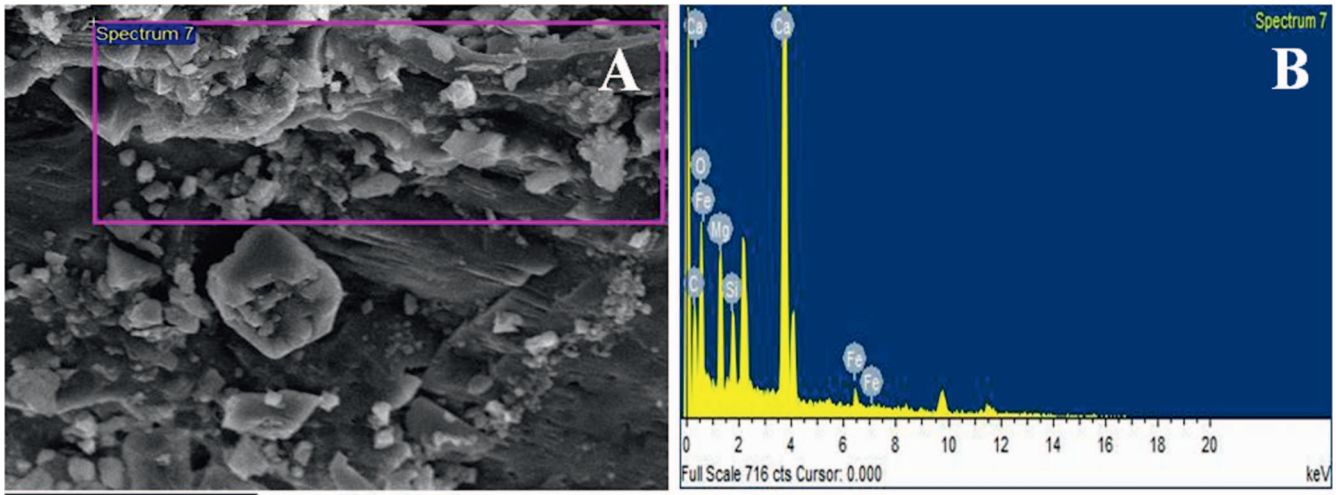
Figure 8. SEM photomicrographs of speleothem samples from the studied caves. (A) Abundant needle-fiber calcites and (B) internal weathered structures of precipitates from *Krem Mawsmai* speleothems. (C) Calcite crystals observed from *Krem Mawmluh* speleothems that are similar in pattern to those generated by isolated bacteria. (D) A large aggregate of the individual bioliths binding with nonglobular carbonate bridges in *Krem Mawjymbuin* speleothems. (E) A large interconnected microbial fiber associated with *Krem Dam* speleothems.



E

Spectrum 2			Spectrum 4		
Element	Weight%	Atomic%	Element	Weight%	Atomic%
C K	9.35	20.24	C K	14.29	21.01
O K	33.31	54.16	O K	62.18	68.62
Ca K	32.68	21.2	Ca K	23.53	10.37
Sc K	0.67	0.38	Totals	100	
Sn L	1.22	0.27			
Te L	10.32	2.1			
Au M	12.45	1.64			
Totals	100				

Figure 9. SEM photomicrographs and EDX spectra of speleothem samples from *Krem Mawsmai*. (A) Presence of spiky calcite, rounded balls of calcite, and microbial filaments. (B) Abundant large needle fiber calcites. (C) Composition of Spectrum 2 in (A). (D) Composition of Spectrum 4 in (B). (E) Semiquantitative analysis of speleothem spectra (C) and (D). The symbol "K" signifies electron shell of the elements.



C

Spectrum 7		
Element	Weight%	Atomic%
C K	14.7	23.79
O K	44.65	54.26
Mg K	6.24	4.99
Si K	2.52	1.74
Ca K	30.06	14.58
Fe K	1.83	0.64
Totals	100	

Figure 10. SEM photomicrograph and EDX spectrum of a speleothem sample from *Krem Mawmluh*. (A) Rhombohedral crystals of calcite accompanied by biofilm and microbial filaments. (B) Composition of Spectrum 7 in (A). (C) Semiquantitative analysis of speleothem spectrum (B). The symbol "K" signifies electron shell of the elements.

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DETECTING VIABLE *PSEUDOGYMNOASCUS DESTRUCTANS* (ASCOMYCOTA: PSEUDEUROTIACEAE) FROM WALLS OF BAT HIBERNACULA: EFFECT OF CULTURE MEDIA

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Abstract: *Pseudogymnoascus destructans* (*Pd*) causes the fungal disease white-nose syndrome (WNS), which has led to high mortality in some hibernating bat species in eastern North America. The ability to detect viable *Pd* in hibernacula is important for understanding the role the environment plays as a reservoir for infectious *Pd*. Previous studies have generally used the high-sugar medium Sabouraud-dextrose (SAB) and have had low yields of viable *Pd* from environmental samples of *Pd*-positive hibernacula. While culture-independent methods (i.e., molecular genetics) have previously shown much better success in detecting *Pd*, these methods cannot determine viability. In 2012 and 2015, we swabbed walls in four hibernacula with WNS-positive bats in New Brunswick, Canada, and cultured the samples using dextrose-peptone-yeast extract agar (DPYA), SAB, and Malt extract (MEA) media. Samples cultured on DPYA produced viable *Pd* 43.7 to 50.0 % more frequently than SAB, with a maximum overall return for DPYA among sites of 62.5 % *Pd*-positive samples over both years. During the initial outbreak of WNS in our study region, *Pd*-positive swabs were produced from 40.0 to 83.3 % of samples on DPYA, whereas SAB produced a maximum of 40.0 %. At one site we detected *Pd* from 83.3% of swabs cultured on DPYA and 0 % on SAB. MEA produced no viable *Pd*. Our figures for *Pd* detection are as high as or higher than previously published culture-independent methods, while also confirming the viability of the *Pd* present. We found that the yield of viable *Pd* from hibernacula walls decreased from 2012 to 2015 as the hibernating bat population decreased due to WNS mortality, but patterns varied amongst hibernacula, and overall, were not statistically different. It is possible that environmental growth of *Pd* contributes to its persistence within hibernacula. We suggest that future studies on the environmental persistence of viable *Pd* discontinue the use of high-sugar media that lack inhibitory fungal growth ingredients, such as SAB and MEA, as they favor fast-growing fungal species that overgrow and mask slower-growing fungi such as *Pd*.

Introduction

White-nose syndrome (WNS) is a fungal disease of hibernating bats that has rapidly spread through the eastern United States and Canada, killing more than 6.7 million bats since it was first reported in 2006 in Albany, New York (USFWS, 2012a). WNS is caused by the fungus *Pseudogymnoascus destructans* (Blehert & Gargas) Minnis & D.L. Lindner, thought to be an invasive species from Europe (Lorch et al., 2011). The ability to detect *Pd* within a hibernaculum is an important part of disease surveillance. Detection often relies on the observation of visible fungal growth and behavioral changes in hibernating bats, but *Pd* can arrive before such changes are noticeable (Janicki et al., 2015). *Pd* surveillance is often conducted with culture-independent methods of molecular genetics, but such approaches cannot discriminate between viable and nonviable fungus. Viable cultures of *Pd* are required by researchers for physiological testing and other studies. Additionally, the ability to detect viable *Pd* in hibernacula is important for understanding the role the environment plays as a reservoir for infectious *Pd*. DNA from various organisms is able to persist long term in the environment, as shown by its recovery from paleontological remains, but the limit of persistence of non-viable *Pd* DNA in bat hibernacula is unknown. Viable *Pd* is known to persist in hibernacula soil in the absence of bats (Lorch et al., 2013), and it may be able to propagate as a saprobe (Raudabaugh and Miller, 2013). Reynolds et al. (2015) successfully grew *Pd* on a variety of sterilized sediments from caves in the laboratory. However, the ability of *Pd* to grow in cave sediments in the presence of native microorganisms has not yet been demonstrated. The relative importance of spores shed from bats in maintaining an environmental reservoir of *Pd* compared to possible environmental growth is unknown. Langwig et al. (2015) found

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higher concentrations of *Pd* DNA on cave walls close to roosting bats compared to locations more than 2 m away, suggesting that bats shed *Pd* spores into their immediate environment. The long-term persistence of these shed spores is unknown. Modelling has shown that *Pd* persists in hibernacula for only three years beyond the loss of bats in the absence of environmental growth, while it persists for decades when environmental growth is possible (Reynolds et al., 2015).

Previous studies have produced low yields of viable *Pd* from *Pd*-positive hibernacula, with only 13 to 33 % positive samples using culture-dependent methods (Lorch et al., 2013; Martinkova et al., 2010; Zhang et al., 2014), as compared to 44 to 91 % with culture-independent methods (Zhang et al., 2014; Langwig et al., 2015). Lorch et al. (2013) found that the probability of detecting *Pd* in a *Pd*-positive hibernaculum was 56 % for each sample using real-time PCR, compared to 14% using culture-dependent methods.

WNS was first observed in New Brunswick, Canada, in 2011 (McAlpine et al., 2011) and had spread to all known hibernacula in the province by 2013 (Vanderwolf et al. 2015). The study reported here had two objectives: to compare the yield of viable *Pd* from hibernacula walls using a variety of culture-media types and to determine if the yield of viable *Pd* from hibernacula walls decreased over time as the hibernating bat population declined due to WNS-associated mortality.

Methods

Swabs were collected from hibernacula where bats (*Myotis lucifugus*, *Perimyotis subflavus* and *M. septentrionalis* with visible *Pd* growth were observed in southern New Brunswick, Canada. Data on physical characteristics of study sites, including location and temperatures, can be found in Vanderwolf et al. (2012). The mean winter (Nov. 1–Apr. 30) temperature in the dark zone of New Brunswick hibernacula is 5.1 °C ± 1.1SD (Vanderwolf et al., 2012). We followed the protocol of the United States Fish and Wildlife Service (2012b) for minimizing the spread of WNS during all visits to caves. Walls were swabbed in three hibernacula, Dorchester Mine, Berryton Cave, and Glebe Mine, both in April 2012 and April 2015. An additional site, White Cave, was sampled in April 2015 because hibernating bats were still roosting there, unlike the other sites. The number of hibernating bats present during swabbing is listed in Table 1, as well as the date of first detection of *Pd* in each site. *Pd* was initially detected at sites by assessing live hibernating bats for the presence of characteristic *Pd* fungal growth. However, lack of visible *Pd* growth does not equate to its absence (Janicki et al. 2015), so visual assessments of *Pd* presence on bats may underestimate arrival date.

Swabs were collected about 2 m above the cave floor deep in hibernacula in areas where bats were known to routinely roost, but always more than 1 m from the nearest roosting bat. Samples were collected from the same areas within hibernacula in 2012 and 2015. At each hibernaculum areas of approximately 20 by 20 cm on the wall were swabbed during each visit, with one applicator used for each area. Swabs were taken with a sterile, dry, cotton-tipped applicator and immediately streaked on the culture medium surface in a petri plate. Diluting streaks were completed within hibernacula within 1 h of the initial streak, after which plates were sealed in situ with Parafilm. A new applicator was used for each swab. Plates were shuffled in the field to ensure media types were not inoculated consecutively. In 2012, dextrose-peptone-yeast extract agar (DPYA; Papavizas and Davey, 1959) and Sabouraud-dextrose agar (SAB) were used, with five plates of each media type for Dorchester Mine and Glebe Mine and six of each for Berryton Cave. In 2015, DPYA, SAB, malt extract (MEA), and modified DPYA were used, with five plates of each media type for each of the four hibernacula. The antibiotics chlortetracycline (30mg/L) and streptomycin (30mg/L) were added to all media. Modified DPYA differed from DPYA in that the agar was autoclaved separately from the other ingredients. Tanaka et al. (2014) found that autoclaving ingredients separately increased the number of bacterial species that could be cultured. MEA consisted of 20 g Malt extract, 1 g peptone, 20 g dextrose, and 20 g agar per liter. DPYA consisted of 5 g dextrose, 1 g peptone, 2 g yeast extract, 1 g NH_4NO_3, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeCl_3 \cdot 6H_2O$, 5 g oxgall, 1 g sodium propionate, and 20 g agar per liter (Papavizas and Davey, 1959).

Plates were incubated inverted in the dark at 7 °C, a temperature that approximates that found in our study sites (Vanderwolf et al., 2012), and monitored over four months, in the manner of Vanderwolf et al. (2013). Initial morphological identifications of *Pd* (using Gargas et al., 2009) were confirmed by sequencing as part of other studies (2012 samples: Khankhet et al., 2014; 2012 and 2015 samples: pers. comm. J. Foster and K. Drees, University of New Hampshire). Permanent dried cultures are housed in the New Brunswick Museum (NBM# F-05262–05282, 05339, 05345, 05404, 05457, 05486).

Since the data were not normally distributed, a Friedman test was used to test the effect of media type and site on the number of *Pd*-positive swabs in 2015. After testing for normality, a general linear model was used to test the effect of media type, site, and year on the number of *Pd*-positive swabs using data from media types and sites sampled in both 2012 and 2015. All tests were performed using Minitab.

Results

Pd was cultured from 40.6 % of wall swabs ($n = 32$) in 2012, and 31.3 % of swabs ($n = 80$) in 2015 (Table 2). When restricting 2015 data to the same hibernacula and media types used in 2012, 26.7 % of swabs ($n = 30$) yielded viable *Pd*, with Dorchester Mine and Berryton Cave decreasing to 10 % and 20 % positive samples from 2012 to 2015, and Glebe Mine increasing to 50 %. These trends were not significantly different between years ($F_{1,11} = 1.26$, $p = 0.299$) or sites ($F_{2,11}$

Table 1. The number of hibernating bats (*Myotis lucifugus*, *M. septentrionalis*, and *Perimyotis subflavus*) present during sampling. Sampling was not conducted in White Cave in 2012. The first detection of *Pd* was determined by the observation of visible fungal growth on hibernating bats.

Site Name	2012	2015	First *Pd* Detection
Berryton Cave	5	0	March 2011
Dorchester Mine	1	1	December 2011
Glebe Mine	174	0	March 2012
White Cave	115	5	December 2011

$= 0.15$, $p = 0.863$), but overall yield was significantly higher on DPYA compared to SAB ($F_{1,11} = 8.51$, $p = 0.022$). Samples cultured on DPYA produced viable *Pd* 43.7 to 50.0 % more frequently than SAB, with a maximum overall return for DPYA among sites of 62.5 % *Pd*-positive samples over both years. During the initial outbreak of WNS in our study region, *Pd*-positive swabs were produced from 40.0 to 83.3 % of samples on DPYA, whereas SAB produced a maximum of 40.0 %. The date of first *Pd* detection in each site did not appear to affect *Pd* yield. However, the date of *Pd* arrival in Dorchester Mine is uncertain, since we observed visible fungal growth on hibernating bats ($n = 140$ bats) on our initial visit to the site in December 2011, while the other sites had been monitored since 2009.

In 2015, *Pd* yield was not significantly different between sites ($S_{3,15} = 4.78$, $p = 0.189$, adjusted for ties), but was significantly different among media types ($S_{3,15} = 9.65$, $p = 0.022$; adjusted for ties). *Pd* yield was higher on DPYA (60 %) compared to SAB (10 %; Table 2). The modification to DPYA (autoclaving the agar separately from the other ingredients) did not increase *Pd* yield compared to unmodified DPYA (11 vs. 12 positive swabs in 2015). Malt medium tended to be quickly overgrown with *Mucor* spp. and *Mortierella* spp., and no *Pd* was obtained.

DISCUSSION

Pd is known to persist in hibernacula soil/sediments in the absence of bats, but healthy bats only occasionally come into contact with the ground (Lorch et al., 2013; Rysgaard, 1942), and *Pd* is not thought to be transmitted aerially (Lorch et al., 2011). Our study shows that viable *Pd* persists on walls of hibernacula that bats regularly come into contact with years after bat populations have declined at these sites or disappeared entirely. Hibernacula walls may act as a source of infection when bats return to hibernate or during swarming activity (Langwig et al., 2015). The number of *Pd*-positive samples did decrease in two hibernacula (Berryton Cave and Dorchester Mine) after the hibernating-bat population declined or disappeared, but increased at the third site (Glebe Mine), although these trends were not significant and low sample sizes complicate interpretation. In Glebe Mine, no more than one bat has been seen since winter 2013 during annual surveys, although the site previously supported hundreds of hibernating bats (Vanderwolf et al., 2012). No more than three bats each have been observed in Berryton Cave and Dorchester Mine during annual winter surveys 2012-2015, although the sites previously had thousands and hundreds of hibernating bats, respectively (Vanderwolf et al., 2012). The reasons for the increase in *Pd* yield in Glebe Mine are unknown. It is interesting to note that the *Pd* yield in Glebe Mine was higher than that from White Cave, where low numbers of bats with visible *Pd*-growth still persist and presumably continue to shed *Pd* spores into the environment. It is possible that environmental growth explains the patterns. Reynolds et al. (2015) found that the greatest growth of *Pd* in the laboratory occurred on cave sediments with high levels of organic matter. The levels of organic matter in our study sites have not been quantified, although our mine sites have old wooden support pillars with mushroom growth that are absent in the caves, and Glebe Mine and White Cave have accumulations of porcupine (*Erethizon dorsatum*) dung. The ability of *Pd* to grow on non-bat substrates in the field and the mechanism for spore dispersal within hibernacula in the absence of bats requires further study.

Verant (2016) found that the prevalence of *Pd* on hibernacula walls increased over time after the first detection of WNS in hibernating bats at sites, although at a slower and lower rate than in sediment samples. Verant (2016) did not detect *Pd* on hibernacula walls until one year after WNS was first detected on bats at the sites. Prevalence was initially low

Table 2. Number and percentage of *Pd*-positive wall swabs taken in four New Brunswick hibernacula April 2012 and 2015 on different media types with the total number of swabs in brackets. DPYA = dextrose-peptone-yeast extract agar. SAB = Sabouraud-dextrose agar. MEA= Malt extract agar. ND = no data.

	2012			2015				
Site Name	DPYA	SAB	Overall Positive	DPYA	SAB	Mod DPYA	MEA	Overall Positive
Berryton Cave	5(6) 83.3%	0(6) 0%	41.7% (12)	2(5) 40%	0(5) 0%	1(5) 20%	0(5) 0%	15.0% (20)
Dorchester Mine	3(5) 60%	2(5) 40%	50.0% (10)	1(5) 20%	0(5) 0%	4(5) 80%	0(5) 0%	25.0% (20)
Glebe Mine	2(5) 40%	1(5) 20%	30.0% (10)	4(5) 80%	1(5) 20%	5(5) 100%	0(5) 0%	50.0% (20)
White Cave	ND	ND	ND	5(5) 100%	1(5) 20%	1(5) 20%	0(5) 0%	35.0% (20)
Overall positive	62.5% (16)	18.8% (16)	40.6% (32)	60.0% (20)	10.0% (20)	55.0% (20)	0% (20)	31.3% (80)

(8 % *Pd*-positive samples from walls and 30 % from sediment using culture-independent methods), but increased to 17 to 75 % from walls and up to 90 % from sediment two years after the first detection of WNS in hibernating bats at each site (Verant 2016). We did not find a lag in *Pd*-detection from hibernacula walls, as prevalence of *Pd* in Dorchester Mine and Glebe Mine was 30.0 % and 41.7 % one month and four months after the first detection of WNS in bats at the sites, respectively. We also did not find such large increases in *Pd*-prevalence more than two years after the first detection of WNS in bats at our study sites. These inconsistencies may reflect differences in the detection of *Pd* DNA using culture-independent approaches compared to culture-dependent methods, which reveal only viable spores, or possibly site differences.

Lorch et al. (2013) and Zhang et al. (2014) found that the probability of detecting *Pd* was higher using molecular methods compared to culture techniques. Viable *Pd* was cultured from only 13.8 % ($n = 195$) of soil samples in *Pd*-positive hibernacula using SAB (Lorch et al., 2013). Zhang et al. (2014) obtained viable *Pd* from 18 % of sediment samples from *Pd*-positive hibernacula in New York using SAB and Rose Bengal medium. In Estonia, 100 % of wall swabs ($n = 4$) on SAB yielded *Pd*, but these swabs were taken within a few centimeters of where one bat with visible white fungal growth had been roosting a few days earlier (Puechmaille et al., 2011). Langwig et al. (2015) found that the percentage of *Pd*-positive samples from cave walls using culture-independent techniques varied with the distance from roosting bats. Swabs taken just under roosting bats were 91 % positive, 66 % were positive 10–20 cm away from bats, and 44 % were positive more than 2 m from bats (Langwig et al., 2015). We swabbed cave walls more than 1 m from roosting bats, if any were present, and obtained results with DPYA that generally match or exceed those obtained with culture-independent methods, while also allowing us to confirm the viability of *Pd*. Our yield of *Pd* from wall swabs using SAB is similar to Lorch et al. (2013) and Zhang et al. (2014).

Discrepancy in *Pd* yield between SAB and DPYA media was less marked when we directly swabbed bats (Vanderwolf et al., 2015), presumably because bats have greater numbers of spores than cave walls. However, Martinkova et al. (2010) cultured *Pd* on SAB from only 33.3 % ($n = 48$) of swabs taken directly from European bats with visible white fungal growth. This may reflect decreased growth of *Pd* on European bats compared to infected bats in North America, or possibly the observed growth on some bats was of a different fungal species than *Pd* (such as *Trichophyton* spp., Lorch et al., 2015).

As pointed out by Cooke (1968), culture media high in soluble sugars, such as MEA and SAB, tend to accumulate extracellular biproducts, leading to premature staling of the medium. We recommend against the use of these media in studies on the environmental persistence of viable *Pd*. We also recommend the use of fungal-growth-inhibiting ingredients, such as sodium propionate and oxgall, as additives to any medium used for this purpose. High-glucose media such as MEA and SAB favor fast-growing species, such as *Mortierella* spp. and *Mucor* spp., which overgrow and mask slower growing fungal species such as *Pd*. Other media types, such as those that mimic the more complex nutrient contents of bats, may produce even higher yields of viable *Pd* from environmental samples collected in hibernacula.

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A NEW METHOD TO ESTIMATE ANNUAL AND EVENT-BASED RECHARGE COEFFICIENT IN KARST AQUIFERS; CASE STUDY: SHESHPEER KARST AQUIFER, SOUTH CENTRAL IRAN

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Abstract: Quantification of recharge is a fundamental issue in evaluation and management of karst water resources. Recharge coefficient (*RC*), which is the ratio of aquifer recharged water (*VR*) to the total precipitation, has been widely used for recharge estimation in karst catchments. Though the *RC* definition is straightforward, in practice, its application on a fixed short time scale (e.g., monthly or annual) is challenging, because of the problems in determination of change in groundwater storage (ΔVS) that is included in *VR*. Some researchers have neglected ΔVS, estimated *VR* as equal to the net aquifer outflows; hence they have over- or under-estimated the *RC*, ignoring the aquifer's antecedent storage condition. In this study, a new method is proposed to estimate the actual event *VR*, and consequently, the event-based *RC*, utilizing the MRC displacement method of Kavousi and Raeisi (2015). Applying the method, *VR* due to a specific event or a combination of events during a hydrological year can be directly estimated by decomposition of the observed hydrograph to the individual hydrographs induced by different events. The method is applicable for karst aquifers with no-flow boundaries, draining by a spring. The calculated event *VR* takes ΔVS into account, enabling an accurate event-based *RC* estimation. The method was applied to the Sheshpeer karst aquifer in Iran. The estimated *RCs* reasonably matched up to the catchment properties and former field studies. Results showed that the annual *RCs* would be underestimated if the ΔVS was neglected, because ΔVS was positive during the study period.

INTRODUCTION

Recharge to karst aquifers has been widely estimated using the ratio of aquifer recharged water (*VR*) to the total precipitation (*VP*) over the catchment during a specific time interval, the so-called *effective infiltration coefficient*, *EIC* (Drogue, 1971; Soulios, 1984; Bonnaci, 2001):

$$EIC = \frac{VR}{VP} \tag{1}$$

Infiltration is the term used to describe the process by which water moves downward through soils and rocks (e.g., Horton, 1933). Some portion of the infiltrated water is retained as soil moisture, which may also be evapotraspirated later, not reaching the saturated zone. Therefore, the recharge amount can be a fraction of the total infiltration amount. Consequently, the term *recharge coefficient*, *RC*, is preferred here instead of *EIC*.

Aquifer recharged water *VR* is the fraction of total precipitation *VP* that reached and would be eventually discharged from the aquifer. Both *VR* and *VP* are expressed in L^3 or just L averaged over the catchment; thus *RC* is a dimensionless coefficient, theoretically ranging from 0 (when $VR = 0$) to 1 (when $VR = VP$). On a global scale, the reported *RCs* for karst aquifers cover the whole range, even approaching the two end members (see for example Ashjari and Raeisi, 2006; Allocca et al., 2014; Fiorillo et al., 2015; Martos-Rosillo et al., 2015). *RC* simply represents the percentage of total precipitation that reached the phreatic zone, reflecting the combined effects of all recharge-prohibiting processes such as water loss through surface runoff or evapotranspiration. The ability to estimate *RC* permits transfer of hydrological information from catchments with adequate hydrological and meteorological measurements to those with insufficient (Ford and Williams, 2007).

Given the catchment area and the spatial variation of precipitation, one can calculate the *VP* over the catchment during a fixed time interval of a year or a month, but estimation of *VR* is not so straightforward. The simplest method to estimate *VR* for a karst catchment with no-flow boundaries, where all recharged water is emerging from a spring, is to consider *VR* as equal to the net spring outflow (V_{Sp}) during the time interval of *VP* estimation (see Bonnaci, 2001). Subsequently, Equation (1) is rewritten as:

$$RC = \frac{V_{Sp}}{VP} \tag{2}$$

However, estimation of *VR* by V_{Sp} is not accurate, because some portion of V_{Sp} during the *RC* calculation interval is, in fact, released from the groundwater storage and might have had residence times of years or even decades, and some

portion of recharge water during the time interval may also remain stored in the aquifer to feed the spring afterward, so V_{Sp} is related to the aquifer's antecedent or subsequent storage condition, such that precipitation events of equal characteristic intensity would not always result in equal V_{Sp}'s; V_{Sp} relevant to a precipitation of 100 mm over a karst catchment would be different if the antecedent spring discharge level was 1 m^3 s^{-1} or 10 m^3 s^{-1}.

For the aquifer boundary condition mentioned for the Equation (2), *VR,* and consequently *RC,* can be more accurately estimated by setting *VR* as equal to V_{Sp} plus change in groundwater storage (ΔVS) during the calculation time interval (see Soulios, 1984):

$$RC = \frac{V_{Sp} + \Delta VS}{VP} \quad (3)$$

ΔVS can be positive (storage replenishing) or negative (storage depleting). Equation (3) gives the exact value of *RC* for the aforementioned aquifer boundary condition, although it suffers from the problem of ΔVS estimation in karst. The widely used water table fluctuation method for ΔVS estimation in unconfined aquifers requires specific yield and water level data at catchment scale (e.g. Rasmussen and Andreasen, 1959; Maréchal et al., 2006; Dewandel et al., 2010), which cannot be reliably determined for karst and hard rock aquifers (Healy and Cook, 2002; Raeisi, 2008).

Equation (3) is only valid for an aquifer with no-flow boundaries where the only discharge point is a spring. The more general form of *RC* equation, considering multiple springs and wells, as well as multiple subsurface inflows and outflows, is (modified from Allocca et al., 2014):

$$RC = \frac{\sum V_{Sp} + \sum V_W + \sum V_{Out} + \sum V_{In} + \Delta VS}{VP} \quad (4)$$

where, $\sum V_{Sp}$, $\sum V_W$ and $\sum V_{Out}$ are the net discharged water through springs, production wells, and groundwater outflows, respectively; and $\sum V_{In}$ is the net groundwater inflows. Equation (4) is the general equation for *RC* estimation, but it has never been used considering all its parameters. Equation (4) is shortened to Equation (3) when only one spring discharges the aquifer and $\sum V_{In}$ and $\sum V_{Out}$ are negligible in comparison to V_{Sp}, ΔVS, and *VP*. However, Equation (3) has not been commonly used either, because of the aforementioned problems in ΔVS estimations for karst aquifers. In practice, Equation (3) is further reduced to Equation (2), assuming large time scales of decades or several years for *RC* calculation, such that the change in groundwater storage became negligible in comparison to V_{Sp} and *VP*. In this way, mean annual *RC* ($RC_{\bar{Y}}$) is calculated (see Soulios, 1984).

$RC_{\bar{Y}}$ just provides a rough estimation of the mean annually recharged water to an aquifer on a long-term scale and is not of practical use for groundwater management. Instead, determination of *RC* during a hydrological year, RC_Y, or even a shorter time scale (monthly RC_M or daily RC_D) is of interest for practical applications (see Bonacci, 2001; Bonacci et al., 2006; Jukić and Denić-Jukić, 2004, 2009; Jemcov and Petric, 2009; Fiorillo et al., 2015). However, at a short time scale of a year (or a month), the amount of change in groundwater storage is likely not negligible in comparison to the rest of the parameters in the *RC* calculation. Consequently, *RC* might be overestimated or underestimated by Equation (2) by neglecting ΔVS when ΔVS is notably negative or positive, respectively.

Bonnaci (2001) estimated the RC_M for Gradole aquifer in Dinaric karst, during 1987 to 1998. Using Equation (2), RC_M of greater than 1 or even infinite was calculated for 24.3% of the cases with little or no precipitation during the month; moreover, it was believed, doubtfully, that the RC_{July} was higher than the RC_{June}. Consequently, Bonacci (2001) modified the RC_M calculation by changing the V_{Sp} definition in the Equation (2). He extrapolated the recession curves during each month to the following three months using a master recession curve, MRC, of Maillet's (1905) simple exponential function. Then, V_{Sp} of a month was calculated as the area bounded between two successive extrapolated MRCs, up to the following three months (see Bonacci, 2001). This way, the effect of change in groundwater storage was partly covered, and some more reasonable RC_M could have been estimated. Nevertheless, Bonacci (2001) calculated the RC_Y using Equation (2), without any further modification to V_{Sp}, in order to consider ΔVS. The reported RC_Ys covered a wide range of 0.356 to 0.763.

In surface-water hydrology, MRC extrapolations are also used for groundwater recharge estimation for stream catchments. The method, so-called MRC displacement (Rorabaugh, 1964), is based on the theory of upward shifting in the streamflow recession curve as a result of recharge events (Rutledge, 1998). The procedure of the method can be summarized as follows. First, a recession curve before and after a streamflow peak is selected and extrapolated to the following time according to the stream's simple exponential MRC. Then a critical time after the peak is calculated, by which the runoff is assumed to have ceased. Finally, groundwater recharge volume is estimated as two times the area bounded between successive MRCs after the critical time (see Rorabaugh, 1964; Rutledge, 1998 for formulas and detailed explanations).

Bonacci (2001) used the simple exponential MRCs in his work that are also widely used in surface-water hydrology. The simple exponential function, known as Maillet's formula, was derived for recession curves by approximate solution of the diffusion equation in porous media (see Maillet, 1905). The formula expresses drainage from a linear reservoir and is usually used to describe the baseflow part of karst spring recession curves, but it commonly fails to match up the entire curves from high to low flow. Semi-log plots of recession curves, log discharge versus time, for many karst springs exhibit two or more linear segments with decreasing order of slopes (e.g. Torbarov, 1976; Milanović, 1976; White, 1988; Baedke and Krothe, 2001; Ford and Williams, 2007), suggesting piecewise exponential functions for the fitting. Segmentation of recession curves has been linked up with

changes in flow regimes (Milanović, 1976; Baedke and Krothe, 2001, Doctor and Alexander, 2005; Doctor, 2008), catchment area, and effective porosity of the declining saturated zone (Bonacci, 1993; Fiorillo, 2011). MRC is here considered as a piecewise-exponential function, namely the segmented exponential MRC (Kavousi and Raeisi, 2015) composed of n segments ending at times $t_i = 1$ to n with n recession coefficients $\alpha_i = 1$ to n, and base discharges $Q_{0(i)} = 1$ to n, that is, exponential segment discharges at time $t = 0$ for each segment.

$$Q_t = \begin{cases} Q_{0\,(1)}\, e^{-\alpha_1 t} & t \le t_1 \quad , \quad Q_t \ge Q_1 \\ Q_{0\,(2)}\, e^{-\alpha_2 t} & t_1 < t \le t_2 \quad , \quad Q_1 > Q_t \ge Q_2 \\ \cdot \\ \cdot \\ \cdot \\ Q_{0\,(i)}\, e^{-\alpha_i t} & t_{i-1} < t \le t_i \quad , \quad Q_{i-1} > Q_t \ge Q_i \\ \cdot \\ \cdot \\ \cdot \\ Q_{0\,(n-1)}\, e^{-\alpha_{n-1} t} & t_{n-2} < t \le t_{n-1} \quad , \quad Q_{n-2} > Q_t \ge Q_{n-1} \\ Q_{0\,(n)}\, e^{-\alpha_n t} & t > t_{n-1} \quad , \quad Q_t < Q_{n-1} \end{cases} \tag{5}$$

Equation (5) implies that the MRC is represented with n straight lines (usually two or three) with slopes of $-\alpha_i = 1$ to n on a semi-log scale (see Figure 1a for a simple example).

In this study, a novel method is proposed to estimate RC_E for an isolated precipitation event that induced a distinctive hydrograph rise and recession. The method is based on the hydrograph decomposition using segmented exponential MRCs (Kavousi and Raeisi, 2015) and is capable of accurate RC_E estimation for a karst aquifer with no-flow boundaries, draining by a single spring. The only required data are the spring's long-term discharge and the spatiotemporal distribution of precipitation over the catchment.

EVENT-BASED RC_E ESTIMATION METHOD

Recharge water from a precipitation event is stored within an unconfined karst aquifer, gradually discharging via the spring, throughout a long period during and after the event. Strictly speaking, each effective precipitation event induces a prolonged individual spring hydrograph, and the individual hydrographs overlap to make the observed spring hydrograph. Thus the observed hydrograph is regarded here as a composite hydrograph that is a combination of several consecutive individual hydrographs induced by numerous previous precipitation events.

The volume of water discharged as an individual hydrograph is, in fact, the water volume of the relevant event that entered the aquifer to be eventually discharged from the spring. Therefore, decomposition of an observed composite hydrograph to its individuals will give the recharge water volume of the individual events. Decomposition of observed composite hydrograph is here performed using the MRC displacement method of Kavousi and Raeisi (2015), applying the following steps:

1. The long-term composite hydrograph is measured.
2. The segmented exponential MRC of the spring is constructed using all prior recorded recessions, from high- to low-flows (see Figure 1a).

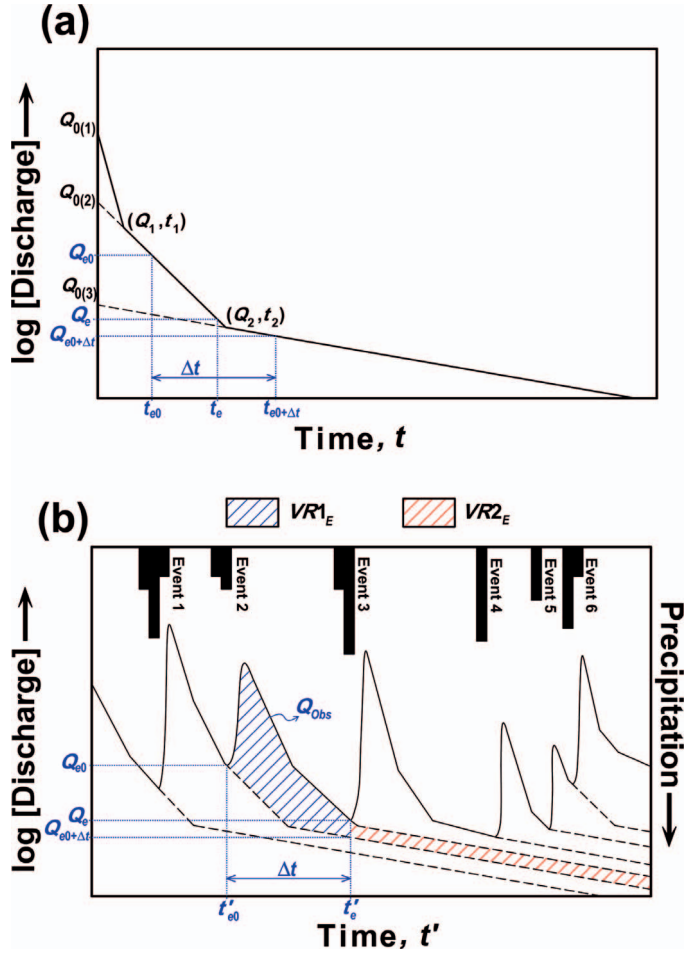

Figure 1. (a) A schematic master recession curve composed of three exponential segments, with parameters in black. (b) Hypothetical precipitation events and resulting observed hydrograph that is decomposed to individual hydrographs by extrapolations based on the MRC in (a) (dashed lines). Recharged water volume (VR_E) for the second event is indicated with hatched areas. $VR1_E$ (red hatched area) is discharged during the observed part of second individual hydrograph, but $VR2_E$ (blue hatched area) is stored in the aquifer and discharged afterward, theoretically until infinite time. Parameters for the second event are specified in blue in both (a) and (b). Note that the vertical axis in both graphs is logarithmic, so that the observed hydrograph and MRCs are represented as straight lines; the base of the vertical axes is not zero.

3. The observed composite hydrograph is decomposed to its individuals by extrapolation of recession curves, by means of the MRC. For this reason, the MRC is horizontally displaced at the end of composite hydrograph recessions (see Kavousi and Raeisi, 2015). Figure 1b shows a hypothetical composite hydrograph that is decomposed to its individuals corresponding to different precipitation events, using the segmented exponential MRC presented as Figure 1a.
4. The event's recharged water volume VR_E is the area bounded by the relevant individual hydrograph, that is calculated by integration, as follows (see Figure 1b):

$$VR_E = \left(\int_{t'_{e0}}^{t'_e} Q_{Obs}\, dt' - \int_{t_{e0}}^{t_{e0}+\Delta t} Q_t\, dt \right) + \left(\int_{t_e}^{\infty} Q_t\, dt - \int_{t_{e0}+\Delta t}^{\infty} Q_t\, dt \right) \quad (6)$$

where, t and t' are the time-scales for the MRC and measured hydrograph; t'_{e0} and t'_e are the times of rise beginning and recession ending on the observed part of the individual hydrograph, respectively; Q_{Obs} and Q_t are the observed discharge and MRC discharge at time t, respectively; t_{e0} and t_e are the MRC-equivalent times for the observed discharges Q_{e0} (at time t'_e) and Q_e (at time t'_{e0}), respectively; and Δt is the time difference between t'_e and t'_{e0} (or t_e and t_{e0}). Figure 1 graphically presents VR_E and the abovementioned parameters on a schematic hydrograph and MRC.

VR_E is the fraction of total precipitation over the catchment that entered the aquifer and is eventually discharged from the spring. Some portion of this water volume was discharged during the observed part of individual hydrograph ($VR1_E$, the blue hatched area in Figure 1b), but some extrapolated portion would be gradually discharged later ($VR2_E$, that is indicated as the red hatched area in Figure 1b). The two terms of Equation (6) are $VR1_E$ and $VR2_E$, respectively.

Providing VR_E , recharge coefficient of the event (RC_E) can be estimated for an aquifer with no-flow boundaries draining by a permanent spring, Equation (1) becomes

$$RC_E = \frac{VR_E}{VP_E} = \frac{VR1_E + VR2_E}{VP_E} \quad (7)$$

where VP_E is the estimated volume of total precipitation over the catchment during the event. The no-flow boundaries may coincide with impermeable layers or groundwater divides. Lots of karst aquifers in the Zagros Mountain Range, and also in other karst regions worldwide, meet the condition.

Equation (7) estimates RC_E of an isolated precipitation event that induced a single distinct hydrograph rise followed by a peak and a recession. However, RC_E for a combination of precipitation events can also be estimated by combining individual hydrographs and precipitation events, treating all of them as a single event, so in case some individual hydrographs or precipitation events are indistinguishable, one would be able to calculate the RC_E of the overall collection of events. This is likely to be the case when the precipitation is mostly snow or the time intervals between rainy periods are short. By combining individual hydrographs for precipitation events during a hydrological year, accurate estimation of RC_Y would also be possible.

Equation (6) can be rearranged to give

$$VR_E = \left(\underbrace{\int_{t'_{e0}}^{t'_e} Q_{Obs}\, dt'}_{V_E} \right) + \left(\underbrace{\int_{t_e}^{\infty} Q_t\, dt}_{VS_E} - \underbrace{\int_{t_{e0}}^{\infty} Q_t\, dt}_{VS0_E} \right) \quad (8)$$

where, V_E is the net spring outflow during the measured part of the individual hydrograph, and VS_{0E} and VS_E are the dynamic reservoir volumes (see Burdon and Papakis, 1963; Ford and Williams, 2007, p. 179) at the beginning and end of the measured part of the hydrograph, respectively. VS_E and $VS0_E$ can be calculated by the following equations, replacing Q_t with the MRC Equation (5):

$$VS_E = \int_{t_e}^{\infty} Q_t dt \;\Rightarrow\; VS_E = \begin{cases} C\left(\dfrac{Q_e - Q_i}{\alpha_i} + \dfrac{Q_i - Q_{i+1}}{\alpha_{i+1}} + \cdots + \dfrac{Q_{n-2} - Q_{n-1}}{\alpha_{n-1}} + \dfrac{Q_{n-1}}{\alpha_n} \right) & Q_e > Q_{n-1} \\ C\left(\dfrac{Q_e}{\alpha_n} \right) & Q_e < Q_{n-1} \end{cases} \quad (9)$$

$$VS0_E = \int_{t_{e0}}^{\infty} Q_t dt \;\Rightarrow\; VS0_E = \begin{cases} C\left(\dfrac{Q_{e0} - Q_i}{\alpha_i} + \dfrac{Q_i - Q_{i+1}}{\alpha_{i+1}} + \cdots + \dfrac{Q_{n-2} - Q_{n-1}}{\alpha_{n-1}} + \dfrac{Q_{n-1}}{\alpha_n} \right) & Q_{e0} > Q_{n-1} \\ C\left(\dfrac{Q_{e0}}{\alpha_n} \right) & Q_{e0} < Q_{n-1} \end{cases} \quad (10)$$

where, C is the unit conversion factor, equal to 86400 when discharge and recession coefficient units are $m^3 \cdot s^{-1}$ and d^{-1}, respectively.

The second term in the right side of Equation (8) is the difference between VS_E and $VS0_E$, that is the change in groundwater storage due to the event (ΔVS_E), according to Raeisi (2008)

$$\Delta VS_E = VS_E - VS0_E \tag{11}$$

ΔVS_E would be positive, if the baseflow discharge level at the beginning of measured part of individual hydrograph is higher at its end, or negative if lower.

Considering Equations (8) and (11), Equation (7) can be written as

$$RC_E = \frac{V_E + \Delta VS_E}{VP_E} \tag{12}$$

Equation (7) is essentially Equation (3) adapted for an event. Thus the proposed method estimates the RC in a short interval of an event RC_E, taking change in groundwater storage into account. Ignorance of a positive or negative ΔVS in an RC_E calculation causes under- or over-estimation.

Application: Sheshpeer Karst Aquifer, Iran

To examine the proposed method of RC_E estimation, Sheshpeer karst aquifer, located in the Zagros Mountain Range, 80 km northwest of Shiraz, south-central Iran, was used as a case study. The Zagros orogenic belt is comprised of three parallel northeast-southwest trending tectonic subdivisions, the Urumieh-Dokhtar Magmatic Assemblage, the Zagros Imbricate Zone, and the Zagros Fold-Thrust Belt (Alavi, 2007). Lithostratigraphic units and tectonic settings of these subdivisions are fairly well described by James and Wynd (1965), Falcon (1974), Stöcklin and Setudehnia (1971), and Alavi (1994, 2007). The Sheshpeer karst aquifer is situated at the border of the Zagros Fold-Thrust Belt and the Imbricate Zone. Extensive studies, including hydrology (Azizi, 1992; Porhemat, 1993), hydrogeology (Pezeshkpour, 1991; Karami, 1993; Raeisi et al. 1993, 1999; Eftekhari, 1994; Raeisi and Karami, 1996; Kavousi, 2008; Raeisi, 2008, 2010; Kavousi and Raeisi, 2015), geomorphology (Kasaeyan, 1990; Marandi, 1990), structural geology (Agha-Amiri, 1993), geophysics (Nakhaei, 1992) and remote sensing (Ebadian, 2002) were carried out on the Sheshpeer catchment.

Hydrogeological Setting

The Sheshpeer aquifer is composed of the calcareous Sarvak Formation (Albian-Turonian) in the northern flank of the Barm-Firooz and Gar anticlines and a portion of the Barm-Firooz anticline southern flank (Raeisi et al., 1993; Raeisi, 2008; Fig. 2). The anticlines are extended in the general direction of the Zagros Mountain Range and are connected by a saddle-shaped plunge. The exposed cores of the anticlines are mainly composed of the karstic Sarvak Formation,

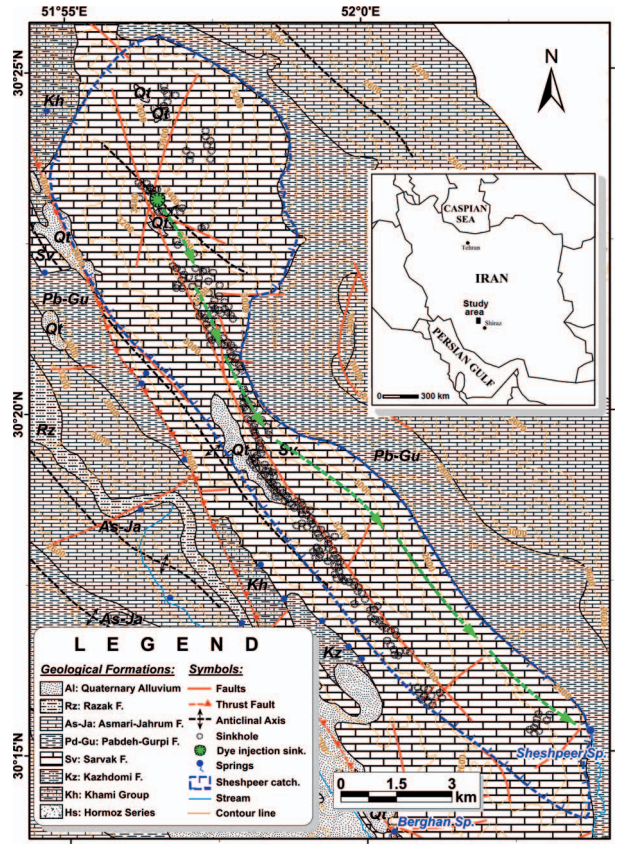

Figure 2. Hydrogeological map of the study area. The green arrows represent the most likely flow path from the dye-injection sinkhole to the spring. The catchment area for the Sheshpeer Spring is outlined in blue.

underlain and overlain by impermeable shale and marl layers of the Kazhdomi (Albian-Cenemonian) and Pabdeh-Gurpi (Santonian-Oligocene) Formations, respectively.

The most important tectonic feature is a major northwest-southeast trending thrust fault (Fig. 2). The northern flank of the anticlines has been brought up by tectonic stresses, and the southern flank has been brecciated so that it is either completely removed or crops out as large rockslide blocks. Several normal and strike-slip faults are also present. The overall tectonic setting of the area has produced suitable conditions for extensive karstification (Raeisi and Karami, 1996, 1997; Raeisi, 2010).

The karst features of the study area are grikes, karrens, small caves, sinkholes, and springs. The most important karst feature is the presence of 259 sinkholes in the Gar and Barm-Firooz Mountains, aligned in a narrow zone on top of the northern flanks from the northern end of the catchment to near of the Sheshpeer Spring (Fig. 2). The sinkholes are primarily of the collapse-doline type and evidently aligned along the strike direction of longitudinal faults. The biggest cave in the catchment has a length of 20 m and is located along a fracture (Raeisi, 2010). Out of twelve springs emerging from the Sarvak Formation in the Gar and Barm-Firooz anticlines, only

Sheshpeer Spring, with a mean annual discharge of 3247 L s^{-1}, is located on the northern flanks (Fig. 2). Berghan Spring is the largest spring in the southern flanks and has a mean annual discharge of 632 L s^{-1}. The mean annual discharges for the rest of the springs range from 1.41 to 68.34 L s^{-1} (Raeisi and Karami, 1996). The outlet level of the Sheshpeer and Berghan Springs are 2335 and 2145 masl, respectively.

All boundaries of the Sheshpeer aquifer are physically no-flow, and all recharged water only emerges via the Sheshpeer Spring (Pezeshkpour, 1991). Groundwater balance indicates a catchment area of about 81 km^2 for the Sheshpeer Spring (Raeisi et al., 1993).The catchment area is in accordance with the area bounded by geological formations, and has a mean ground elevation of 2998 masl. The aquifer boundaries are based on the following observations (Raeisi, 2008): (1) The northern flank of the Gar and Barm-Firooz anticlines have been brought up by tectonic stresses, such that the aquifers of the northern and southern flanks have been disconnected by the underlying impermeable Khazdumi Formation that crops out in some portions of the anticline core (Fig. 2). Tracer tests have confirmed this hydrogeological disconnection. (2) The northwest and northeast boundaries of the catchment are surrounded by the impermeable Pabdeh-Gurpi Formations. Groundwater flow through the Sarvak Formation under these formations is not possible, because karstification is not expected to occur under an 800 m thick layer of the Pabdeh-Gurpi Formations, there are no outcrops of the Sarvak Formation in adjacent parallel anticlines, and tracer tests confirmed the hydrogeological disconnection with the springs of the adjacent anticlines. (3) Sinkholes are only located in the catchment of Sheshpeer Spring, and the uranine tracer injected in a sinkhole 18 km away only emerged from this spring.

Hydrological Data

The hydrograph of the Sheshpeer Spring was measured for almost three hydrological years from 1990–1991 to 1992–1993 (Pezeshkpour, 1991; Karami, 1993; Eftekhari, 1994). Kavousi and Raeisi (2015) reconstructed ten years of daily discharge data for the spring by means of a multiple time-series regression model (Phillips and Durlauf, 1986) based on the recorded discharge at two hydrometric stations downstream of the spring and also the precipitation at a climatological station. The segmented exponential MRC determined for the spring based on the thirteen-year hydrograph is (Kavousi and Raeisi, 2015)

$$Q_t = \begin{cases} 9469.5\ e^{-0.0482t} & t \le 5.87 \quad , \quad Q_t \ge 7137.2 \\ 7725.2\ e^{-0.0135t} & 5.87 < t \le 38.26 \quad , \quad 7137.2 > Q_t \ge 4612.3 \\ 6607.8\ e^{-0.0094t} & 38.26 < t \le 105.24 \quad , \quad 4612.3 > Q_t \ge 2457.9 \\ 3529.7\ e^{-0.0034t} & 105.24 < t \le 172.31 \quad , \quad 2457.9 > Q_t \ge 1951.6 \\ 2419.0\ e^{-0.0012t} & t > 172.31 \quad , \quad Q_t < 1951.6 \end{cases} \tag{13}$$

where the units of discharge, recession coefficient, and time are L s^{-1}, d^{-1}, and d, respectively.

The climate type of the study area is Mediterranean, characterized by mild, dry summers and cool, wet winters. Precipitation events are normally isolated, occurring during late fall, winter, and early spring. There is no precipitation during the rest of the year. The precipitation in winter is mainly in the form of snow.

To estimate spatial distribution of precipitation over the Sheshpeer catchment, ten precipitation-gauge stations were selected. Stations were selected based on their availability and locations parallel to the northwest-southeast general direction of Zagros Mountain Range and also Mediterranean frontal systems, which are the main source of precipitation in the region (see Sabziparvar et al., 2015). Figure 3 shows the spatial distribution of the stations on a Triangular Irregular Network map that highlights the topographical features. Preliminary studies indicated that the short-term precipitation timing and amount at stations far in the northeast and southwest of the Sheshpeer catchment, perpendicular to the general direction of Zagros and also Mediterranean frontal systems, are not similar to those of the stations close to the catchment. Therefore, stations far in the northeast and southwest of the Sheshpeer catchment were not considered, since they are not representative for the catchment precipitations. Alijani (2008) proved that the Zagros Mountains block the moist air masses from entering central Iran to the northeast and east.

Shekarak station, which is located on the Barm-Firooz southern flank in the vicinity of the Sheshpeer catchment, was installed by Shiraz University to measure climatological parameters and facilitate water-balance calculations. Although the station is the most representative for the Sheshpeer catchment, it was only active during hydrological year 1991–1992. Precipitation data at the Komehr and Sepidan stations were also not available during 1991 to 1993, and thus, were reconstructed based on the data from adjacent Chubkhale and Berghan stations. The linear correlation coefficients of precipitation amounts for Komehr with Chubkhale and Sepidan with Berghan stations were 0.93 and 0.98, respectively. These are very close to 1, showing the accuracy available for reconstructing the missing data.

Results and Discussions

The proposed method is able to estimate a recharge coefficient RC_E for an isolated precipitation event that induced a distinctive hydrograph rise and recession and also for a

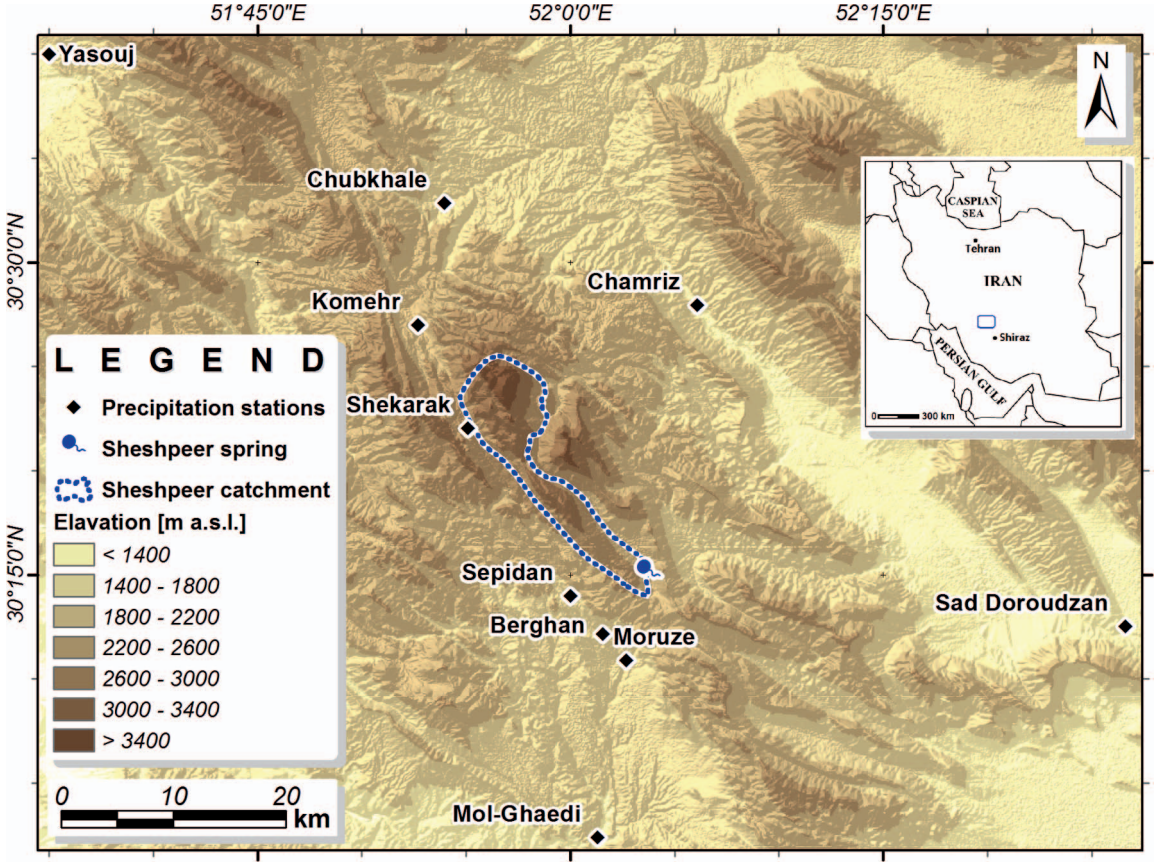

Figure 3. Spatial distribution of precipitation gauge stations on the TIN map. Sheshpeer Spring and its catchment are indicated.

combination of precipitation events such as RC_Y for a hydrological year. For the Sheshpeer catchment, two RC_Y and nine RC_E can be calculated.

Sheshpeer aquifer has a single spring and all its boundaries are no-flow. Measured spring discharge comprises hydrographs of two hydrological years 1990–1991 and 1991–1992 and some part of the hydrological year 1992–1993. Therefore RC_Y can be calculated just for hydrological years 1990–1991 and 1991–1992. But the overall hydrograph can be decomposed to nine individual hydrographs by means of Equation (13), the spring's segmented exponential MRC (see Fig. 4). It should be pointed out that the first individual hydrograph of the hydrological year 1992–1993 (92-93-1 in the figure) consisted of some spring discharge with indistinguishable precipitation events; therefore, the overall precipitation during the series of events is treated as the VP_E, and the overall minor peaks are treated like a single individual hydrograph in the VR_E calculations.

Cumulative precipitation amount corresponding to each individual hydrograph or hydrological year was determined at all gauging stations and was used to estimate the total precipitation over the catchment. Multilinear-regression models were used to estimate the spatial distribution of precipitation over the catchment. The MLR models have previously been used for mountainous regions (e.g., Marquínez et al., 2003; Naoum and Tsanis, 2004; Um et al., 2010). Cumulative precipitation P and geographical position altitude Z, latitude X, and longitude Y of the stations were used as response and predictor variables, respectively. Different alternative MLR models were examined by stepwise regression to find the statistically most robust model. The best MLR models for the second event of hydrological year 1991–1992, all events of hydrological year 1992–1993, and the long-term period of 1972 to 2012, utilize Z and Y, Z only, and Z and X, respectively, while the best MLR models for the rest are dependent on all three predictors. Table 1 presents the MLR models for the events, the hydrological years, and also the long-term mean from 1972 to 2012. Adjusted R^2, which is an indicator of explanatory power of models, is above 0.7 for all the MLR models, except for two events in the hydrological year 1992–1993.

Spatial distribution of precipitation over the catchment was calculated with GIS, using the corresponding MLR model and the digital elevation model of the region. The calculated mean precipitations over the catchment, P_{Mean}, are also listed in Table 1. Figure 5 shows the spatial distribution of mean annual precipitation over the catchment for the long-term period of 1972 to 2012. The minimum, mean, and maximum

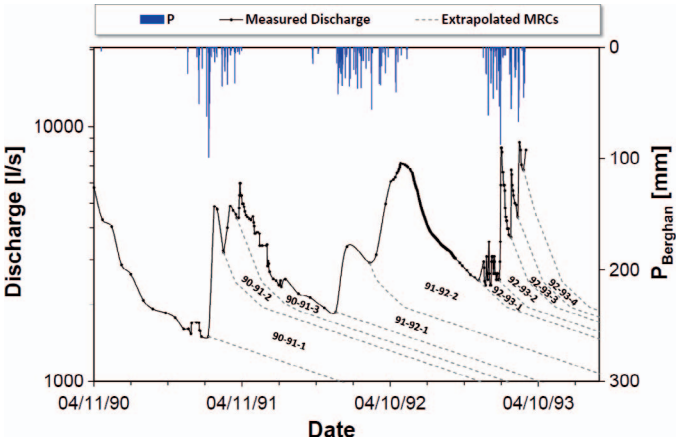

Figure 4. Sheshpeer Spring hydrograph and the corresponding precipitation hyetograph at the Berghan station. Extrapolated master recession curves (dashed lines) are appended at the end of observed recessions, decomposing the measured hydrograph into components. The number of the individual event hydrograph for each hydrological year is indicated; for example, 91-92-2 is the second individual hydrograph during hydrological year 1991–1992; these numbers are used in Table 1 and Figure 6. Note that the vertical axis for discharge is logarithmic.

precipitation over the catchment for this period of years are 887.6, 1256.3, and 1628.7 mm y^{-1}, respectively. It should be pointed out that the MLR model of long-term precipitation has not been used for the *RC* calculations in this study, but is just given to provide a sense of precipitation distribution over the catchment, which is highly dependent on its mountainous landscape.

The amount of V_{Sp}, *VS*, *VS0*, ΔVS, *VR*1, *VR*2, and *VR* for the hydrological years and also the nine events were calculated using Equations (6) and (8) to (11) and are shown in Table 2. The changes in groundwater storage for all the hydrological years and events are positive, except for the third and second events of the hydrological years 1990–1991 and 1991–1992, respectively. *VP* over the Sheshpeer catchment are also computed by GIS (Table 2). Finally, *RC* of the events and hydrological years were estimated using Equations (7) or (12) and are shown in the Table 2 and Figure 6.

The calculated RC_E has a wide range of variation, and the RC_E for the last event of each hydrological year is larger than the preceding RC_E during the same year. $RC_{91\text{-}92\text{-}2}$ even goes slightly beyond the feasible range to 1.08. RC_E for the hydrological year 1992-1993 are also smaller than those of former hydrological years. The fact that the Sheshpeer catchment is mountainous and most of the precipitation is snow is most likely the main reason for this behavior. Porhemat and Raeisi (2001) reported that about 65% of the total precipitations was snow in hydrological year 1991-1992. Field observations show that snowmelt happens on the peak of Gar and Barm-Firooz Mountains even in May–June. Therefore, some portion of snowpack from initial events in a hydrological year could melt and recharge the aquifer during the following events' period. So, according to Equation (7), the RC_E is underestimated for early snowfall events, since the VP_E is overestimated, as all equivalent water of early snow precipitation does not enter the aquifer during the event. This phenomenon is the main reason for RC_E underestimation for early snowfall events, as well as RC_E overestimation for the subsequent events. On other hand, the antecedent moisture condition was definitely lower for the initial events of each hydrological year, requiring some precipitation water to compensate for the soil-moisture deficit, which in turn reduces the VR_E *and* RC_E for the initial events of each year. Consequently, the calculated RC_Ys can be considered as more accurate than RC_Es for our case study.

Porhemat (1993) directly measured runoff and snow storage and estimated snow melt and sublimation by regionally calibrated equations in three small catchments near the Shekarak station, near the Sheshpeer catchment (see Fig.

Table 1. Calculated multilinear-regression models for the events, hydrological years, and long-term average, together with the adjusted R^2 for the fit and the resulting mean precipitation over the Sheshpeer catchment.

Hydrological Year	Event/Period	Regression Formula	Adjusted R^2, %	Catchment P_{Mean}, mm
90–91	1	$P_{90\text{-}91\text{-}1} = 21368 + 0.45455Z - 359.3X - 105.0Y$	89.5	876.3
	2	$P_{90\text{-}91\text{-}2} = 786 + 0.210704Z - 95.21X + 128.52Y$	92.6	371.3
	3	$P_{90\text{-}91\text{-}3} = -4989 + 0.17508Z - 34.07X + 215.26Y$	94.7	298.5
	Overall	$P_{90\text{-}91} = 17165 + 0.8404Z - 488.6X + 238.7Y$	93.3	1542.8
91–92	1	$P_{91\text{-}92\text{-}1} = 6136 + 0.34067Z - 258.4X + 232.8Y$	79.6	794.6
	2	$P_{91\text{-}92\text{-}2} = -13740 + 0.52921Z + 436.6Y$	89.6	1091.9
	Overall	$P_{91\text{-}92} = -3488 + 0.8627Z - 315.1X + 631.4Y$	86.0	1889.5
92–93	1	$P_{92\text{-}93\text{-}1} = -105.66 + 0.13546Z$	69.6	302.4
	2	$P_{92\text{-}93\text{-}2} = -157.33 + 0.23976Z$	75.9	564.9
	3	$P_{92\text{-}93\text{-}3} = -114.28 + 0.12462Z$	66.2	261.1
	4	$P_{92\text{-}93\text{-}4} = -191.36 + 0.20766Z$	78.5	434.2
1972–2012	41 years	$P_{1972\text{-}2012} = 17030 + 0.5215Z - 333.7X$	79.6	1256.3

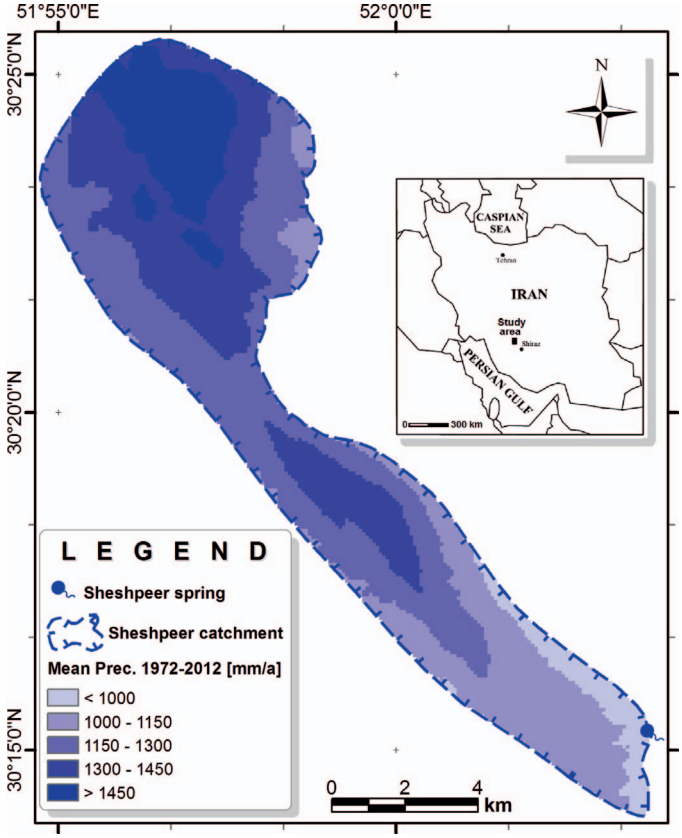

Figure 5. Spatial distribution of mean annual precipitation over the Sheshpeer catchment during 1972 to 2012 based on multilinear regression from precipitation gauges in the vicinity of the catchment.

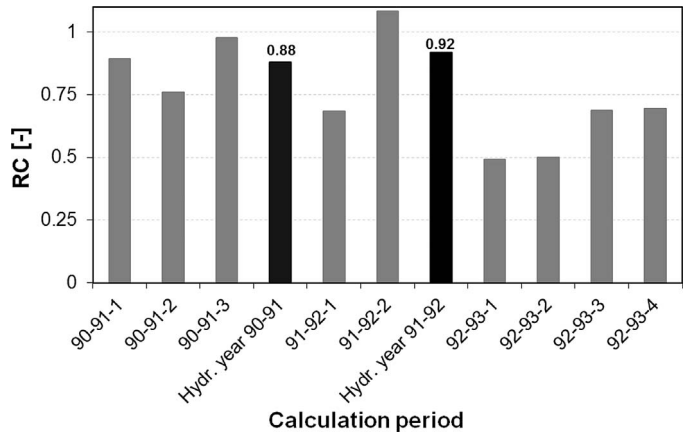

Figure 6. Estimated recharge coefficient RC_E for events (gray bars) and hydrological years RC_Y (black bars) for the Sheshpeer aquifer during hydrological years 1990–1991 to 1992–1993.

3). The average sublimation, runoff, and recharge from snowmelt for the selected catchments during hydrological year 1991-1992 were estimated to be 5.2, 25.3, and 69.5 percent, respectively. Porhemat stressed that in the Sheshpeer catchment, which is covered by 259 sinkholes, the *RC* could be as high as ~95%, so that almost all the snow contributed direct recharge. The estimated RC_Ys for the 1990–1991 and 1991–1992 years found in the current study are 88 and 92 percent, respectively, which are very close to the estimated value by Porhemat. The small discrepancy can be caused by several things. The multilinear-regression model we used might have incorrectly estimated the precipitation. The RC_Y in the current study accounts for all the processes that prevented precipitation from reaching the saturated zone. Porhemat considered sublimation as the only alternative to recharge in the catchment during snow-melting. Although recharge of the Sheshpeer aquifer is limited to a 5 or 6 month wet season, the catchment is a natural pasture (Raeisi and Karami, 1996) and almost 35% of the karstic Sarvak Formation is covered by soil (Karami, 1993). Therefore evapotranspiration might account for some water loss, especially during growing season. Spring discharge was measured weekly or even monthly in some periods, especially during baseflow, which might have

Table 2. Estimated *RC* for events (RC_E) and hydrological years (RC_Y) calculated in this study. For the definitions of the parameters entering into the result, see the text. RC^*_Y is the annual RC_Y calculated neglecting ΔVS_Y.

Year	Event	Duration	*VS*, Mm^3	*VS*0, Mm^3	ΔVS, Mm^3	V_{Sp}, Mm^3	*VR*1, Mm^3	*VR*2, Mm^3	*VR*, Mm^3	*VP*, Mm^3	*RC*	RC^*_Y
90–91	1	12/21/1990 – 02/19/1991	154.9	104.0	50.9	12.5	7.7	55.7	63.3	71.0	0.89	···
	2	02/20/1991 – 03/22/1991	165.7	154.9	10.9	11.9	4.3	18.5	22.8	30.1	0.76	···
	3	03/23/1991 – 11/30/1991	129.7	165.7	−36.0	59.6	11.0	12.5	23.6	24.2	0.98	···
	···	Hydrologic year	450.3	424.6	25.7	84.0	23.0	86.7	109.7	125.0	0.88	0.67
91–92	1	12/1/1991 – 01/11/1992	152.3	129.7	22.5	21.6	8.7	35.4	44.1	64.4	0.69	···
	2	01/12/1992 – 11/14/1992	148.4	152.3	−3.9	100.3	55.7	40.7	96.5	89.1	1.08	···
	···	Hydrologic year	300.6	282.0	18.6	121.9	64.4	76.1	140.6	153.1	0.92	0.80
92–93	1	11/15/1992 – 12/20/1992	148.5	148.4	0.1	12.0	2.0	10.1	12.0	24.5	0.49	···
	2	12/21/1992 – 01/30/1993	159.3	148.5	10.8	12.1	6.4	16.5	22.9	45.8	0.50	···
	3	01/31/1993 – 02/18/1993	166.1	159.3	6.8	7.7	2.7	11.8	14.5	21.1	0.69	···
	4	02/19/1993 – 03/04/1993	181.7	166.1	15.7	8.7	3.7	20.7	24.4	35.2	0.69	···

reduced the accuracy of V_{Sp} and consequently RC in the current study.

The effect of ignoring change in groundwater storage in the RC calculations was also investigated. RC^*_Y in Table 2 is RC_Y neglecting change in groundwater storage, that is, considering $\Delta VS_Y = 0$. Ignoring ΔVS_Y causes RC^*_Y to be less than RC_Y; the fact that the ΔVS_Y is positive for both hydrological years is the reason for this result. RC^*_Y would be greater than RC_Y if ΔVS_Y was negative.

Conclusions

Knowledge of recharge coefficient RC during a hydrological year or a shorter time scale is of central importance in karst water evaluation and exploitation studies. Determination of the change in groundwater storage ΔVS is a prerequisite for a reliable estimation of RC on short time scales, especially in case ΔVS is significant in comparison to the other parameters in the RC equation. A new method is proposed to estimate event-based RCs for a single or a combination of precipitation events with distinctive hydrograph spikes. The basis of the method is decomposition of the measured hydrograph to its individual components by displacement of a segmented exponential master recession curve to the end of recession curves. The method is applicable for karst aquifers with no-flow boundaries draining by a permanent spring, where the overflow and underflow components are absent or negligible.

It was proved that the estimated event-based RC considers the event ΔVS to be central to calculating event-recharged water VR. However, it should be pointed out that the definition of VR in the proposed method is quite different from that in the literature. The proposed method considers VR of an event that discharges for infinite time, not during a fixed time interval. Some portion of the event's recharge is discharged during the event's peak in the measured hydrograph, but some portion, which is extrapolated, remains in the aquifer to supply the spring at later times.

Event and annual RCs for the Sheshpeer karst catchment in Iran were calculated for nine isolated precipitation events and two hydrological years. Since most of the precipitation over the catchment is snow, calculated RCs for hydrological years were more accurate and more in accord with former field studies. It was shown that the annual RCs would be underestimated by over ten percent if the positive ΔVS during the study period was ignored.

In surface-water hydrology, groundwater recharge is estimated as an event-based process (e.g., Rutledge, 1998). Karst aquifers may contain well-developed conduit systems that are sometimes regarded as underground rivers (e.g., White and White, 1989), hence calculation of event-based RC for karst catchments appears to be more reasonable than its calculation for fixed, short time intervals such as months. The latter would be especially unacceptable in arid and semi-arid regions, where the precipitation events are limited to certain periods, although permanent springs are discharging without any interruption.

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INVENTORY OF BATS AND CULTURABLE PROTEOBACTERIA FROM CUEVA LAS ESCALERAS (TÁCHIRA, VENEZUELA): EVIDENCE OF POTENTIAL HUMAN HEALTH RISKS

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Abstract: Caves are common roosts used by bats as permanent places for the settlement of stable colonies because caves are minimally affected by environmental conditions. Although Venezuela is a cave-rich country, with almost 700 caves formally described in the 2006 speleological inventory, these are still unknown in terms of their whole biological composition. Particular groups of animals have been described for some of these caves, but a list of culturable proteobacteria found inside any cave in the country has never been reported. During an inventory of bats in a small cave within a recreational park, we decided to perform a first look at the diversity of its bacteria and determine whether some of them have been reported as present in other caves, other environments, or even bats. Identification of bacteria was possible by amplifying and sequencing 16S rRNA genes from cultivated samples. Twenty-three clonal samples of bacteria from Cueva Las Escaleras (Pregonero, Táchira state, Venezuela) were obtained and analyzed. All but one sample belonged to the phylum Proteobacteria, and most of them have been reported to be potentially pathogenic to humans. From these identified bacteria, some (*Achromobacter denitrificans*, *Proteus mirabilis*, and *Microvirgula aerodenitrificans*) showed resistance to five widely used antibiotics. These results are important, as some bacteria found inside this cave may contaminate the water that flows from the cave and runs to the stream that crosses the park, forming ponds that surround lunch places. We detected a potential threat for public health at local and regional scales because many visitors use this water for drinking and washing hands and faces, and the cave itself is used for urination, defecation, and sexual activities.

Introduction

Venezuela is a cave-rich country. De Bellard Pietri (1966), in a review of 713 caves of the country, classified them according to geographical location and provided the following figures: 333 in the central region (north) of the country, 91 in eastern Venezuela, 227 in the west, 32 south to River Orinoco, and 30 in the insular region. There are no caves in the Llanos (central plains). The number of caves later increased to 989 as reported by the same author in the *Atlas Espeleológico de Venezuela* (de Bellard Pietri, 1969). However, only 650 Venezuelan caves were officially included in a recent speleological inventory (Urbani et al., 2006). The vast majority of caves in Venezuela originated from dissolution. In terms of their biodiversity, Venezuelan caves display a rich array of different species of animals, particularly insects and arachnids. Some caves can contain up to a hundred different animal taxa (Galán and Herrera, 2006); among vertebrates, Venezuelan caves host a wide range of fishes, birds, and bats, the latter mainly from the families Emballonuridae, Phyllostomidae, Mormoopidae, Desmodontidae, Natalidae, Furipteridae, Vespertilonidae, and Molossidae (Galán et al., 2008, 2009; Galán and Herrera, 2006). Caves are commonly used as roosts by bats, as they are places for the establishment of stable, long-lasting colonies of these gregarious mammals (Kunz and Lumnsden, 2003). Conditions in caves are, in general, more stable than any other natural roost, since caves beyond the entrance are minimally affected by transient environmental conditions such as rain and wind.

Campbell et al. (2011) stated that many bacteria found in caves might be non-native species that have been transported into caves via water, air, or animals, and that their effect on the original environment is unknown. Despite Venezuela having a high density of known and unknown caves (Galán, pers. comm.), there is almost no literature regarding cave bacteria in this country. After a careful search for microbial studies in Venezuelan caves, we were able to find only one study (Barton et al., 2014). The authors analyzed the microbial activity of Roraima Sur Cave (Roraima Tepuy, Bolívar, Venezuela), working with DNA extracted from pooled samples of sediments from which 16S rRNA amplicons were obtained, cloned, and subsequently sequenced. In endolithic bacterial communities at the entrance of the cave, an unusual community structure was characterized by the dominance of

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Actinomycetales (mostly *Pseudonocardia* sp.) and Alphaproteobacteria (represented by nitrogen-fixing Beijerinkiaceae and *Methylocella*); deeper in the cave, the authors found that the microbial communities were dominated (82–84%) by a unique clade of Ktedonobacterales (Chloroflexi). From the Archaea, dominance (100%) of phylotypes from the Thaumarchaeota group was also demonstrated. This study involved an orthoquartzite cave, and it was based entirely on non-culture methods.

During an inventory of bats in a small cave within a recreational park, we decided to perform a first look at the diversity of culturable bacteria present in this cave. Although bats and other animals can be major sources of bacteria, humans also seem to be very frequent visitors based on the direct observation of human waste. Humans can be negatively affecting this cave, bringing bacteria on the soles of their shoes and by dumping feces, urine, blood, semen, and other organic waste. An important step to fully understanding the actual effect of bacteria on bats and other cave-organisms is to characterize the bacteria associated with this type of roost. The goal of this study was to determine what culturable bacteria might be present in Cueva Las Escaleras, and to provide a list of bats roosting in this cave. Moreover, if humans are frequent visitors to this cave, we would initially expect to find that the presence or absence of bacteria species showing association to humans. Finally, it would be important to determine whether some bacteria present in this cave have been reported as potentially dangerous or pathogenic.

Methods

Study Site

The cave is located within a recreational park called Las Escaleras, and it is known as Cueva Las Escaleras (8°00′18.8″ N, 71°43′41.8″ W, 1320 masl). The cave is found approximately 4 km southeast of the town of Pregonero, Municipio Uribante, Estado Táchira, Venezuela (Fig. 1). The vegetation of the area is mainly that of human-disturbed habitats, followed by open savanna of oligotrophic origin and small patches of secondary forest surrounding rivers and creeks (Ramoni-Perazzi et al. 2014). Mean temperature of the study site is 20 °C (Molina-Chacón, 1983). Annual precipitation averages 1636.9 mm (INAMEH, 2008), occurring in a unimodal pattern, with a period of water deficit from January to March, and a period of water availability the rest of the year, with maxima in July and August (Molina-Chacón 1983). The cave is about 38 m long and it is divided in two chambers, the second of which is then divided in two, forming a Y. The cave is frequently visited by people.

Sampling Procedure: Bats

Bats were captured to determine the inventory of bats living within the cave. All sampling protocols were performed following guidelines of the American Society of Mammalogists for capture, handling, and care of mammals (Sikes et al. 2011). Bats were captured using both hand nets and 12 m long, 38 mm mesh, 50 denier, four-shelf mist nets (Avinet, Dryden, New York, USA; Kunz et al. 2009) between 11:00 and 16:00 h. All individuals were released in the study site immediately after their species was determined.

Sampling and Collection of Bacteria

During three field trips in February 2010 the cave was sampled for bacteria. The floor, ceiling, and walls of the cave were scratched with syringes, and the resulting dust directly streaked onto Petri dishes containing agarized Luria-Bertani rich medium and fungicides (see below). To collect airborne bacteria, four Petri dishes, one per corner of the sampled area at the beginning of the left side of the Y, were left open during the time required, about 45 min, for cave sampling. Using a cooler, inoculated dishes were brought to the lab, where they were incubated at a constant temperature of 37 °C under aerobic conditions up to 72 h. To avoid fungal growth, agarized plates were amended with Terraclor (860 μg/mL) and Benlate (200 μg/mL). Terraclor is a 75% pentachloronitrobenzene (PCNB) soil fungicide (Chemtura, Middlebury, CT), while Benlate is the 50% commercial product of the systemic fungicide benomyl (Dupont, Wilmington, DE). Selected clones, based on shape, size, and color were kept in stabs and at –80 °C for long-time storage.

Bacteria Cultivation and Purification

Inoculated dishes were incubated for 24 to 72 hours after collection, and then kept under aerobic conditions at 4 °C until use. Based on macromorphological differences among colonies, such as size, color, elevation, border, and shape, selected colonies were streaked again for further purification. Colonies were reisolated in the same medium and observed under the microscope after Gram staining to check for purity and Gram's reaction (Gerhardt et al., 1994). Five isolated, purified clones from each original colony were stored at –80 °C and used to streak master Petri dishes, one for every cave part: soil, walls, ceiling, and air.

Phenotypic Characterization of Bacteria

Isolated colonies of all sampled bacteria were described in terms of color, shape, texture, borders, opacity, and other properties. Additionally, all samples were tested by Gram's reaction and the KOH test. Samples proven to be pure were subjected to further characterization using an API gallery battery of tests (API20E). Assay for antibiotic resistance was carried out in the same LB medium supplemented, in separate plates, with ampicillin (50 μg/ml), streptomycin (50 μg/ml), gentamicin (50 μg/ml), kanamycin (30 μg/ml), or tetracycline (12.5 μg/ml).

Amplification of the 16S rRNA Gene by Colony PCR

One day before the amplification of the 16S rRNA gene by PCR, each individual colony was reisolated as before and

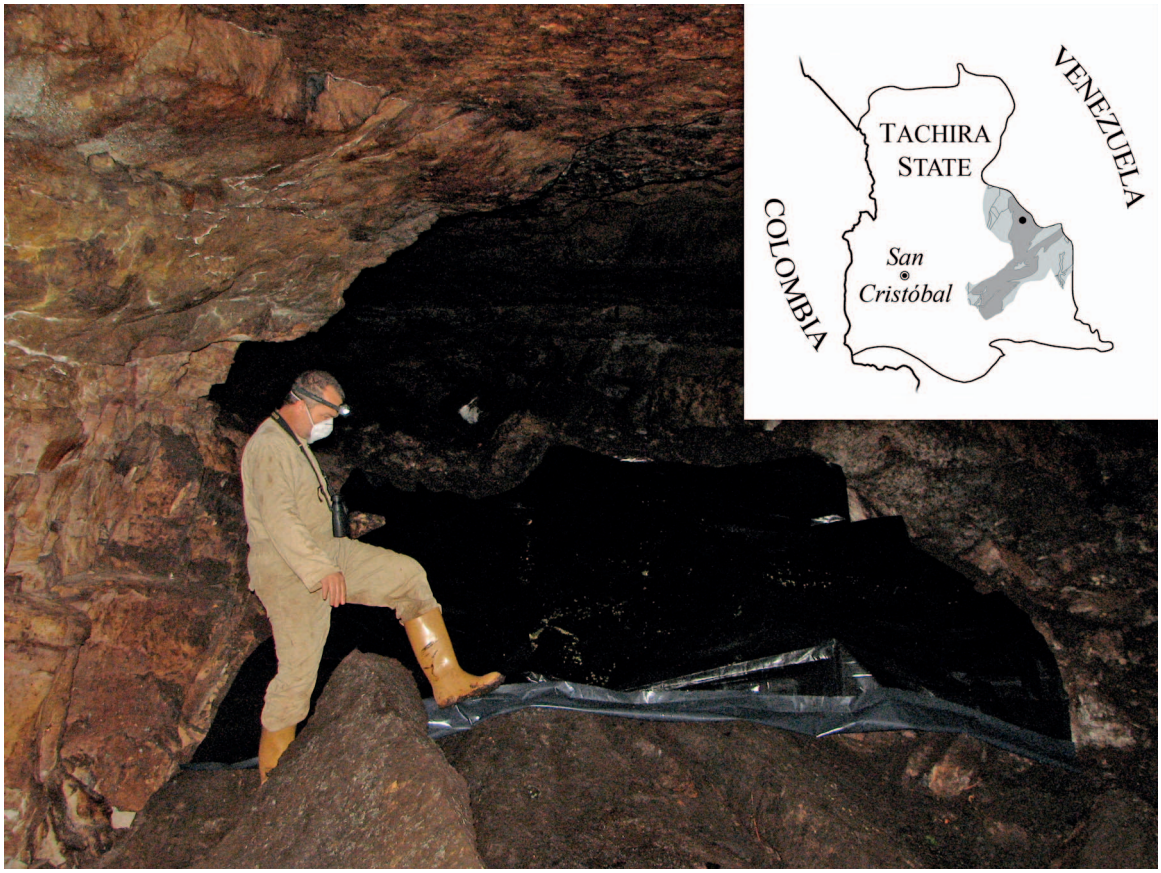

Figure 1. Inside Cueva Las Escaleras, Pregonero, Municipio Uribante, Táchira state. Top-right: Location of the municipality and the distribution of its geologic units following Garrity et al. (2006). The Lower Cretaceous Río Negro Formation, which is composed of limestone, shale, calcareous sandstone, and conglomerate, is highlighted in darker gray.

grown overnight at 37 °C. Fresh cultures were always used in all amplification protocols. Reaction mixtures for PCR amplification of the 16S rRNA gene (Dekio et al., 2005) consisted of 10 µL of the 1X GoTaq Green master mix (Promega, Madison, WI) supplemented with the universal primers 27F: 5′AGAGTTTGATCCTGGCTCAG3′ and 1492R: 5′GGTTACCTTGTTACGACTT3′ (Batison et al. 2009). Once the reaction mixture was prepared, the colony to be tested was gently touched with a micropipette tip and washed in the reaction mixture tube. PCR amplification was performed according to the following program: an initial denaturation step at 95 °C for 10 min, followed by 30 cycles of denaturation at 94 °C for 45 sec, annealing at 51 °C for 45 sec, and extension at 72 °C for 90 sec. A final extension step at 72 °C for 10 min was also included (Batisson et al., 2009; Dekio et al., 2005). Amplification products' quantity, quality, and size were checked by agarose gel electrophoresis and digitally recorded as previously recommended (Sambrook and Russell, 2001). Single, clear bands were salt and ethanol-precipitated and sent for sequencing, both strands, without further purification to the sequencing facility of Instituto Venezolano de Investigaciones Científicas (IVIC, Caracas). All amplicons were sequenced with the amplification primers cited before plus internal primers directed towards the ends of the amplicon as reported by Rogall et al. in 1990 (Pa:5′-AGAGTTTGATCCTGGCTGAG-3′, and Pe:5′-CCGTCAATTCTTTGAGTTT-3′). In general, each amplicon produced four to six sequence reads of high quality; a few produced only two workable reads.

Bioinformatics Analysis

A contig for every sequence per sample was obtained using all derived reads per amplicon with BioEdit (Hall, 1999), and the contig compared with equivalent sequences available in public databases (GenBank) by BLAST using default parameters (Altschul et al., 1990). An *a priori* criterion of quality of similarity of 98% or higher (Pei et al. 2010, Stackebrandt and Ebers 2006) was used as the threshold value of success and identification if the query coverage was also equal to or higher than 98%. An *a posteriori* criterion of higher than 99% similarity with 100% coverage was set later. Moreover, sequences identified this way were also subjected to other two criteria of identity by similarity: best BLAST species matches were corroborated with the RDP's Classifier

Table 1. Identity of the bacteria sampled at Cueva Las Escaleras (Pregonero, Táchira, Venezuela) based on the sequencing and analysis of the 16S rRNA gene.

Sample[a]	Contig Length, nt	Best Candidate Species	GenBank (this Work)
M1, W	1413	*Pseudomonas* sp.	KT792722
M2, W	1411	*Pseudomonas* sp.	KT792723
M3, W	1402	*Achromobacter denitrificans*	KT792724
M4, W	1407	*Pseudomonas monteilii*	KT792725
M5, S	1420	*Citrobacter freundii*	KT792726
M6, S	1407	*Pseudomonas monteilii*	KT792727
M7, S	...	Excluded (sequences too short)	...
M8, S	1409	*Pseudomonas putida*	KT792729
M9, S	1412	*Escherichia coli*	KT792730
M10, S	1457	*Pseudomonas putida*	KT792731
M11, S	1412	*Pseudomonas xiamenensis*	KT792732
M12, S	1412	*Raoultella electrica*	KT792733
M13, S	1441	*Paenibacillus lactis*	KT792734
M14, S	1417	*Klebsiella oxytoca*	KT792735
M15, S	817	*Klebsiella oxytoca*	KT792736
M16, A	1462	*Proteus mirabilis*	KT792737
M17, A	1414	*Proteus mirabilis*	KT792738
M18, A	916	*Proteus mirabilis*	KT792739
M19, A	866	*Proteus mirabilis*	KT792740
M20, A	920	*Proteus vulgaris*	KT792741
M21, A	877	*Microvirgula aerodenitrificans*	KT792742
M22, A	850	*Providencia rettgeri*	KT792743
M23, A	1424	*Proteus mirabilis*	KT792728

[a] S, soil; W, wall and ceiling; A, air

(Wang et al., 2007) and with the EzBioCloud (Kim et al., 2012) algorithms. In Table 1, accession numbers for the bacteria molecularly analyzed in this work is provided, along with their putative identity in those cases were the three criteria of identification were concordant (all but one case). Finally, alignments were performed using CLUSTALX2 (Larkin et al., 2007), and relatedness among bacteria analyzed by neighbor-joining using MEGA6 (Tamura et al., 2013) with 1000 replicates under the K2P model.

Results

Cueva Las Escaleras is a recreational place for the inhabitants and visitors of the town of Pregonero, and humans leave remnants of burnt logs, toilet paper, cans, plastic bottles and residues, feces, used condoms, and female hygiene products that were observed during our sampling visits. These remains become scarcer as light diminishes deeper inside the cave to the place where the bacterial samples were taken, close to a bat colony. We were able to recover bacteria from the scratched ceiling, walls, and floor of the cave (Fig. 2). Plates left open to the air, a third of all samples, also yielded bacteria. In total, we worked with twenty-two isolated and well molecularly identified bacteria. Colony PCR allowed for the reliable identification of the samples selected, which is why we changed our *a priori* criterion of identification to make it the more stringent 100% coverage and more than 99% similarity. Only two samples (M1 and M2), with contigs derived from five and six independent sequencing reactions (1413 and 1411 nt long, respectively, but identical between them) provided reliable information only to the genus level (97% similarity to *Pseudomonas* spp. with a coverage of 100%). This sample might represent a new *Pseudomonas* species. Additional biochemical tests not shown were concordant with the molecular identity of the bacteria subjected to this analysis, which is summarized in Table 1 and Figure 3. All but one of the bacterial samples belongs to the phylum Proteobacteria (two to the β group, and nineteen to the γ group); the other bacterial species was *Paenibacillus lactis* (phylum Firmicutes).

Surprisingly, all bacteria were resistant to ampicillin, and thirteen of the samples were resistant to at least one of the other antibiotics tested; three bacterial samples (*Achromobacter denitrificans*, *Proteus mirabilis,* and *Microvirgula aerodenitrificans*) were resistant to the five antibiotics tested in independent assays (Table 2).

Five species of bats were identified inside Cueva Las Escalerass: the large fruit-eating bat (*Artibeus amplus*), the short-tailed fruit bat (*Carollia perspicillata*), the common vampire bat (*Desmodus rotundus*), the hairy-legged vampire bat (*Diphylla ecaudata*), and the Luis Manuel's tailless bat (*Anoura luismanueli*) (Fig. 4).

Figure 2. Bacteriological sampling in Cueva Las Escaleras for airborne bacteria (A) and by wall and ceiling scratching (B).

Discussion

As an initial attempt to characterize the bacterial flora present in Cueva Las Escaleras, a molecular approach based on the amplification and sequencing of the 16S rRNA bacterial gene was used in this study, taking as a cutoff value of identification more than 99% similarity between our sequences and those reported at public databases. Results were concordant by three identification molecular, independent criteria, including the unassigned *Pseudomonas* sp. reported here.

As reported elsewhere, the main group of bacteria found in caves is the Proteobacteria, particularly when they are identified by molecular tools; Actinobacteria, however, constitute the bacteria most frequently found in these environments, which seem to represent a habitat particularly favorable for members of this group (Jurado et al., 2010). All but one of the bacteria identified in this work are Proteobacteria.

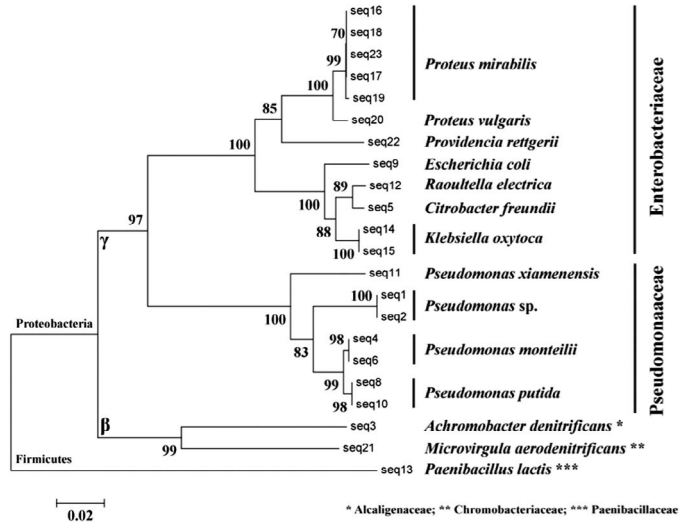

Figure 3. Cladogram of the bacteria of Cueva Las Escaleras reported in this work after running MEGA6 (Tamura et al., 2013) by neighbor-joining with 1000 replicates under the K2P model.

Almost all the bacteria samples molecularly identified in this study (Table 1) are recognized as potential human pathogens. *Achromobacter denitrificans*, which is a ubiquitous bacteria commonly found in soil and aquatic environments, has been reported to cause pneumonia in humans (Aundhakar et al., 2014); *Citrobacter freundii*, although a rare opportunistic nosocomial pathogen, is able to cause neonatal meningitis among other illnesses (Badger et al., 1999; Chen et al., 2002; Tschäpe et al., 1995); *Escherichia coli*, a very well know pathogen, has also been reported in other caves visited by humans, like the Lascaux Cave in France (Bastian et al., 2010), six caves in northern Alabama and northwestern Georgia, USA (Campbell et al. 2011), and diverse caves in Mizoram in northeast India (De Mandal et al., 2014) to name a few; *Klebsiella oxytoca*, which may cause colitis and sepsis, can be present in human stools (Högenauer et al., 2006); *Microvirgula aerodenitrificans*, a denitrifying bacteria originally isolated from activated sludge (Cleenwerk et al., 2003; Patureau et al., 1998), although not deemed as pathogenic, can also be found associated with bacteremia in immunosupressed patients (Murphy et al., 2012); *Proteus mirabilis*, widely distributed in soil and water is responsible for approximately 90% of all *Proteus* infections in humans, particularly of the urinary tract; and *Providencia rettgerii*, also common in soil and water, can cause opportunistic infections in humans, including the urinary tract and eyes, as well as traveler's diarrhea, abdominal pain, fever, and vomiting (Yoh et al., 2005). Among the *Pseudomonas* species (Anzai et al., 2000) found in Cueva Las Escaleras, we were able to identify by 16S rRNA sequencing *P. xiamenenesis* and from the *putida* group, *P. putida* and *P. monteilii*. The latter was originally isolated from clinical samples including bronchial aspirates, and it is believed to be a rare opportunistic pathogen or colonizer

Table 2. Antibiotic resistance shown by isolated bacteria sampled at Cueva Las Escaleras (Pregonero, Táchira, Venezuela).

Sample	Best Candidate Species	Antibiotic Resistance[a]
M1	*Pseudomonas* sp.	Amp
M2	*Pseudomonas* sp.	Amp
M3	*Achromobacter denitrificans*	Amp, Str, Gnt, Kan, Tet
M4	*Pseudomonas monteilii*	Amp
M5	*Citrobacter freundii*	Amp
M6	*Pseudomonas monteilii*	Amp, Tet
M7	Excluded in sequencing	Amp
M8	*Pseudomonas putida*	Amp
M9	*Escherichia coli*	Amp, Str, Gnt, Kan
M10	*Pseudomonas putida*	Amp
M11	*Pseudomonas xiamenensis*	Amp
M12	*Raoultella electrica*	Amp, Tet
M13	*Paenibacillus lactis*	Amp, Str, Gnt, Kan
M14	*Klebsiella oxytoca*	Amp, Gnt, Kan, Tet
M15	*Klebsiella oxytoca*	Amp, Tet
M16	*Proteus mirabilis*	Amp, Tet
M17	*Proteus mirabilis*	Amp, Str, Gnt, Kan, Tet
M18	*Proteus mirabilis*	Non tested
M19	*Proteus mirabilis*	Amp, Kan, Tet
M20	*Proteus vulgaris*	Amp, Kan, Tet
M21	*Microvirgula aerodenitrificans*	Amp, Str, Gnt, Kan, Tet
M22	*Providencia rettgeri*	Amp, Gnt, Kan, Tet
M23	*Proteus mirabilis*	Not tested

[a] Determined by lack of growth after 24-48 h of incubation under aerobic conditions at 37 °C on solid LB media containing ampicillin (Amp) 50 μg/ml, streptomycin (Str) 50 μg/ml, gentamicin (Gnt) 50 μg/ml, kanamycin (Kan) 30 μg/ml, or tetracycline (Tet) 12.5 μg/ml

(Elomari et al., 1997). We found another pseudomonad that is closely related to *P. pseudoalcaligenes*, but a similarity of 97% does not allow conclusion about specific identity. Two different samples were identical among themselves at the sequence level.

Another bacterium found in this study, a Firmicutes, was *Paenibacillus lactis*, an organism originally isolated from raw and heat-treated milk (Schedelman et al., 2004), but that can also be found in bacterial communities in environmental samples (da Mota et al., 2005). We found no reference dealing with pathogenicity to humans of this bacterium or the recently described *Raoultella electrica* (Kimura et al., 2014).

We contend that the bacteria reported are not indigenous to the cave but carried into it by humans because, first, humans leave man-made litter in the cave along with feces, urine, menstrual blood, and semen; second, the bacteria found in other caves, including some with recreational uses but with better visiting strategies and cave protection, do not resemble the bacteria we report in that the ones present in Las Escaleras are almost all pathogenic for humans; third, bacteria isolated from caves as their indigenous habitats and considered pathogenic mostly belong to the Actinobacteria (particularly those of the genera *Nocardia*, *Mycobacterium*, *Gordonia*, *Rhodococcus,* and *Streptomyces*; Jurado et al., 2010), while others are members of the Alphaproteobacteria groups such as *Inquilinus limosus* or *Aurantimonas* spp., members of the genus *Afipia*, or *Staphylococcus aureus,* none of them found in this work; and finally, although not completely conclusive, of forty-two species of bacteria in a catalog of bacteria (González-Quiñones et al., 2014) found on the skin of *Sturnira lilium* and *S. bogotensis* bats (Chiroptera: Phyllostomidae), only two (*E. coli* and *C. freundii*) were also present in this cave.

Bats found inside Cueva Las Escaleras are using it as a day roost, since all of them were captured during their daylight resting period. Two of the species of bats (*Carollia perspicillata* and *Desmodus rotundus*) are common (Linares, 1987) and widely distributed in Venezuela (Linares, 1998). *C. perspicillata* is the most common and widespread bat species in Venezuela (Linares 1998). Since it feeds on at least fifty different fruits, it is an important seed disperser in many moist evergreen and dry deciduous forests, usually below 1,000 masl (Fleming, 1988). *C. perspicillata* roosts in caves, forming groups of no more than one hundred individuals. The vampire bat (*D. rotundus*) is a common species that frequently uses caves as roosts. It forms stable, long-lasting colonies.

The other three species (*Artibeus amplus*, *Diphylla ecaudata*, and *Anoura luismanueli*) are more geographically restricted and less common, and all three might need caves as primary roosts (Handley, 1987; Linares, 1998). The large fruit-eating bat (*A. amplus*) is a species commonly found in caves (Handley, 1987; Ruiz-Ramoni, 2010), and its large

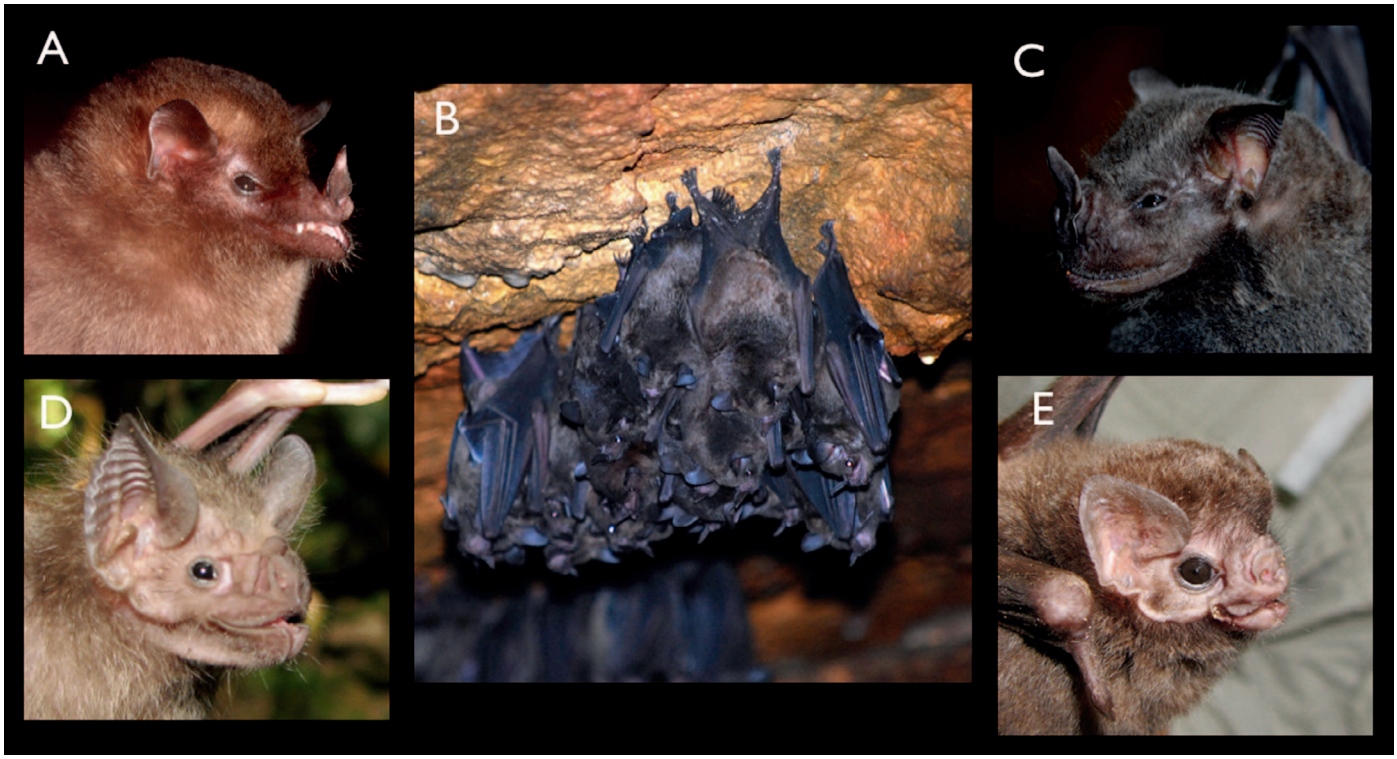

Figure 4. Bats captured and identified at the study site, Cueva Las Escaleras, Táchira state, Venezuela. A, *Anoura luismanueli*; B, *Carollia perspicillata;* C, *Artibeus amplus*; D, *Desmodus rotundus*; E, *Diphylla ecaudata*.

colony in Las Escaleras cave is a stable group throughout the year (Ruiz-Ramoni, 2010). Luis Manuel's tailless bat (*Anoura luismanueli*) is an uncommon species of nectarivorous bat from Los Andes, and it is also found in caves. The hairy-legged vampire bat (*D. ecaudata*) is the only one of three extant species of vampire bats that feeds exclusively on the blood of birds. It is a rare species, one of the few species of monogamous bats (and mammals), and is monotypic (Linares, 1998). Bat diversity in this small cave is remarkable.

Although the presence of these bats in the cave could suggest that they are not being adversely affected by the potentially pathogenic bacteria, it is of utmost importance to stop using it as a recreational room for human activities. It is still unknown whether potentially pathogenic bacteria could cause sickness or death of bats. Although bats are still living there despite these bacteria, the natural environment in this cave has been affected, and it is not possible to determine its consequences without further research. For example, potentially pathogenic bacteria might be affecting vampire bats (*Desmodus rotundus*), because this species commonly walks on the ground, especially after a meal. Vampire bats might not be able to fly when they finish feeding on blood, and they usually walk or run on the ground when returning to their roosts. The contact of their bodies with the contaminated soil could affect their own lives and that of other members of the colony due to allogrooming.

Protecting a cave from antropogenic disturbances is of utmost importance not only to preserve its biodiversity, but also as a source of discoveries. For example, observations in this particular cave resulted in the first and only report of leucism, a pigmentation disorder, in *A. amplus* (Muñoz-Romo et al., 2014), and also the discovery that folivory was a permanent phenomenon in some species (Ruiz-Ramoni et al., 2011); it had been considered a rare and occasional phenomenon since its discovery (van der Pijl, 1957).

As stated by Galán and Herrera (2006), it is imperative to protect karst regions, including the conservation of their species, many of which are unique and hence invaluable in terms of the biodiverse richness of Venezuela and the planet. Also, the findings of diverse vertebrate fossils in Venezuelan caves, including from various bats (Rincón, 2004), adds to the importance of these particular environments for the understanding not only of our present biodiversity, but also that of our distant past, as well as of the ecological requirements of an important group of bats.

We consider the bacteriological status of Cueva Las Escaleras a potential public health threat because water from the cave floor is running directly into the stream that serves this recreational park. Furthermore, bacteria found in this work are also present in the air, making more worrisome their potential health risks to humans. The bacteria assemblage derived from human presence and activities in caves might be affecting the natural bacterial communities in ways that are not yet completely understood (Campbell et al., 2011).

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DRAINAGE AND SIPHONING OF A KARSTIC SPRING: A CASE STUDY

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Abstract: The Fuentetoba Spring, with a mean flow of 210 L s^{-1}, releases the discharge from an aquifer that comprises three hydraulically-connected synclines. The spring has a very irregular flow of between 8 L s^{-1} and 3,400 L s^{-1} due to the predominant water circulation that is non-Darcian turbulent flow as an underground torrent in the vadose zone, as well as through well-developed karstic conduits in the phreatic and epiphreatic zones, as attested to by speleological explorations. The long response times to recharge by the Fuentetoba karstic system, seen in the spring's hydrograph, are controlled by regional factors. Nevertheless, certain responses that have very long time lags under high water conditions might be governed in the final stretch of the flowpath by mechanisms of pressure or siphoning of the floodwave, and perhaps by constrictions or plugs of sediment in the conduits.

Introduction and Objectives

The analysis of flow variation in springs is important for two reasons. The quantification and prediction of spring flow is an essential prerequisite for managing groundwater resources in an aquifer, and the intensity, duration, and nature of precipitation are the variables that most influence the shape of the hydrograph. Analysis of flow variations can elucidate some of the characteristics of the aquifer that influence the relation between recharge and discharge of water: the different flow states, storage, inertia, and flow recession, and their relationship with structural geological features. Spring discharge is dependent on catchment characteristics such as size and slope, recharge style, drainage network density, geological variability, vegetation, and soil (Ford and Williams, 2007).

The temporal distribution of natural recharge and corresponding transit time of the water through karst aquifers can be a complicated issue since, in addition to depending on the intensity and duration of the recharge events, as well as the antecedent soil moisture, it depends on the existence of significant heterogeneity in the conduits' geometry and connections between them, and on the hydraulic parameters of the vadose and phreatic zones (Halihan and Wicks, 1998, Geyer et al. 2008).

Karst flow is usually subdivided into conduit flow, fracture flow, and matrix flow. Rapid responses in the hydrograph under high flow conditions are usually explained by conduit flow and fracture flow. The beginning segment of the recession curve and its steeper slope are usually explained by drainage through fractures and the rock matrix, while only the transmissivity of the matrix plays a part in the shallower slopes of the recession curve when the flow is very slow (Shevenell, 1996). However, the phreatic and epiphreatic conditions can change over time, and the geometry of the groundwater flow may change depending on the particular conduits that are used under high- or low-water conditions.

All these factors can lead to a situation where the temporal distribution of the discharge does not exactly correspond to the recharge. This has been observed in certain karst systems, particularly during periods of high water and storm events. Various possible mechanisms are cited to explain this type of hydrograph.

The flow of some karst springs can rapidly change from slight or absent to very great. This effect can be explained by the impulsion under pressure that occurs during periods of high recharge to old, ponded water that is stored close to the spring. This phenomenon has been observed in several karst systems (Yevjevich, 1981). Other intermittent phenomena are explained by the existence of a siphon that operates only under high-water conditions when the groundwater level lies above the mouth of the outlet. In other cases, the siphon can operate along an outflow conduit, carrying the ordinary groundwater flows that are ponded up in a reservoir behind the spring. When the water reaches the mouth, all the water that was stored in the reservoir is expelled due to the vacuum created. There are many examples of springs associated with siphons (Milanovic, 2007). The phenomenon of siphoning (i.e., regular evacuation through an inverted U-tube) occurs only in very special cases, such as at the source of the river Mundo in Spain (Rodríguez-Estrella et al., 2002). Mangin (1974) studied the intermittent phenomena and made numerous experiments using small-scale models. Other unusual cases have been cited, such as Gelodareh Spring (Iran), where the existence of a siphon-flow system explains the multiple periodic peaks of the breakthrough curve and the lack of tails (Karimi and Ashjari, 2009).

Sara (1977) and Urzendowsky (1993) explained that in Big Spring in Kings Canyon National Park a sediment plug exists that blocks flow through the main outflow conduit. Under high-water conditions this blockage is released when the water

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level behind it rises and produces a sufficient hydraulic head to remobilize the deposits and allowing for the evacuation of the water impounded behind them. As the water level falls, sediment deposition begins again, and the conduit is plugged, so continuing the cycle. Halihan et al. (1998) explain that drainage in the Devil's Cave system during storm events is governed by the presence of constrictions in the network of conduits, and they were able to model the hydrograph of the outlet spring in a satisfactory manner by considering a reservoir/constriction model.

The case studied in this paper is the karst system of Pico Frentes, in the Spanish Iberian Range. This system, draining through the Fuentetoba Spring, has a catchment of approximately 26 km^2 that is the object of our investigation (Fig. 1). It is currently used to supply water to a small village and various groups of houses. The Fuentetoba karst system is an unconfined karst aquifer. It is geometrically well-defined, opening the opportunity to make direct observations of the active and abandoned conduits in the unsaturated zone within the stream caves by means of conventional caving. We also accessed the phreatic conduit of the Fuentetoba spring, as well as the underground river in the cave associated with this spring, by cave diving. These investigations showed us what the karstic aquifer is really like inside, albeit in a partial and incomplete way. The recent exploration (Sanz Pérez et al., 2012) of these apparently independent cavities that converge at the Fuentetoba Spring increases the chances of elucidating the drainage from this karstic system, even though we are dealing with fragments of conduit networks that must belong to a much larger subterranean drainage system.

The organization of these conduits is comparable to the drainage network of a surface river system (Bakalowicz, 2005). Flow in a karstic aquifer exhibits a double (or triple) system, characterized by the interaction of the diffuse and conduit domains. In the conduits, flow can be rapid (Shuster and White, 1971; Atkinson, 1977). The flow in the conduits can be laminar or turbulent, depending on the Reynold's number. In karstic aquifers drained by large springs, the flow is usually organized in high-permeability channel networks (Worthington and Ford, 2009), which are suited to mathematical modelling (Worthington, 1999, 2009).

The aim of this investigation was to analyze the drainage of Fuentetoba Spring and the variable response of the aquifer to recharge, especially in situations where siphoning occurs. In this respect, hydrograph analysis, tracer tests, and direct observations of the cave hydrology were all useful.

Methods

Karstic aquifers have unique characteristics and a complexity that differentiates them from other types of aquifers. This means that the classical hydrogeological research approach may be inappropriate or insufficient (Bakalowicz, 2005). Specific techniques are required to explore and study them (White, 2002 and 2003; Ford and Williams, 2007; Goldscheider and Andreo, 2007).

In the case of Fuentetoba Spring, the presence of only one borehole in the entire aquifer limits many aspects of hydrogeological understanding. However, a hydrogeological study to define the aquifer and quantify the flow from its springs, along with the establishment of a water balance using a mathematical rainfall-runoff model, has led to a basic understanding of this karstic aquifer (Rosas et al., 2016). Using this prior knowledge of the specific characteristics together with available information resources, we applied and combined the methods outlined below to advance the understanding of this karstic drainage system.

A gauging station was installed to measure the spring flow at Fuentetoba Spring over the hydrological years of 2010-11 and 2011-12. The spring was gauged along the discharge stream. The calibration curve for the gauging station was established by direct gauging using flowmeters under various hydrological situations, resulting in a known relation between water levels and flow. The hydrograph of Fuentetoba Spring was analyzed. Tracer tests can be done (for example, Käss, W, 1998; Geyer et al., 2007; Benischke et al., 2007; Perrin and Luetscher, 2008; Goldscheider et al., 2008; Segovia et al., 2011) to characterize local hydrogeological properties, such as the possible effect of siphoning or lags in flow. In the case of Fuentetoba Spring, three tracer tests were performed in 2012 and 2013 during low-water periods that were followed by recharge events, using uranine, together with point injections of NaCl. The injection point was in the subterranean river 3,000 m straight-line distance from Fuentetoba Spring, specifically in the final stretch where it flows through a syncline that exhibits a unique hydrogeological behavior.

Less conventional methods, such as direct mapping by cave divers and conventional caving and mapping of the conduit network were used both in the epiphreatic and the saturated zones. These techniques allowed us to better understand the hydrological functioning of the karst medium and certain peculiarities of this system. These speleological surveys were undertaken over the last four years under various hydrological conditions. Numerous speleological explorations and topographical surveys were done in the 3,000 m stretch that is currently known of the Majada del Cura Cave network. These expeditions included point gauging of the subterranean river under both high and low water situations (Fig 1). Four cave diving surveys were completed in the underwater parts of the Majada del Cura cave network (Figs. 1 and 2). Five cave diving surveys were made in the outflow conduit of Fuentetoba Spring (Fig. 3). Further time was spent looking for new access routes into the other conduits between the underground river and Fuentetoba Spring, either by detailed surveys of existing potholes or looking for new caves. A description of this conduit network is included in the section Description of the Study Area, while the hydrological observations are included in the Results section.

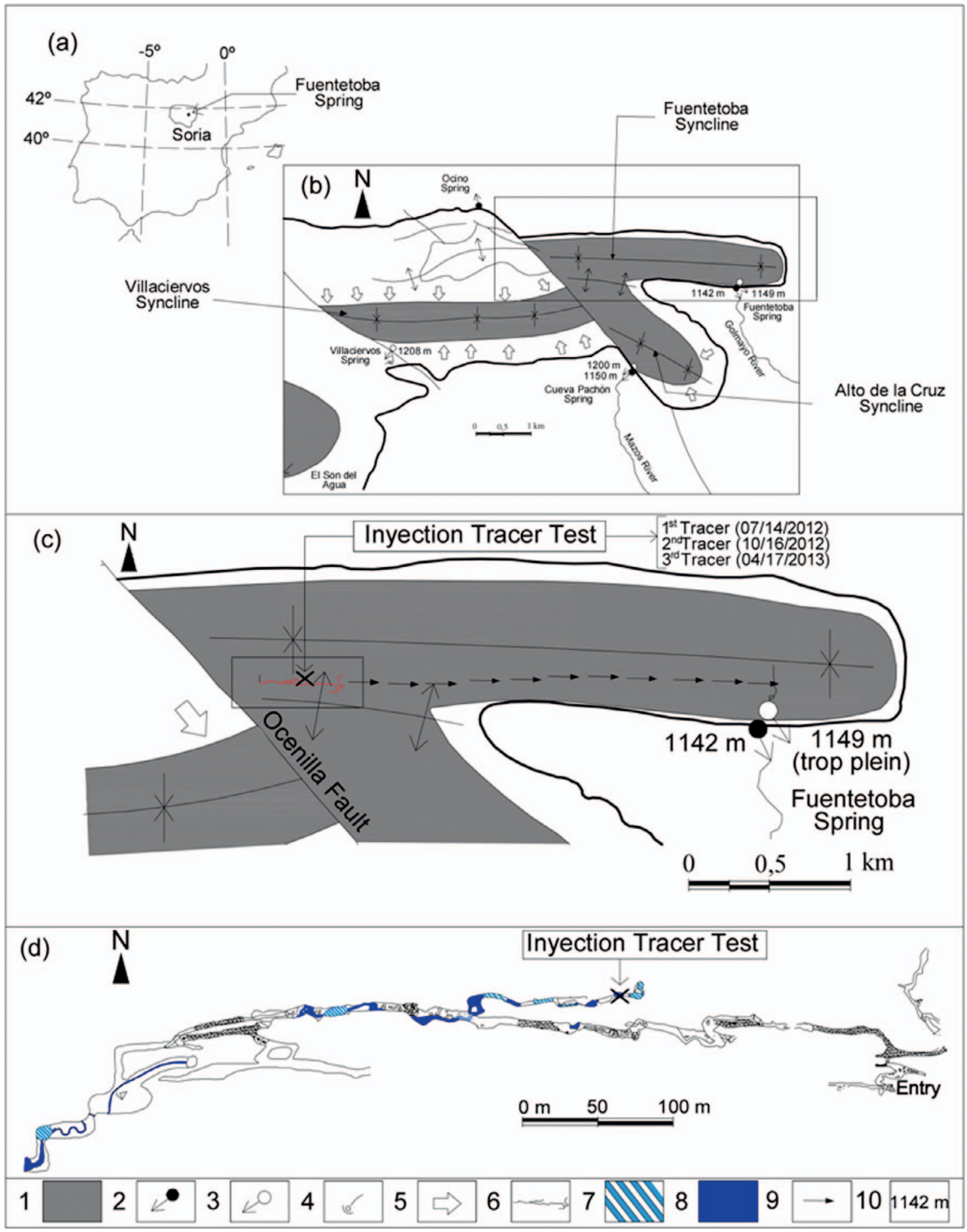

Figure 1. (a) Location of the study zone. (b) Karst system of Fuentetoba Spring. (c) Network of explored underground river and groundwater path confirmed by tracer studies in the Fuentetoba syncline. (d) Detail plan view of the conduits and galleries of the underground river. 1. Outcrop of the limestone-marl contact. 2. Permanent spring. 3. Overflow spring. 4. Ephemeral spring. 5. Lateral recharge in unsaturated zone of the syncline flanks. 6. Network of explored galleries of the underground river. 7. Siphons. 8. Underground river. 9. Flow of groundwater confirmed using tracers. 10. Elevation in meters.

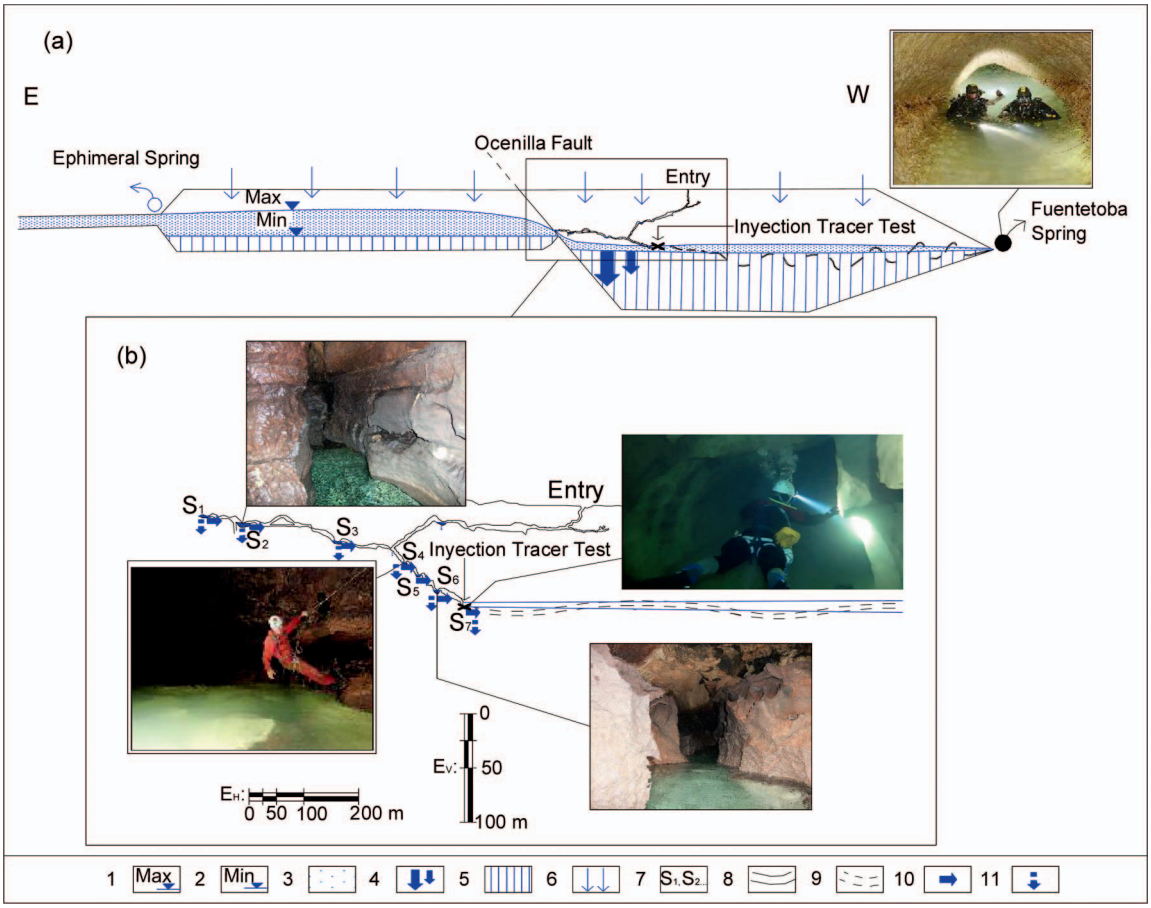

Figure 2. A. (a) Schematic hydrogeological section running east-west. (b) Profile of network of explored and inferred conduits of the underground river that emerges at the Fuentetoba Spring. 1. Maximum phreatic level. 2. Minimum phreatic level. 3. Epiphreatic zone. 4. Flow under pressure during high-water. 5. Permanent reserves. 6. Natural recharge. 7. Siphons [sumps] explored by divers. 8. Network of explored conduits. 9. Schematic and inferred network of conduits. 10. Flow direction of underground river. 11. Filtrations in the underground river.

DESCRIPTION OF THE STUDY AREA

CLIMATE AND VEGETATION

The climate of this area is Mediterranean, with a relatively cold winter and an average annual rainfall of 574 mm, peaking in the spring. The spatial distribution of rainfall on the plateau is very uniform, and it is not uncommon for winter precipitation to fall as snow. The harsh continental climatic conditions of the area, the impoverished soils that are unfit for cultivation, and the large infiltration capacity of the karst gives the plateau its marked aridity that has conserved the extensive woodlands of Spanish Juniper (*Juniperus thurifera*), this being one of the trees most resistant to such conditions.

STRATIGRAPHY AND TECTONIC STRUCTURES

From a stratigraphic point of view, the oldest deposits in the area belong to the Weald Facies and comprise conglomerates, sands, limonites, and purple clays. Altogether, this series can exceed 200 m thick. Above it lies the Albian Utrillas Facies, consisting of 150 m siliceous, terrigenous deposits in a white kaoliniferous matrix. Above these are fossil marls from the Cenomanian and Turonian, reaching 101 m thick. The upper part of the Turonian and the Coniacian-Santonan-Campanian comprise some 200 m of nodular limestones (IGME, 1980, 1982 and Navarro, 1991), which project upwards to form the scarps along the northern edge of the sierra, such as Pico Frentes. Overlying these series and concordant with the earlier geological formations are the Garumnian facies, already transitioning into the Tertiary (Fig. 3).

Structurally, the folds, which were generated during the Alpine Orogeny, follow an east-west alignment. There is a large asymmetrical syncline, the Villaciervos syncline, whose northern limb dips gently and is more developed than the southern limb. Beyond, to the northeast, is a small anticline. This succession of folds has been displaced by the Ocenilla Fault. This fault is a dextral strike-slip fault with a horizontal displacement of 1,500 m, though it also has a vertical

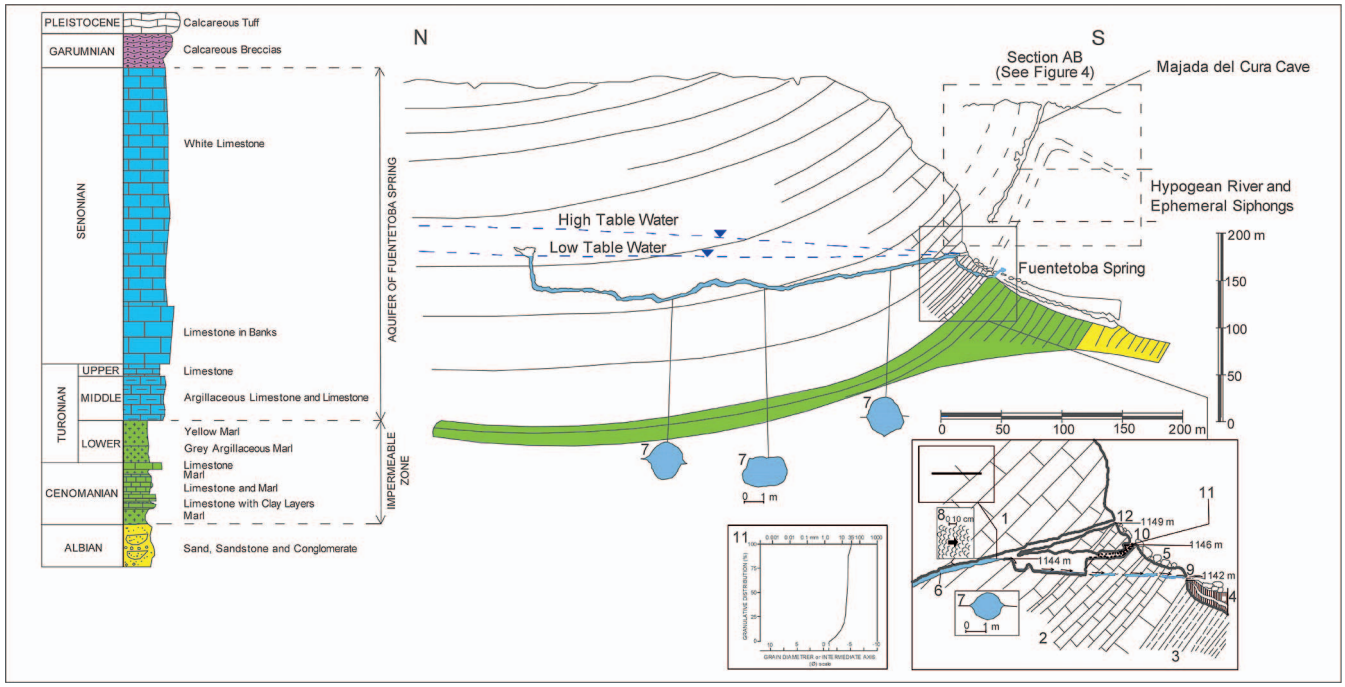

Figure 3. Conduit of the Fuentetoba Spring and stratigraphic column in the Fuentetoba Syncline Aquifer. 1. Limestone in banks. 2. Limestone. 3. Marl. 4. Tufa. 5. Blocks. 6. Conduit. 7. Conduit section. 8. Scallops. 9. Spring. 10. Lower spring overflow. 11. Granulative distribution. 12. Upper spring overflow.

displacement of 40 m, as deduced from the structure contours, and the sunken block is the eastern one. Beyond the Ocenilla Fault, from north to south, are three folds: the Pico Frentes or Fuentetoba Syncline, followed by an anticline with steep limbs, and finally the syncline of the Sierra de La Llana and Alto de Peña Cruz, along whose southern edge emerges the Cueva Pachón Spring (Figs. 1 and 4). Fuentetoba Spring, meanwhile, emerges on the southern edge of the northern syncline.

The Coniacian-Santonan-Campanian limestones make up a clearly permeable hydrostratigraphic layer of considerable thickness that overlies low-permeability marls. The Weald and Utrillas facies are considered to be of low to moderate permeability (Fig. 3).

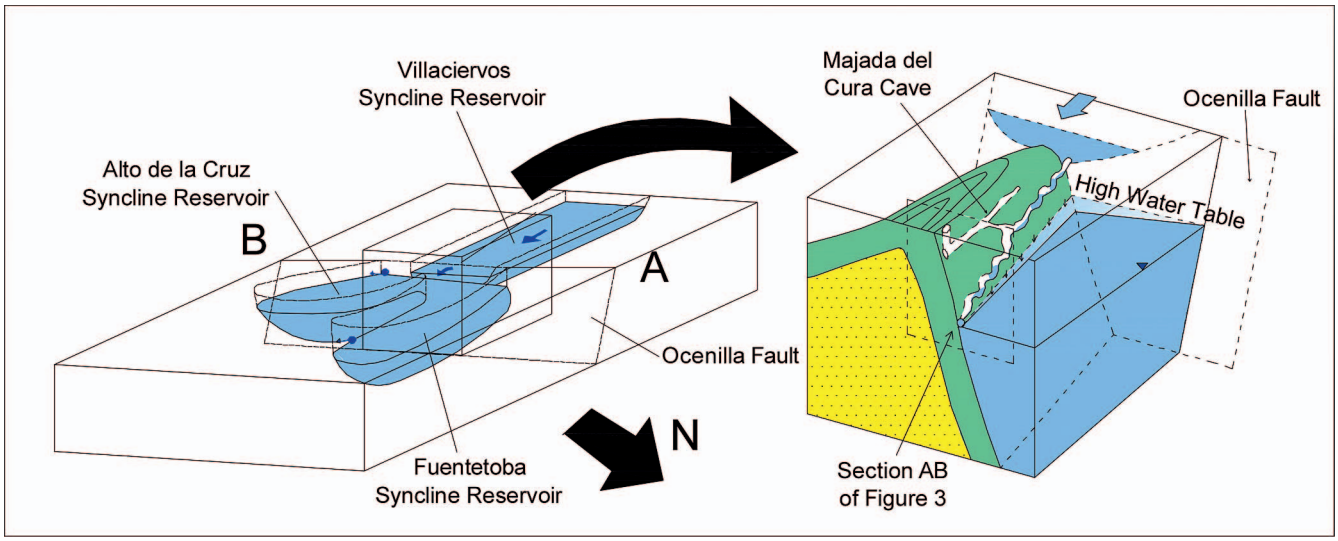

Figure 4. A conceptual three-dimensional model showing water flow in the three syncline reservoirs and the detailed geological location of Majada del Cura subterranean river.

TOPOGRAPHY, GEOLOGY, AND GROUNDWATER FLOW

The aquifer feeding Fuentetoba Spring extends over an area of 26 km^2. Its geometry combines a syncline on the western side of the Ocenilla Fault and the succession of a syncline-anticline-syncline on the eastern side of this fault (Figs. 1b, 1c, and 4). The fold geometry is very well defined and placed the aquifer reservoirs mainly in the three hydraulically connected synclines. Together, they have a total groundwater storage capacity of between 5×10^6 m^3 and 7×10^6 m^3. The last syncline, called Pico Frentes, before arriving at Fuentetoba, holds between 2.76×10^6 m^3 and 3.68×10^6 m^3 of permanent reserves (the permanent water in Figure 2) that accounts for more than 50% of the whole aquifer (Rosas et al., 2016).

This calcareous unconfined aquifer is elevated in the manner of a meseta, and its edges are very precise, since all its edges outcrop on the slopes of the fairly impermeable Cenomanian-Turonian marls that form the base of the aquifer. The Fuentetoba Springs emerge where the impermeable marls occur at lower elevation (1,140 m) and at the source of the river Mazos (1,150 m). Recharge to this unconfined aquifer and peneplain is autogenic and diffuse. The recharge area of the Villaciervos syncline is around 20 km^2, the Fuentetoba syncline covers 4.75 km^2, and the syncline of Alto de la Cruz covers 4.25 km^2.

Groundwater flows along the base of the synclines towards the Fuentetoba Springs (210 L s^{-1}) and source of the river Mazos (50 L s^{-1}). This river has a highly variable regime and low inertia, with several small discharges arising under high-water conditions. Thanks to the field data (Rosas, 2013) and hydrograph simulations of these springs using a mathematical rainfall-runoff model (Rosas et al., 2016) the mean water-balance was calculated in detail for a 20-year time series, as follows: rainfall 16.86×10^6 m^3 (100 %), natural recharge 8.35×10^6 m^3 (49.53%), EVT 8.50×10^6 m^3 (50.41%), groundwater pumping 0.01×10^6 m^3 (0.06%), surface runoff 0 m^3, and groundwater transfers to other aquifers 0 m^3.

The structure of the aquifer takes the form of a syncline in the west whose axis dips towards the east. This conditions the convergence of flows and the accumulation of water at its heart, directing groundwater flow towards the east. An appreciable portion of the limb of this syncline lies outside the saturated zone (Figs. 1b and 1c and 4), but water that infiltrates during recharge is efficiently returned to its core. Part of the groundwater is stored in the syncline of Alto de la Cruz, as has been demonstrated by prolonged tracer tests (Rosas et al., 2016), while water infiltrating into the catchment itself also collects in this syncline. However, the majority of flow is towards the Fuentetoba syncline, once it has passed the Ocenilla Fault. In this case, it undoubtedly passes through a number of galleries, including the Cave of Majada del Cura, which closely follows the contact with the limestone-marl beds as far as Fuentetoba Spring (Figs. 1c and 1d). Water stored in the Alto de la Cruz syncline is also directed toward Fuentetoba, except on the threshold of the anticline axis that separates these folds. Only the southern part of the syncline must feed the spring that is the source of the river Mazos (Fig. 4).

The situation is summarized in Figure 1 and Figure 4, showing the three hydraulically-connected synclines that act as groundwater reservoirs. The largest in size and the one with the largest recharge area is in the west, though it has relatively smaller storage capacity because it lies away from the discharge points and so its level fluctuates more. In contrast, the two smaller synclines to the east have thicker saturated zones and less variable levels, since they are situated close to the system's outlets.

CAVES, SUBMERGED CONDUITS, AND SUBTERRANEAN RIVERS

A number of smaller caves have been recognized in the karst system, including both potholes and caves, but two important ones will provide information about the epiphreatic and phreatic zones of the karst. One of the caves, Majada del Cura, is oriented east-west, and at 3 km it is relatively long and its lower galleries are active. The present-day vadose circulation is in the form of an underground river. In the stretch that clearly flows towards its emergence at Fuentetoba, the river forms a series of waterfalls and rapids interrupting longer sections of mostly free flow, though with a number of local siphons. Seven siphons have been identified in the explored galleries under low-water conditions but there may be other sections that also siphon during high-water periods (Figs. 1d and 2). The river is the main collector of other subterranean inflows into the karst massif. The water flows along the contact between the steeply inclined limestone and marly-limestone beds at the edge of the southern limb of the Pico Frentes syncline (Fig. 4). This gallery lies near the top of the phreatic zone, where speleogenesis is maximized. Its existence provides a natural drain and impedes any rise in water level after significant recharge events.

The other collector is a submerged cave, a pressure conduit associated with the outflow channel that drains the flow through Fuentetoba Spring. The cave is oriented N-S, is 350 m long, and descends through the unsaturated zone of the syncline aquifer to 45 m below the level of this spring (Fig. 3). A characteristic of the large springs of the Upper Cretaceous aquifers in this zone is the presence of phreatic conduits associated with them; the spring at Fuentona de Muriel emerges from a conduit that is more than 110 m deep and 400 m long (Sanz Pérez and Medina Ferrer, 1987), the spring at La Galiana flows from a conduit with some 400 m explored-length (Segovia et al, 2011), and the Fuente Azul de San Pedro de Arlanza in Burgos emerges from a vertical phreatic conduit more than 100 m deep.

RESULTS

HYDROLOGY OF THE MAJADA DEL CURA CAVE

Over four years of exploration (2011–2015), a variety of observations have been made that are of hydrological interest.

In very rainy spells, normally dry siphons in the intermediate galleries are flooded, temporarily impeding access to the deeper, dry galleries that lead to the underground river. The water filters slowly through the base of these flooded chambers and, after a few weeks, they dry out, restoring access to the cave network. The underground flow inside the cave is torrential, and the course includes rapids and waterfalls with siphons and lagoons in between. It drops some 70 m over a reach of 700 m towards the lowest point in the eastern end of the cave, a mere 5 or 10 m above the level of Fuentetoba Spring, even though this point is still some 2.5 km away from the spring (Fig. 4).

The underground flow has been point-gauged under various conditions, and the flow has varied from a few liters per second up to 500 L s^{-1}, although the highest floodwaters have not been recorded. The overall perception gained over the years is that the flow through this cave forms an appreciable proportion of that emerging through Fuentetoba Spring. However, we cannot dismiss the possibility that other secondary supply conduits exist. Nevertheless, during a tracer test inside the cave under low-water conditions (and on other occasions as well), it was observed that the flow through the cave, at the time less than 10 L s^{-1}, was greater than what was emerging at the spring.

Under moderate and high water conditions, the level is seen to rise up to the roof of the galleries and a number of siphons become flooded, so impeding conventional explorations. Moreover, some of the intermediate and upper siphons are flooded. Smooth cave walls with erosion scallops are common, typical of phreatic conduits. In the intermediate stretches of the gallery, there are marks on the walls in some of the chambers that indicate the height that some of the lakes can reach, which is 3 m above the watercourse in some instances. At certain points there are recent terraces of rounded 2 cm gravel 1 m above the present-day course of the underground river, while elsewhere there are flat deposits of sand, with current ripples above pebbles. We have occasionally seen them half a meter below the water surface. There are also potholes or marmites around some subcurrent rimstone dams or gours that are filled with the same kind of pebbles as on the terraces. Some are found some 2.5 m above the actual vadose channel and are generated by flood waves whose mechanical erosive action (corrasion) has been recorded in the breaks of slope in the watercourse at these calcite paleogours due to their relative softness. These geological formations have been preserved unaltered by the ordinary floods of these years when explorations were made, and they provide proof of violent and extraordinary floods in the past. Given the cross-section and slope of the river bed, such floods, which are still possible today, could have easily exceeded 3,000 L s^{-1}.

Once the rain stops, the flow of the underground river rapidly diminishes, and the water level in the lagoons and siphons falls very quickly. During speleological explorations, the water level in this cave is notorious for falling several centimeters or even decimeters from one day to the next, though only rarely is there no flowing water. It has been observed that where the river is above the phreatic level, there is loss due to filtration through the floor that is more significant the higher the gallery lies.

As can be seen from Figure 2, the closest stretch of the explorable underground river to Fuentetoba Spring lies 2.5 km away in a straight line. Its galleries are a series of more or less open U-shaped siphons, which increase in number in the direction of flow and obstruct conventional exploration. Thus it appears that this is the overall trend until they emerge. This is not surprising, since, as mentioned above, there is another submerged conduit that feeds the spring, which almost certainly represents the final stretch of the hypogean river. Observations made during periods of drought show that the water in the siphons closest to Fuentetoba Spring is practically stagnant and the phreatic level is barely 5 to 10 m above the level of the spring, despite being so far away, with a calculated hydraulic gradient of 0.1% to 0.3%, which is insufficient to allow a rapid flow of the groundwater.

The Conduit of Fuentetoba Spring

Fuentetoba Spring (elevation 1,140 m) emerges from a natural flooded gallery. The cave diving explorations were done during the long summer period of low water, when the rising current in the gallery was practically imperceptible. Under these conditions, the divers could proceed without danger. The level of the upper part remained constant at 2 m above the external spring. However, during the spring floods, the upper part of the conduit became inaccessible for conventional speleological explorations.

The conduit that leads to the spring has been explored for a distance of 350 m, with a maximum elevation of 45 m, above the spring, at an elevation of 1,095 m. Over 350 m it rises almost vertically, up to zero level (Fig. 3). This rising exit conduit is tubular, which is very typical of deep phreatic circulation in the saturated zone of typical karstic aquifers. It is a single conduit with a quasi-cylindrical cross section extraordinarily constant in form. Its diameter is 2 m and it dips 20° towards the north. The conduit penetrates quite far, reaching half the depth of the saturated zone at the heart of this small syncline, and thus, it allows efficient drainage of this part of the aquifer. Figure 3 shows the profile of this conduit within the Fuentetoba Syncline.

This cavity reflects the stratification. In the beginning, it would have been established in a particular bank of thick, homogeneous limestone that lies above one of the first marly intercalations that form the impermeable base of the aquifer. It roughly follows the dip direction of the beds, although its inclination is less than the dip of the strata. The conduit developed within this calcareous layer, almost invariably towards the center of the syncline, along a length of 350 m. The end of the gallery is a single tube, whose vertical termination reaches as far as the water level, where it opens out into a chamber.

Throughout the shallower part of the conduit (Fig. 3) and under drought conditions when Fuentetoba Spring is dis-

charging around 10 to 50 L s^{-1}, the water level lies some 2 m above the spring. This is because the water escapes sideways through a crevice, discharges into an underground chamber, and emerges between the blocks and debris on the slopes of the Sierra de Pico Frentes (Fig. 3). However, the dry conduit persists for a few meters more until it opens onto the hillside. This outlet, situated 5 m above the spring and 2 m above the ponding (low-water) level of the conduit, contains water during very large floods (between 1,500 L s^{-1} and 2,000 L s^{-1}), acting as an overflow route and disgorging a flow of up to 100 L s^{-1}. Under such severe flood conditions, another outflow also comes into play. This lies below the overflow passage and communicates with the chamber referred to above (Fig. 3). It is funnel-shaped, and so water spurts out under pressure. Sediment on the bed of this spring is made up solely of limestone cobbles between 1 and 35 cm diameter. The pebbles are extraordinarily well rounded and polished. As the spring flow diminishes, the water level in the conduit and spring falls rapidly by several decimetres a day in line with the very fast emptying regime of this resurgence.

The main conduit contains no sediment deposits, except in its lower part where there are a few rounded pebbles. The walls of the tube have been subject to corrosion and have been sculpted all over with dissolution scallops, ranging from centimeters to decimeters in size (mean 20 cm). This has produced smooth surfaces that have got an undulating micro-morphology. The fact that the gallery contains no fillings indicates an active high-velocity circulation dominated by corrosion, in which sedimentation of sand and clays is impeded.

FUENTETOBA SPRING DISCHARGE HYDROGRAPH

In our case study, we assumed that the whole flow draining through the karst system was monitored over two hydrological years (2010–2011 and 2011–2012), though there may have been additional, diffuse subterranean outflows through fractures penetrating the marly base of the aquifer and transferring water to the sands of the Utrillas Facies and perhaps also small filtrations through the tuffs around the springs.

Over the two-year study period, a wide variety of hydrological situations occurred, ranging from prolonged drought to intense rainfall, which enabled diverse characteristics of this drainage to be analyzed. During the hydrological year 2010–2011, mean flow at this spring was around 200 L s^{-1}, falling to 8 L s^{-1} during the dry season and rising to as much as 3,400 L s^{-1} after flood events. Thus, the spring's flow is highly irregular, is sensitive to the dry season, and has a relatively rapid response in one or two days to rainfall and snowmelt (Fig. 5). The sharp hydrograph peaks also indicate major development of karstic conduits, where conduit permeability may be predominant (Bonacci and Zivaljevic, 1993; Bonacci, 1993; Worthington, 1999).

After any dry period, short or long, we have observed that there is a delay of one or two days before the spring reacts to normal rainy periods. This lag is interpreted to reflect the time for the bulk of the wave in the vadose zone to arrive from the two synclines of Villaciervos and Alto de la Cruz. The rapid pressure pulses through the karstic conduits in the saturated zone are hardly noticeable in the ascending limb of the hydrograph peaks. However, these pressure pulses are prominently manifest in the siphoning behavior of the spring, as is observed from the operation of the underwater gallery and the overflow passage associated with it.

The hydrograph of the spring manifests two kinds of emptying. In the first kind, the bulk of the groundwater empties very quickly under a predominantly turbulent regime, as indicated by the slope of the recession curve on semi-logarithmic paper ($\alpha_1 = 0.18$ d^{-1}). The mean flow at the beginning of the recession curve is 800 L s^{-1} (70×10^3 m^3 d^{-1}). The second kind of emptying is the curve that occurs with very low flows, normally below 25 L s^{-1}. It represents the emptying through both large and small conduits, as we were able to observe in the cave-dive surveys. The slope of the hydrograph is very shallow ($\alpha_2 = 0.008$ d^{-1}), which means that the spring does not stop flowing. The mean flow at the start of the recession curve with α_2 is 23 L s^{-1} (2,000 m^3 d^{-1}),

Several composite hydrograph recessions have been observed. Following the usual recession curve analysis (Ford and Williams, 2007), the expression obtained for this case is

$$Q_t = Q_{01}\, e^{-\alpha_1 t} + Q_{02}\, e^{-\alpha_2 t} = 70 \times 10^3\, e^{-0.18t} + 2,000\, e^{-0.008t} \quad (1)$$

where flow Q is expressed in m^3/day and time t in days.

$$V_1 = \frac{Q_{01}}{\alpha_1} = \frac{70 \times 10^3}{0.18} = 4.0 \times 10^5\ m^3 \quad (2)$$

$$V_1 = \frac{Q_{02}}{\alpha_2} = \frac{2,000}{0.008} = 3.0 \times 10^5\ m^3 \quad (3)$$

$$V = V_1 + V_2 = 7.0 \times 10^5 m^3 \quad (4)$$

DISCHARGE HYDROGRAPH OF THE SIPHONING FUENTETOBA SPRING

Figure 5 shows the gauged and simulated hydrograph (Rosas et al., 2016) of the Fuentetoba spring for the hydrological years 2010–2011 and 2011–2012, the latter obtained using the unicellular mathematical model CREC (Guilbot, 1975). It indicates that when precipitation and natural recharge are small, the gauged outflow at the spring is less than simulated. However, the gauged outflow is lower than the simulation when precipitation is greater. These are relatively small, but clearly visible differences as seen from the B(–) and D(–) expanded periods in Figure 5. One must take into account that CREC is a model of regional implementation based on water balance on soil and black-box with reservoirs. The model does not include various things observed in other karst systems, such as constriction

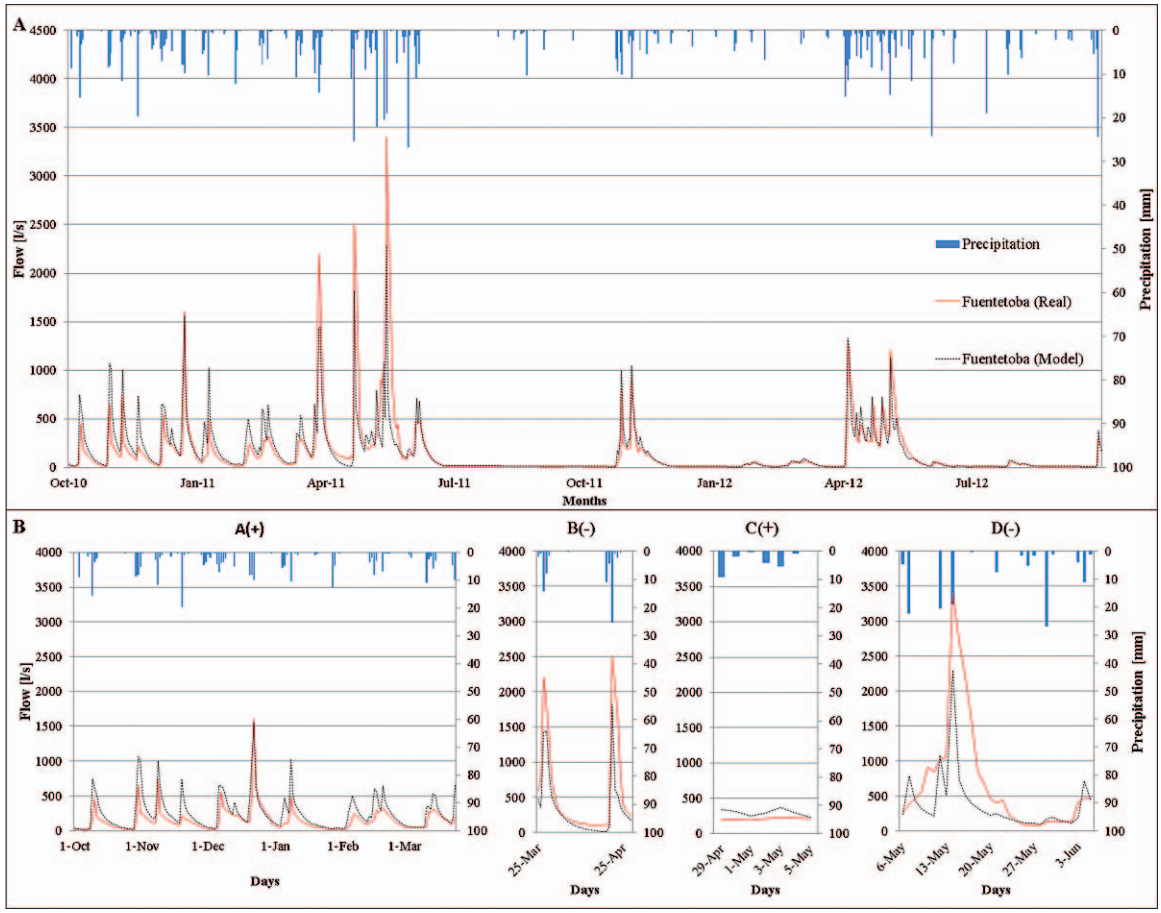

Figure 5. A. Actual and simulated hydrographs of the Fuentetoba Spring for 2012-2013 with precipitation record for comparison. The periods of water storage and water deficit are expanded. They are interpreted as being due to impulsion under pressure and siphoning. B. Showing the fact that there is an ebb-and-flow phenomena originated some distance away.

phenomena, plugs of sediment in the conduits, siphoning, or any other mechanism involving long response times to recharge.

It has also been observed that during precipitation events when there is intense and significant recharge, the volume of water issuing from the spring is greater than the amount predicted by hydrograph modelling; see the periods highlighted (–) in Figure 5. Between March 25, 2011 and April 18, 2011, the actual spring flow was 0.33×10^6 m^3 more than calculated by the model. Between the May 9, 2011 and May 20, 2011, the measured discharge was 0.68×10^6 m^3 more, and between May 30, 2011 and June 7, 2011 the gauged flow was 0.36×10^6 m^3 higher. These periods of anomalous flow are short, one to two weeks at most, and this phenomenon occurred three times in 2011. Given the strong karstification of the aquifer and its very low inertia, it would be reasonable to think that the same volume of water recharged would be expelled after a short time, but not a smaller or larger amount.

A relatively important issue in applying the mathematical model was the impossibility of simulating some of the peaks under high and low flow conditions in detail. For the purposes of the hydraulic balance, this does not matter much. However, we believe that the anomalies may be attributable to the peculiarity of the hydrogeological karst and that the model failed because it is designed to simulate behavior of karstic aquifers.

This invites the question whether the aquifer has a larger catchment than inferred. However, this is not the case, since its recharge area is very well defined (Rosas, 2013). It cannot be fully explained by the cumulative effect of a snowmelt, shifted in time, because precipitation falling as snow is not considerable and this phenomenon is repeated consistently. According to the mathematical model, while during two events there was no snow, much of the reserves (approximately 1.0×10^6 m^3) were produced corresponding with several snowfalls (December 2009 and March 2010).

Our explanation for both phenomena together is that part of the water coming from rainfall during dry periods is stored in the aquifer and that this same water emerges from the aquifer during wet periods. This is suggested by some of the direct observations made inside the caves (see above) of galleries in the unsaturated zone that temporarily retain the water in

Table 1. Injections conditions for the uranine tracer test at Cave of Majada del Cura, 2,500 m from Fuentetoba.

	Tracer Injection Period				
Injection No.	Date	Time, h	Tracer	Tracer Mass, g	Stream Flow, L s^{-1}
1	July 14, 2012	1400	uranine	35	3.12
			NaCl	20,000	
2	Oct. 16, 2012	1900	uranine	35	5
3	May 17, 2012	1800	uranine	100	4

hanging lagoons and the presence of a reservoir of quasi-stagnant groundwater in low-water periods in the last of the three synclines at Fuentetoba. Moreover, we have confirmed the establishment of siphons with associated air chambers and lagoons upstream of the spring, which would serve as reservoirs prone to siphoning.

We observed that this groundwater reservoir in the Fuentetoba Syncline is rapidly increased in high-water periods due to the inflow from the underground river in Majada del Cura cave and other unknown sources. It provokes an excessive rise in the phreatic level and in the hydraulic head in the submerged galleries and so increases the water velocity and volume issuing from the spring. We also confirmed this hydrology using tracer tests.

Discussion of the Tracer Results

Three chemical tracer tests were done during 2012 and 2013 (Table 1) to verify the connection between the discharge points and the existing springs, as well as to measure the groundwater flow velocities. The tracer used was uranine, classically employed in karst environments (Käss, 1998), as well as NaCl once. The quantities of tracer used were 35 g, 35 g, and 100 g of uranine each for the three tracer tests, and 20 kg of NaCl for the first tracer test. Given that there are no permanent surface watercourses and only rarely ephemeral ones, the chosen point of injection was the underground river in the Majada del Cura Cave.

The Fuentetoba Spring was monitored by taking two samples per day. Water samples were stored in 100 ml plastic bottles and refrigerated in darkness to minimize any microbial or photo degradation. Spectrometry was done very shortly after sampling. To determine the true uranine concentration in each test, all samples were analyzed and compared with a spring water sample taken before the survey, and care was taken to separate the various tests sufficiently over time to avoid interference from earlier tests.

Three tests were done in the underground river in the cave at Majada del Cura during low-water periods, although later, there were sudden changes due to rainfall. The tests were repeated several times because at the time we did not suspect any siphoning behavior and the monitoring time was set too short and no tracer was detected at the outlet. It was expected that the tracer would reappear rapidly, corresponding to the velocity of the underground river under turbulent regime and, in any case, with the same velocity as measured in the similar aquifer of Fuentona de Muriel, where flow velocity is 500 m/day under low-water conditions and 3,000 m/day under high-water conditions (Pérez and Sanz, 2011). However, this was not the case.

In terms of the three tests conducted in the Majada del Cura cave, which lies some 2,500 m from the Fuentetoba Spring, we made the following interpretation. The first test was done during the dry season when there was very little flow in the underground river. The tracer water was held back in the galleries of the syncline or travelled very slowly and almost certainly emerged after a rainy period. During the second test, the tracer was again held back and was remobilized by a new flood flowing through the system several days after the injection. The same thing occurred during the third test following a rainy spell after the test. The tracer appeared later. We assume the recharge into the aquifer from this rain event carried the remaining diluted tracer along the hypogean river and was held up in the standing-water zones (Fig. 6).

There are no outflows between the injection point into the subterranean river and Fuentetoba Spring. The recovery of the tracer was high (85%) but incomplete; this could well have been due to measurement errors or small, unmonitored and unquantifiable seepages in the vicinity of the spring.

Overall, the tracer tests demonstrated that in the last of the three synclines, before issuing from Fuentetoba Spring, part of the groundwater flow became ponded in siphons and lagoons due to the low-water conditions. It emerged later when there was a significant impulsion of recharge water. The tracer tests also delimited this (siphoning) phenomenon to the 3,000 m stretch between the end of the explored part of the cave and the spring, through the whole of the small, elongated Fuentetoba syncline. However, we cannot discount that there may be other siphons operating upstream of the cave, as stated above. At least in this segment of the aquifer, it does not make sense to speak of groundwater velocity, because this velocity depends on when the recharge event occurs, a characteristic observed in other karstic aquifers (Field and Pinsky, 2000; Goldscheider N., 2005; Goldscheider N., 2008).

Discussion

The long response times to recharge into the Fuentetoba karst seen in the hydrograph of the spring are a result of regional effects that have been simulated in a reasonably satisfactory way. The prolonged tracer tests that were done in

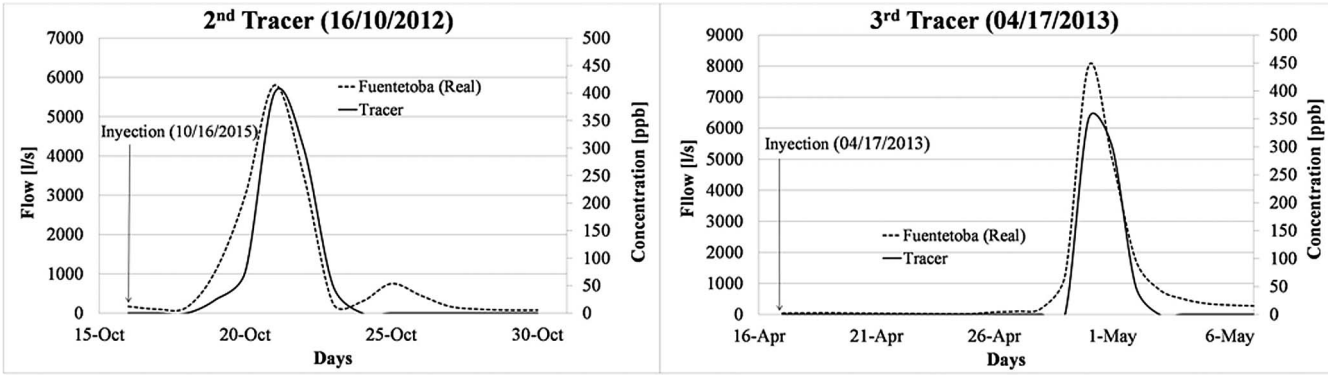

Figure 6. Curves of concentration against time for the tracer tests undertaken in the Fuentetoba Syncline, between the underground river and the spring.

the Villaciervos syncline that demonstrate the connection with Fuentetoba Spring have given the best overall representation of the drainage of this system (Rosas et al., 2016).

Nevertheless, some responses with a very long time-lag that manifest under high-water conditions are seen in a qualitative way in the hydrograph, but they could not be quantified using numerical modelling. The mechanism governing this response must be a non-Darcian physical model under a turbulent flow regime that occurs following rainy periods in the final stretch of the flowpath within this karst system, that is to say, in the Fuentetoba syncline. Its location has been narrowed down to this final stretch by the results of the short-distance tracer tests that were done in the conduit of the subterranean river in the extreme west of this syncline. It is not only the tracer tests that verify the phenomenon, but also the fact that if these ebb-and-flow phenomena (Figure 5.B) originated some distance away, their effects would be diminished by the time they reached the spring.

The characteristics of the system through the conduits were made evident by the ponding of the tracer water for days, weeks, and months and then a rapid emptying during periods of flood. Nevertheless, the spring continued to issue a smaller flow during the recession phase, which must be due to matrix flow through the syncline along hydraulic gradients that are distinct from those of the conduit flow.

Figure 7 explains the model proposed for the drainage of this karstic system. It is a model of three successive reservoirs that exist as hydraulically connected synclines by means of a subterranean river. It has to assume that the synclines accommodate different types of flow (matrix, fracture, and conduit flow), as if there were other virtual reservoirs with high and low permeability. The reality is undoubtedly more complex. For example, during the speleological explorations, there was evidence of water storage in the unsaturated zone conduits as small hanging pools that are seasonal by nature and that empty quite slowly. This is what happens in the temporal lake in Cueva de Villaciervos in the epikarst zone, where the bed of the lake is clayey, as indicated by Sanz and López (2000).

The general outline for this network indicates that an active channel hydraulically connects the various parts of the aquifer, behaving under moderate and high-water conditions as a drain from the synclines of Villaciervos and Alto de la Cruz, towards the Fuentetoba syncline. In detail, it can be seen how the preferential flowpaths of the hypogean circulation have become well established along a particular layer of limestone that abuts the sharp crest of the Fuentetoba anticline (Figures 3 and 4). These limestone layers, running east-west and dipping between 45° and 65° on its northern limb, determine that the conduit network is very rectilinear and follows the direction of the strata (Fig. 3). The limestone is exactly the same as the one at Fuentetoba Spring. It is very probable that the network of explored galleries continues in a straight line eastward towards this spring, which is the likely final destination of the hypogean river, having flowed through a succession of numerous siphons (Figs. 2 and 7)

Figure 2 explains how this phenomenon could occur. It can be observed that, although the recharge of quite rainy spells is uniformly spread out, 50% of the recharge surface of the entire aquifer lies over the syncline farthest from Villaciervos. In this section, there is an inordinate elevation of the phreatic level due to the concentration of recharge water coming from the limbs of the fold lying above the unsaturated zone. Under very high water periods, the phreatic level overtops the topography of a valley and creates the ephemeral spring of Las Fuentes (Villaciervos Spring in Fig. 1). However, the level in the Fuentetoba syncline does not vary because of its proximity to the outlet and the lack of a significant hydraulic gradient, which means that the velocity in this section of the aquifer is low; under low-water conditions, it can be practically zero.

We have not yet observed pressure propagation from these pulses of local recharge, since the hydraulic connection between these synclines is not very large (as is clear from Fig. 1b) because it is impeded by the throw of the Ocenilla Fault. The existence of the underground river in the cave indicates

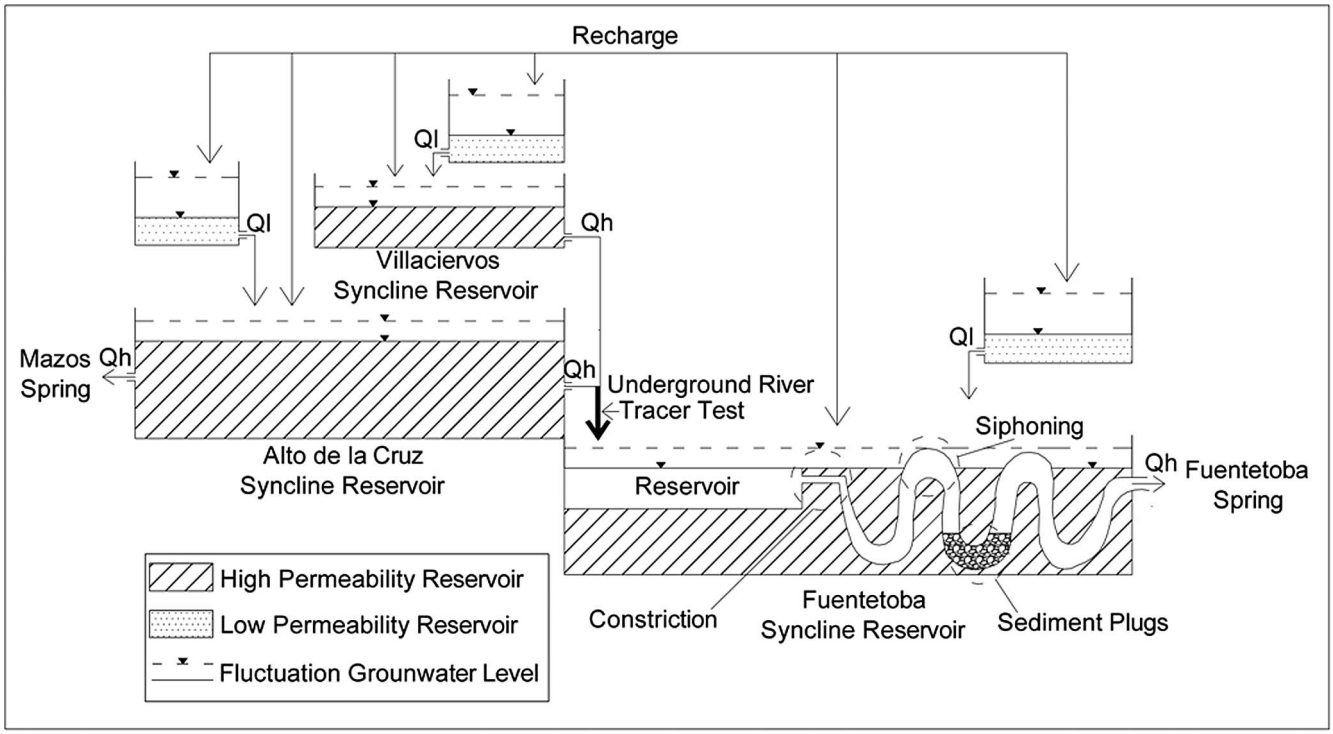

Figure 7. Simplified schematic model in which the three reservoirs in the three synclines in the karst system of Fuentetoba Spring are shown. It is assumed that the flow in each one is distributed in two deposits of high and low permeability. In the last reservoir (Syncline Fuentetoba) there are three potential hypothesis of the delayed flow: siphoning, sediment plugs, and passage constrictions, or a combination of those. Qh: Outflow coming from the high permeability reservoir; Ql: Outflow coming from the low permeability reservoir. The location of the tracer injection in the underground river is shown.

that there is basically a free-flowing, fluvial regime as far as the start of the Fuentetoba Syncline.

It has been observed how this current increases rapidly a day or two after heavy rain. This hypogean network behaves in a similar fashion to a dendritic drainage network of a torrential river, capturing the recharged water into the synclines farther upstream. The flood wave coming from the syncline farthest from Villaciervos passes through the Ocenilla Fault and is redirected through the cave.

During periods of recession, the water flowing along the subterranean river is 4 L s^{-1} more than what emerges at Fuentetoba Spring. Our interpretation is that on these occasions the water is being impounded or stored before it reaches the spring.

From the end of the explored section of this subterranean river, a syncline extends over the 3 km distance to Fuentetoba. The impounded groundwater is calculated to be between 2.76 $\times$ 10^6 m^3 and 3.68 $\times$ 10^6 m^3 (Rosas et al., 2016), has practically no gradient, and is stagnant. Here, there must be numerous submerged conduits and siphons where the movement and response to floods must be activated to a large extent through pressure propagation. When the bulk of the kinematic flood of the subterranean stream enters this area, which is as yet unexplored, it should produce an elevation of the water level in the siphons and intermediate lagoons, flooding them, especially the narrow passages. At this final area, there are various possible explanations for the anomalous behavior of the system's drainage and, in fact, a combination of these could be acting, since no one possibility contradicts any other.

Observations over the final accessible stretch of the conduit that leads to the spring confirm that there are no sediment plugs, nor even any bottom sediment, and it is not known for this spring to expel sediments or debris. Its waters are almost always very clear, at least over the four years of observations. Outside, there are neither terraces nor deposits that have been expelled by the spring, only calcareous tuffs. The transport of sediments by the subterranean river under normal flood conditions must be very small, as observed during the speleological surveys, since the current is not turbid, nor does it contain a significant sand load. It should be noted that the catchment of the subterranean river is autogenous and that there is no external sediment source. Nevertheless, there are occasional hanging banks of sand or rounded pebbles that have been deposited during exceptional floods, which testify to the presence of a sediment load that must be deposited in the lower parts of the conduits of the lowest syncline of Fuentetoba. There have been no direct observations of

changes in water level in the final stretch of the underground torrent, nor marks or sediment terraces that would belie a sudden rise in level caused by a plug farther downstream. However, since the network of galleries that must exist between the explored part of the cave containing the underground river and the spring, nearly 3 km away, is unknown, we cannot reject the possibility that gravel or sand plugs are present at the base of other, unexplored siphons, which are not ejected by the spring but enter suspension and later settle out again.

Another possible explanation would be the existence of constrictions in the conduits, as actually happens in several siphons of this subterranean torrent, where the variations in water level in the associated reservoirs upstream can be as much as 2 or 3 m during high water periods. A similar thing could also occur farther downstream. However, there is no physical evidence of this happening, nor have we seen any large oscillations in water level in the final stretch of the underground torrent. Neither have we encountered sediments due to a decrease in flow upstream of the hypothetical constrictions. Nevertheless, to dismiss the constrictions hypothesis, the network of unexplored galleries needs to be better understood.

The 700 m or so of explored subterranean river contains seven small siphons, and the explored section of the submerged conduit just upstream of the spring contains the beginning of a large siphon. These observations suggest that the network of conduits in the intermediate section is likely linked by means of a series of siphons. If there were inverted siphons in the unexplored stretch, they could be primed by stream flow during periods of flood, producing suction of water from the lagoons and flooded galleries, so emptying a large volume of ponded water in a short time. This would mean that the flow discharged would be greater than the volume coming from the recharge wave. This could plausibly be the predominant mechanism, without needing to dismiss the other mechanisms described above.

Moreover, given the dimension of the outlet at Fuentetoba Spring, the outlet must be very sensitive to pressure variations, and an excess-pressure of a few centimeters at its base would be enough to provoke an upward flow as far as the overflow outlet. The known tube behind the spring is about 350 m long and reaches 42 m deep. It discharges about 50 L s^{-1} with an average hydraulic diameter of 1.6 m. With greater discharges the flow becomes stronger.

The increases in water level due to the recharge from intense rainfall events or snow melt, means that there is an increase in hydraulic head between the lower and upper ends of the tube. This increases the flow and its velocity. We conclude that the system presents very large variations in both flow and pressure, and that small differences in pressure caused by the transmission of pressure waves due to recharge into the syncline are sufficient to mobilize the old, stagnant water held in the outlet-conduits of the spring.

According to Curl (1966) the mean paleovelocity can be calculated from the scallop length and the hydraulic diameter of the outflow conduit. For Fuentetoba Spring, this gives a result of 10 to 15 cm s^{-1}, which is faster than the critical velocity for laminar flow in a cylindrical conduit 1.5 m in diameter and with a 15° slope. In other words, the paleoregime of this conduit was turbulent, and its velocity would be approximately equivalent to a flow of between 800 L s^{-1} and 900 L s^{-1}.

This expulsion of old stagnant water under pressure close to the outlet spring has been observed in many karstic systems (Yevjevich, 1981), but the siphoning phenomenon, the regular evacuation through an inverse U-shaped siphon, occurs only rarely, such as at the source of the river Mundo in Spain (Rodríguez-Estrella et al., 2002). This explains the surplus flows and the remobilization of semi-stagnant water which, in our case, was tinged with fluorescein from the tracer tests. The reservoirs emptied by the siphoning are refilled following non-torrential rain events, then they discharge again. A simple emptying calculation of an assumed 3,000 m long gallery with a 10 m^2 cross-section, a similar continuation to the Cave of Majada del Cura, would involve 30,000 m^3. The Fuentetoba siphon alone would give a volume of around 1,500 m^3. It is easy to conjecture a network of conduits through the entire syncline that would explain the calculated volumes of between 0.33×10^6 m^3 and 0.68×10^6 m^3 issuing from the siphons in 2011.

Conclusions

The overall drainage of the karst in the Fuentetoba Spring system and the wide amplitude of the hydrograph following recharge are determined by regional effects and by the complex geology comprising three synclines that are hydraulically connected by means of a fault and a subterranean river. Certain responses with a very long time-lag manifest under high water conditions and suggest a system with a quite rare hydrological behavior.

To understand the karstic drainage system of Fuentetoba Spring better, we monitored the emerging flow. In the interior of a cave, we made direct observations by conventional caving and by cave diving of an important and representative part of the conduits in the karst.

The hydrograph of the spring has a very pronounced variability, with sharp peaks and rapid emptying. There is no inertia, and the spring does not maintain hydrodynamically significant volumes for any period of time. This is not only a consequence of the small recharge area of the aquifer, but also of the network of conduits that carry predominantly turbulent flow through a very well developed karst system.

The flow in the vadose zone is characterized by an underground river that is torrential in nature and consists of free-flowing sections over a series of rapids, waterfalls, and lakes, with intervening stretches of forced phreatic flow in sumps that are most significant in the epiphreatic zone. In the phreatic zone, forced deep conduction through large conduits seems to predominate. The outflow at the spring is through a

large-diameter conduit with a high discharge capacity, and the flow quickly goes from laminar to turbulent regime and is very sensitive to changes in the groundwater head motivated by flood events. Erosion marks along this outflow tube and associated gravel deposits indicate high velocities and flows exceeding 1000 L s^{-1}.

According to the hydrograph analysis and the tracer tests, part of the recharge water during dry periods is stored through the dry period in the last of the three synclines, where the subterranean stream ponds up. This water is later released during wet periods. The responses to these recharge events following low water periods could be governed in the final stretch by mechanisms of impulsion under pressure or by siphoning of the flood wave, since we assume that the siphon geometry continues through the unexplored part of the karst. However, other possible mechanisms cannot be rejected, such as constrictions and sediment plugs in the conduits.

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LACK OF CAVE-ASSOCIATED MAMMALS INFLUENCES THE FUNGAL ASSEMBLAGES OF INSULAR SOLUTION CAVES IN EASTERN CANADA

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Abstract: The biogeography of cave fungi and factors that influence community composition are poorly known. The movement of animals into caves from the outside environment is thought to be one factor that affects cave mycota. Islands often have different faunal assemblages from the mainland, and this may affect the fungal diversity of island caves. In 2014 we swabbed walls in three natural solution caves on Anticosti Island, Quebec, to determine the composition of cave fungal assemblages present relative to well-studied nearby mainland sites. At least one of these caves, Grotte à la Patate in Anticosti National Park, appears to support overwintering bats. Culture-dependent methods were used to establish pure cultures, and fungi were identified by a combination of morphology and genetic sequencing. A total of 54 fungal taxa were identified, with a mean of 7.4 ± 3.9 taxa per swab. The most common taxa isolated were *Penicillium* spp., *Pseudogymnoascus pannorum* sensu lato, *P. roseus*, *Trichoderma* sp., *Cladosporium* spp., *Thysanophora* spp., *Mucor* sp., and *Trichosporon dulcitum*. *Pseudogymnoascus destructans* (*Pd*), the causative agent of the fungal disease white-nose syndrome in bats, was not detected, and we conclude that *Pd* was not present in the three sampled caves as of summer 2014. Two of the caves did not appear to be suitable bat hibernacula based on microclimate, although diverse fungal assemblages were detected on the walls. Several other fungal taxa common to bat hibernacula on the mainland, in addition to *Pd*, were lacking from Anticosti Island caves. We suggest that fungal assemblages on Anticosti Island are influenced by the absence of non-volant cave-visiting mammals on the island, particularly porcupines (*Erethizon dorsatum*) and raccoons (*Procyon lotor*), both frequent cave associates elsewhere in Maritime Canada.

Introduction

Little is known about the biogeography of fungi in caves and those factors that influence the composition of the mycological community in such habitats. Fungi present in caves are generally introduced from the non-subterranean environment by water, air, and fauna; it has yet to be determined if endemic cave fungal species exist (Vanderwolf et al., 2013a). Insects, mammals, and their associated dung are thought to influence the diversity of cave mycota by introducing spores from the surface environment and by providing a substrate for fungal growth within caves (Min, 1988; Dickson, 1975). There is evidence that some fungal species, when present in caves, are associated with specific fauna. For instance, several fungal species are known to be associated with insects in caves, such as entomopathogenic fungi (Yoder et al., 2009).

Anticosti Island, located in the Gulf of St. Lawrence, Quebec, Canada, 35 to 74 km from the mainland, is populated by only five non-volant native mammals, although at least twelve other mammal species have been introduced, either successfully or unsuccessfully, since European settlement of the region (Cameron, 1958). Acoustic surveys conducted in Anticosti National Park in August–September 2007 have confirmed the presence of native bats, including *Myotis* spp. (*M. lucifugus* or *M. septentrionalis*), *Lasiurus cinereus*, and *L. borealis* (Plamondon 2009). Several caves on Anticosti Island have been reported as potential bat hibernacula, but winter access is difficult, and bats have been observed overwintering in only a single cave on the island, Grotte à la Patate (Julien Mainguy, Ministère des Ressources naturelles et de la Faune, per. comm. to DFM April 2010). Nonetheless, an extensive karst topography suggests that other bat hibernacula may exist on the island. Non-volant mammals that are known to frequently enter caves on the adjacent mainland, including *Erethizon dorsatum* (porcupine), mustelids, and *Procyon lotor* (raccoon), are absent on Anticosti Island (Newsom, 1937; Cameron, 1958; Gaetan Laprise, Québec Ministère des Forêts, de la Faune et des Parcs per comm. to KJV and DFM). However, deer mice (*Peromyscus maniculatus*), known to enter mainland caves in the region, are present (Newsom, 1937; Cameron, 1958; Trevor-Deutsch, 1973; Darmon et al., 2013). The arthropod diversity in caves on Anticosti Island is unknown.

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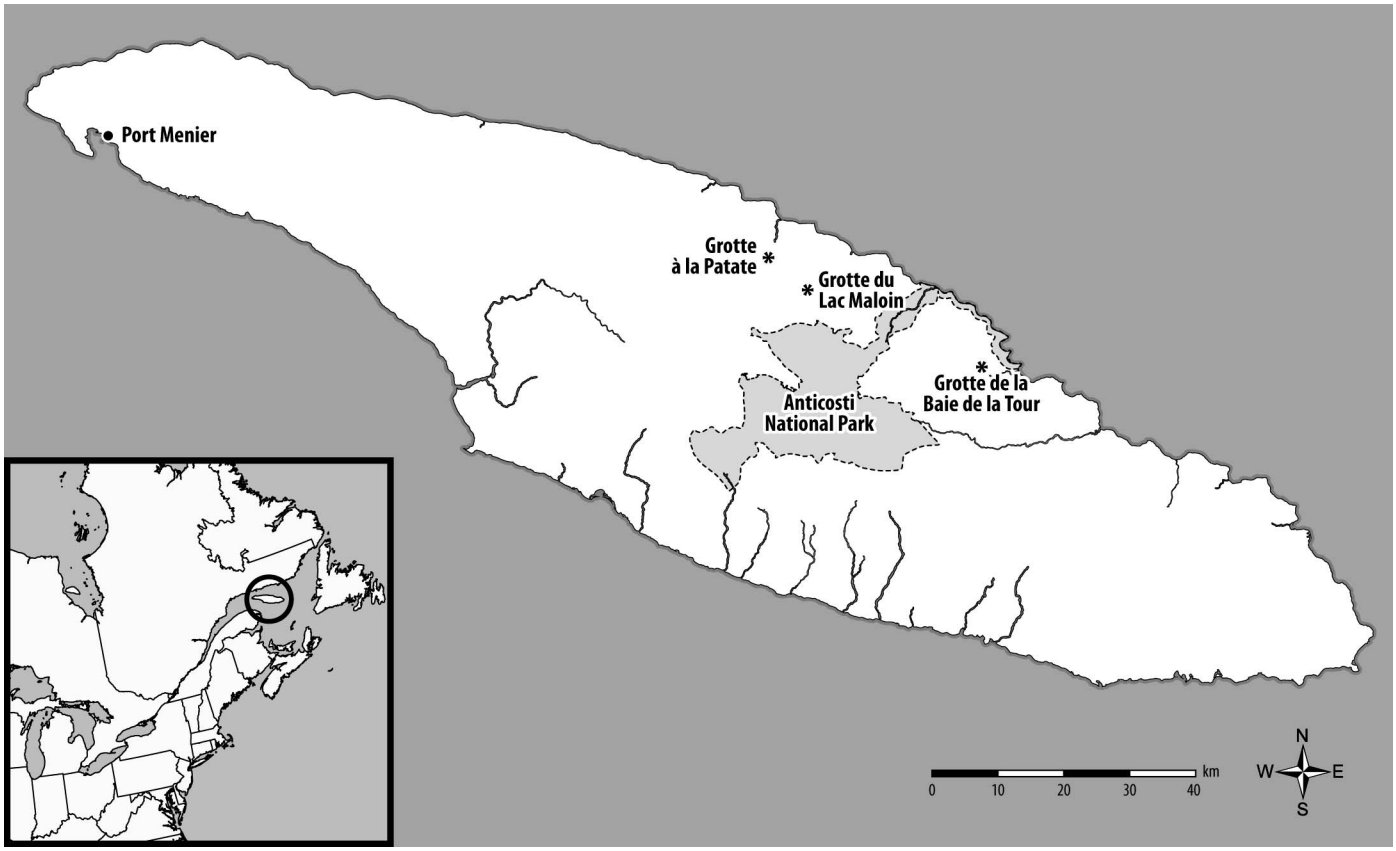

Figure 1. Anticosti Island, Quebec, with the location of the three study caves marked with asterisks. Port Menier is the only permanent settlement on the island.

The fungal assemblage in caves on the mainland in nearby New Brunswick has been relatively intensively studied (Vanderwolf et al., 2013b, 2016a). Our objectives were to document the mycota in caves on Anticosti Island and compare these to previous studies in caves in New Brunswick and to assess factors that may influence the fungal assemblage in the caves, such as microclimate and the presence of mammals, running water, and arthropods.

Methods

Site Description

Anticosti Island is 7943 km^2 in area, with a bedrock of Silurian and Ordovician limestone. The island is separated from the mainlands of Quebec and New Brunswick by marine straits 35 to 74 km wide. Although the human population of Anticosti Island can double in the summer (Danièle Morin, Quebec Ministry of Natural Resources, per. comm. to KJV and DFM), the only permanent settlement is Port-Menier, on the western end of the island, which supports a year-round population of about 250 people. The mean temperature on the island 2010–2014 was 3.48 °C ± 1.1SD, with a minimum average of –10.3 ± 4.0 °C in February to a maximum of 16.9 ± 0.9 °C in July (Environment Canada, 2015).

We visited three natural limestone caves on Anticosti Island in July 2014 (Fig. 1). We followed the protocol of the United States Fish and Wildlife Service (2012) for minimizing the spread of *Pseudogymnoascus destructans*, the agent of the fungal disease white-nose syndrome in bats, during all visits to caves on Anticosti Island. Point measurements of cave temperature and relative humidity were taken in each cave using a Kestral 3000 Pocket Weather Meter (part# 0830FOR; Nielsen-Kellerman, Boothwyn, PA). Each cave was surveyed for the presence of bat carcasses, guano, and signs of invertebrates and other vertebrates.

Grotte à la Patate, located in Anticosti National Park (N49.6545°, W62.9503°), was visited July 7, 2014. This cave is the third largest in Quebec, with at least 625 m of passage and the largest underground room (Fig. 2) in the province (map in Roberge et al., 1985; Lauriol et al., 1987). The cave walls are muddy and composed of fine-grained siltstone and Ordovician limestone. An active stream, with multiple small waterfalls along its length, flows through the main passage and out the large entrance. Approximately 50 to 70 roosting bats (likely *Myotis lucifugus* and *M. septentrionalis*) were reported in the cave October 1988, specifically in an offshoot from the main passage with no running or standing water (Danièle Morin, Québec Ministère des Forêts, de la Faune et des Parcs, per. comm. to KJV and DFM). The offshoot starts approx-

Figure 2. (A) entrance and (B) passage in Grotte à la Patate. Note that the entrance and large main passage are readily accessible to terrestrial mammals.

imately 55 m from the entrance. Regular public tours of this cave are conducted by national park staff during the summer months, and the location is well publicized in the tourist literature. Approximately one thousand tourists visit the park during the summer, several hundred of whom visit the cave annually (Anticosti National Park staff, per. comm. to DFM and KJV).

Grotte de la Baie de la Tour (N49.5043°, W62.4980°) was visited July 9, 2014. The cave has approximately 270 m (Danièle Morin, Québec Ministère des Forêts, de la Faune et des Parcs, per. comm. to KJV and DFM) of narrow, high passage with an active stream and multiple small waterfalls along its length. Debris and foam (Fig. 3) high on the walls suggests the cave passage fills with water in spring and after heavy rains. The cave is well marked and close to a road, with a small viewing platform overlooking the entrance. Waterfalls spanning the entrance to the cave likely discourage most visitors.

Grotte du lac Maloin (N49.61042°, W62.87001°) was visited July 11, 2014. This cave has about 50 m of mostly low, narrow passage with an active stream featuring multiple small waterfalls and several flowstone features (Fig. 4). The integrity of the entrance and speleothems indicates the cave is seldom visited. Detritus observed on the cave ceiling suggests the entire passage floods on occasion.

Fungal Sampling

Walls in the dark zone of the caves were swabbed with a sterile, dry cotton-tipped applicator. In Grotte à la Patate swabs were taken at the back of the offshoot some 70 m from the entrance (Roberge et al., 1985), an area where roosting bats had been reported and where we observed bat guano on the walls and floor. In Grotte de la Baie de la Tour and Grotte du lac Maloin swabs were collected as far into the cave as was accessible; estimated at 50 to 100 m and about 30 m, respectively, from the entrances. Each applicator was rubbed multiple times over a different 10 by 10 cm area of the wall, with a new applicator used for each swab. After swabbing, the applicator was immediately streaked across the agar surface in a petri plate. Diluting streaks were completed in the cave

Figure 3. Entrance (A) to and passage (B) in Grotte de la Baie de la Tour. Note foam from recent high water high on the cave wall near the ceiling, circled. Foam selectively traps the conidia of a wide variety of fungal species, while severe intermittent flooding in this cave probably precludes use by hibernating bats.

within 1 h of the initial streak, after which plates were sealed in situ with Parafilm (Pechiney Plastic Packaging, Chicago, IL). Dextrose-peptone-yeast extract agar (DPYA) was used (Papavizas and Davey, 1959), infused with the antibiotics chlortetracycline (30 mg/L) and streptomycin (30 mg/L). We have previously documented this as a superior medium for isolating *P. destructans* from the environment (Vanderwolf et al., 2016b).

In the laboratory, samples were incubated, inverted, in the dark at 7 °C in a low-temperature incubator (Model 2015, VWR International, Mississauga, ON, Canada), to approximate the subterranean environment. Samples were monitored over four months until no new cultures had appeared for three weeks on a plate or the plate had become overgrown with hyphae. Once fungi began growing on the plates, each distinct colony was subcultured to a new plate. DPYA without oxgall and sodium propionate was used for maintaining pure cultures.

DNA Extraction

Fungal plugs were collected using sterile techniques from the pure fungal cultures. Total genomic DNA was extracted using plant-DNA extraction protocol (Ivanova et al., 2008) with minor modifications. In brief, ethanol-fixed tissue was transferred in a tube rack and dried at 56 °C. Dried tissue was homogenized with a TissueLyser (Qiagen GmbH, Hilden, Germany) using 3 mm tungsten carbide beads (Qiagen) and sterile sand at 30 Hz for 1 min. A volume of 100 µL of ILB buffer with Proteinase K (700 mM GuSCN, 30 mM EDTA pH 8.0, 30 mM Tris–HCl pH 8.0, 0.5% Triton X-100, 5% Tween-20, and 2 mg/mL Proteinase K) and was added to each sample. Samples were incubated at 56 °C for 1 hour, and 200 µL of PBB1 buffer (Ivanova et al., 2008) was added to each sample followed by an incubation at 65 °C for 30 min. A volume of 150 µL of each lysate was transferred into a well in a 1 mL Acroprep 96-well plate with 1 µm glass fiber media (Pall Life Sciences, Ann Arbor, MI, USA). The wash stages followed

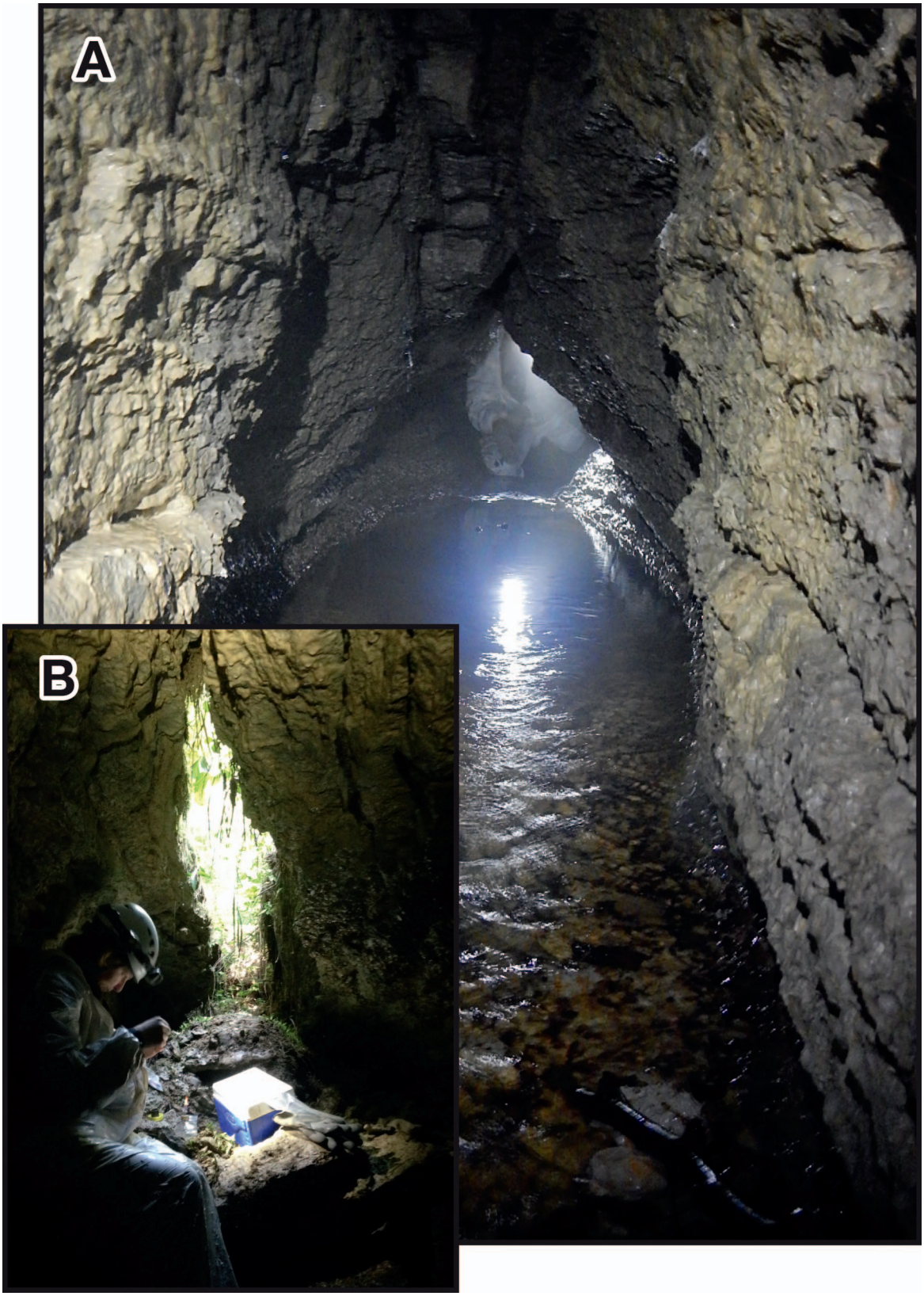

Figure 4. (A) Passage in and (B) entrance to Grotte du lac Maloin. Most passage is low with active development by a stream.

Table 1. Temperature and relative humidity in three natural solution caves on Anticosti Island, Quebec in July 2014. The approximate distance from the cave entrance that dark zone readings were taken is also reported. ND = no data.

	Dark Zone			Twilight Zone		Outside	
Cave	Temp, °C	RH, %	Distance, m	Temp, °C	RH, %	Temp, °C	RH, %
Grotte à la Patate	8.8	83.3	End of offshoot, 70	ND	ND	ND	ND
	8.1	99.4	Main passage, 50	ND	ND	ND	ND
	12.1	99.4	Main passage, 70	ND	ND	ND	ND
Grotte de la Baie de la Tour	15.1	88.1	50−100	13.6	85.4	17.8	100
Grotte du lac Maloin	14.9	81.3	30	17.0	100	25.8	41.2

standard protocol. DNA was eluted in 50 μL of 10 mM Tris-HCl pH 8.0.

PCR Amplification and Sequencing

Fungal primers ITS-1F (Gardes & Bruns, 1993) and ITS 4 (White et al., 1990) were used for amplification of ITS1, 5.8S, and ITS2 regions. All PCR reactions had a total volume of 12.5 μL and included: 6.25 μL of 10% trehalose, 2.00 μL of ultrapure water, 1.25 μL 10× PCR Platinum Taq buffer [500 mM KCl, 200 mM Tris–HCl (pH 8.4)], 0.625 μL MgCl2 (50 mM) (Invitrogen, Life Technologies), 0.125 μL of each primer (0.01 mM), 0.0625 μL of each dNTP (10 mM), 0.3 U of Platinum DNA Polymerase (5 U/μL) (Invitrogen, Thermo Fisher Scientific), and 2.0 μL of DNA template. The thermocycle profile for ITS region consisted of 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. PCR products were visualized on a 2% agarose gel using an E-Gel96 Precast Agarose Electrophoresis System (Invitrogen). Bidirectional sequencing was done using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific) on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Life Technologies) as described in Hajibabaei et al. (2005). Bidirectional sequences were assembled in CodonCode 4.2.2 (CodonCode Corporation) and manually edited.

Data Analysis

Identifications were carried out by comparing the micro- and macromorphological characteristics of the microfungi to those traits appearing in the taxonomic literature and compendia (Domsch et al., 2007; Seifert et al., 2011) and using molecular techniques described above. Desiccant-dried cultures are preserved in the New Brunswick Museum (NBM# F-05200–05245, 05283–05290). All collection data and specimen images for fungi identified by molecular techniques were uploaded to BOLD project Barcoding Bat-Associated Cave Fungi. Sequences were compared against the reference sequence records available in Barcode of Life Data Systems and the National Center for Biotechnology Information's megablast, excluding uncultured/environmental sample sequences. Nucleotide BLAST search results were visualized using MEGAN (v. 5.10.3). Additionally, the Mothur algorithm was utilized to assign posterior probability of sequence match to a local database of ITS sequences downloaded from Genbank (NCBI). Cumulative results were visualized using Tableau 7.0.

Results

Environmental

Grotte à la Patate had the coolest dark-zone temperatures compared to the other two caves (Table 1). Dark zone temperatures in Grotte à la Patate are similar to those recorded in bat hibernacula on the nearby mainland (Vanderwolf et al., 2012). Although bat guano was present, signs of dead bats or bat bones were not observed in Grotte à la Patate. No signs of occupancy by bats were observed in the other two caves. Signs of other mammals were not observed in the caves. The high summer temperatures recorded in the dark zones of Grotte de la Baie de la Tour and Grotte du lac Maloin in July, combined with short passage lengths, suggest that winter dark-zone temperatures are unstable and that the microclimate in these caves is unsuitable for hibernating bats.

Few arthropods were observed in Grotte à la Patate, and invertebrates were not detected in Grotte de la Baie de la Tour. Various dipterans were observed throughout Grotte du lac Maloin, and the cave-associated spider *Meta ovalis* was present at the entrance.

Fungi

Fungi were isolated from all 22 swabs taken in the three caves, producing 183 isolates with a mean of 7.4 ± 3.9 fungal taxa per swab (Table 2). *Pseudogymnoascus destructans* was not detected. The most common of the 54 fungal taxa isolated were *Penicillium* spp. (isolated from 81.8% of swabs), *Pseudogymnoascus pannorum* sensu lato (54.5%), *P. roseus* (50.0%), *Trichoderma* sp. (40.9%), *Cladosporium* spp. (36.4%), *Thysanophora* spp. (36.4%), *Mucor* sp. (31.8%), and *Trichosporon dulcitum* (31.8%). Grotte à la Patate appeared to have the lowest fungal diversity amongst the caves, despite more intensive sampling (Table 2).

Table 2. Fungal taxa cultured from wall swabs in three natural solution caves on Anticosti Island, Quebec. The number of positive swabs for each fungal taxon in each cave is shown. D = identified by DNA, M = identified by morphology.

	Cave		
Fungi	Grotte de la Baie de la Tour	Grotte du lac Maloin	Grotte à la Patate
Ascomycota			
Capnodiales			
Cladosporium sp.	3 M	2 M	1 D, 1 M
C. cladosporioides (Fresen.) G.A. de Vries	0	0	1 D
Chaetothyriales			
Phialophora sp.	1 D	0	0
Dothideales			
Scleroconidioma sphagnicola Tsuneda, Currah & Thormann	1 D, 2 M	0	1 M
Eurotiales			
Penicillium sp.	4 M	5 M	9 M
P. cf. janthinellum Biourge	0	2 M	3 M
P. cf. thomii Maire	1 M	0	0
Penicillium subgenus biverticillium	0	1 M	0
Thysanophora sp.	1 M	0	0
Thysanophora sp. 1	4 M	1 M	2 M
Thysanophora sp. 2	1 M	0	0
Helotiales			
Identified to order only	2 D	0	0
Cadophora sp.	1 D, 1 M	0	0
Catenulifera sp.	1 M	0	0
Chalara longipes (Preuss) Cooke	0	1 D	0
Cistella acuum (Alb. & Schwein.) Svrcek	0	1 D	0
Hypocreales			
Acremonium sp.	0	0	1 M
Beauveria sp.	0	1 M	0
Cosmospora obscura Rossman & Samuels	1 D	0	0
C. viridescens (C. Booth) Grafenhan & Seifert	1 D	1 D	0
Fusarium sp.	2 D	0	0
Hypocrea pachybasioides Yoshim. Doi	0	0	1 D
Isaria fumosorosea Wize	0	2 D	0
Lecanicillium sp.	1 M	0	0
Neonectria obtusispora (Cooke & Harkness) Rossman, L. Lombard & Crous	1 D, 2 M	1 M	0
Tolypocladium inflatum W. Gams	0	2 D	0
Trichoderma sp.	3 M	1 M	5 M
Volutella rosea Sacc.	0	1 D	0
Incertae sedis			
Oidiodendron truncatum G.L. Barron	0	0	1 D, 1M
Pseudogymnoascus pannorum senso lato (Link) Minnis & D.L. Lindner	0	4 M	8 M
P. roseus Raillo	3 M	1 D, 3 M	2 D, 2 M
Verticillium sp.	2 M	2 M	0
Microascales			
Doratomyces stemonitis (Pers.) F.J. Morton & G. Sm.	1 M	1 M	1 D, 3 M
Kernia sp.	0	0	1 D
Onygenales			
Identified to order only	0	1 D	0
Aphanoascus canadensis Currah	0	1 D	0
Arachniotus sp.	0	1 D	0
Arachniotus ruber (Tiegh.) J. Schrot.	0	0	1 M

Table 2. Continued.

Fungi	Cave		
	Grotte de la Baie de la Tour	Grotte du lac Maloin	Grotte à la Patate
Gymnoascus reesii (Tiegh.) J. Schröt	0	0	1 D
Trichophyton sp.	0	1 M	0
Phyllachorales			
Plectosphaerella cucumerina (Lindf.) W. Gams	0	1 D	0
Pleosporales			
Didymella sp.	1 D	0	0
Microsphaeropsis sp.	1 M	0	0
Phoma sp.	3 D, 1 M	0	0
P. novae-verbascicola Aveskamp, Gruyter & Verkley	1 D	0	1 D
P. radicina (McAlpine) Boerema	1 D	1 D	0
Sordariales			
Chaetomium sp.	0	0	1 D
C. globosum Kunze ex Fr.	0	0	1 D
Mammaria sp.	0	0	1 M
Xylariales			
Truncatella angustata (Pers.) S. Hughes	0	2 D	0
Basidiomycota			
Unidentified	0	1 M	1 M
Tremellales			
Trichosporon dulcitum (Berkhout) Weijman	0	0	1 D, 6 M
Zygomycota			
Mortierellales			
Mortierella sp.	1 M	0	0
Mucorales			
Mucor sp.	3 M	0	4 M
Umbelopsis angularis W. Gams & M. Sugiyama	0	1 D	0
U. isabellina (Oudem.) W. Gams	0	1 M	0
sterile			
	4 M	2 M	3 M

Notes: Number of swabs; Grotte de la Baie de la Tour = 6; Grotte du lac Maloin = 5; Grotte à la Patate = 11.
Total fungal taxa; Grotte de la Baie de la Tour = 26; Grotte du lac Maloin = 26; Grotte à la Patate = 24.
Mean number of fungal taxa per swab ± SD; Grotte de la Baie de la Tour = 9 ± 4.8; Grotte du lac Maloin = 9 ± 5.3; Grotte à la Patate = 6.0 ± 2.2.
Range in number of fungal taxa per swab; Grotte de la Baie de la Tour = 2 − 14; Grotte du lac Maloin = 2 − 17; Grotte à la Patate = 2 − 9.

Discussion

We isolated the lowest diversity of fungi in Grotte à la Patate, although sample size was largest for this cave compared to the other two caves sampled. Sampling was conducted deeper within the cave at Grotte à la Patate than at the other two sites, and this may explain the comparatively low fungal diversity. Other studies have found that fungal diversity decreases with increasing distance from cave entrances (Kuzmina et al., 2012; Mulec et al., 2012). Most of the fungal taxa documented during this study have previously been found in caves in other regions (Vanderwolf et al., 2013a). Several fungal genera isolated during this study, such as *Cladosporium, Penicillium*, and *Mucor*, are ubiquitous in non-cave environments (Domsch et al., 2007). Sugita et al. (2005) found that *Trichosporon* spp. were commonly isolated from bat guano in caves in Japan, and we cultured *Trichosporon dulcitum* exclusively from Grotte à la Patate, the only cave on the island where bat guano was observed. Entomopathogenic genera, such as *Isaria, Beauveria,* and *Tolypocladium* (Domsch et al., 2007), were exclusively isolated from Grotte du lac Maloin, where arthropods were more abundant than the other two caves. Fungal genera commonly associated with plants or plant litter, such as *Umbelopsis, Catenulifera, Phoma, Cadophora, Fusarium, Truncatella, Phialophora, Verticillium, Didymella, Neonectria, Plectosphaerella cucumerina, Volutella,* and *Chalara* (Meyer and Gams, 2003; Domsch et al., 2007; Bogale et al.,

2010; Arzanlou et al., 2012), were more common in Grotte du lac Maloin and Grotte de la Baie de la Tour. We suggest this is because Grotte du lac Maloin and Grotte de la Baie de la Tour are shorter than Grotte à la Patate and more prone to outside influences. Additionally, stream foam is known to concentrate fungal conidia (Bärlocher and Marvanová 2010) and was present high on the walls in Grotte de la Baie de la Tour. Conifers are the dominant forest cover on Anticosti Island, and several of the fungal genera isolated are frequently associated with conifer litter, such as *Scleroconidioma, Thysanophora, Cistella, Cosmospora*, and *Neonectria* (Iwamoto et al., 2005; Koukol, 2009; Grafenhan et al., 2011; Koukol et al., 2012). Some of the isolated fungi are coprophilous, such as *Aphanoascus canadensis, Gymnoascus reesii,* and *Doratomyces stemonitis* (Currah, 1985; Domsch et al., 2007).

Previous reports of bats in Grotte à la Patate, our own observations of bat guano in this cave, and microclimate data for Grotte à la Patate relative to elsewhere in the region (Vanderwolf et al., 2012) together suggest this cave is, or was, an active bat hibernaculum and capable of supporting a fungal assemblage that includes *Pseudogymnoascus destructans*. Since we have previously cultured *P. destructans* from walls in caves on the mainland, even where bats have apparently been extirpated (Vanderwolf et al., 2016b), we conclude that *P. destructans* was not present in the three island caves as of summer 2014. *P. destructans* is thought to be primarily transmitted by bats and has rapidly spread throughout northeastern North America from its epicenter in New York (Turner et al., 2011). Although McLeod et al. (2015) suggested that bat hibernacula on islands in the Gulf of St. Lawrence, Canada, might have the potential to provide an eastern North American refuge from *P. destructans*, they found that marine straits were only a partial barrier to bat movement. As of the 2015-2016 hibernation period, *P. destructans* had not been documented on Newfoundland, but it has been present since 2013 on Prince Edward Island and Cape Breton Island, other large Gulf of St. Lawrence islands (Heffernan, 2016). Oceanic straits may present a partial barrier for the movement of mainland bats to Anticosti Island, and this may slow the transmission of *P. destructans* and provide a temporary refuge for bats hibernating on the island. Although we did not detect *P. destructans* in caves on Anticosti Island, the closely related *P. pannorum* and *P. roseus* were relatively common. *Pseudogymnoascus pannorum* is common in caves worldwide (Vanderwolf et al., 2013a), and *P. roseus* is generally associated with soil and wood, particularly in conifer forests (Currah, 1985; Sigler et al., 2000).

Of those fungal genera isolated from cave walls on Anticosti Island, eighteen are identical to those cultured from cave walls in New Brunswick bat hibernacula (unpublished data). Most of the fungal taxa isolated from Anticosti cave walls that we have not isolated previously from cave walls in New Brunswick have been cultured from bats in New Brunswick caves (Vanderwolf et al., 2013b, 2016a). While we found *Thysanophora* sp. more abundant on cave walls on Anticosti Island relative to those in New Brunswick, several fungal taxa abundant on walls and bats in New Brunswick caves were absent from caves on Anticosti Island. These include taxa such as *Leuconeurospora polypaeciloides*, *L. capsici*, *Phaeotrichum hystricinum*, *Humicola* cf. UAMH 11595, *Microascus* spp., *Preussia* sp., *Trichosporiella* sp., and *Arthroderma silverae*. In New Brunswick *L. polypaeciloides*, *L. capsici*, *P. hystricinum*, *Humicola* cf., and *Microascus* spp are more abundant in caves in which mammal dung is present (Vanderwolf et al., 2013b), while *Preussia* sp., *A. silverae*, and *P. hystricinum* are associated with mammal dung in surface environments (Cain, 1956; Domsch et al., 2007; Currah et al., 1996). In addition to overwintering bats (*M. lucifugus*, *M. septentrionalis*, *Perimyotis subflavus*), caves in the Maritimes are frequented, rarely to habitually, by a variety of mammals, including *Peromyscus maniculatus*, *Rattus norvegicus* (Norway rat), *Castor canadensis* (beaver), *Mustela* sp. (weasel spp.), and especially *Erethizon dorsatum* and *Procyon lotor* (McAlpine, 1977; McAlpine et al., 2011; Vanderwolf et al., 2012, 2013b). It appears that the lack of these latter two particular mammal species on Anticosti Island, frequent cave associates elsewhere in Maritime Canada (Calder and Bleakney, 1965; McAlpine, 1979; Moseley, 2007), may influence fungal assemblages present in Anticosti solution caves.

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TERRESTRIAL FILAMENTOUS FUNGI FROM GRUTA DO CATÃO (SÃO DESIDÉRIO, BAHIA, NORTHEASTERN BRAZIL) SHOW HIGH LEVELS OF CELLULOSE DEGRADATION

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Abstract: Subterranean environments are oligotrophic. However, few studies have investigated the composition and function of their terrestrial mycobiota. This study examined the functional role of filamentous cave fungi in cellulose degradation. Soil samples and dry sediment were collected in the surface epigean environment and two sites in the entrance and twilight zones inside Gruta do Catão in the São Desidério karst area, state of Bahia, Brazil. Fungi were cultured from the samples, and the total organic carbon, culturable microorganisms, and carbon and nitrogen microbial biomasses were estimated. All fungal strains were evaluated for cellulase production in carboxymethylcellulose synthetic medium, and the enzymatic indices were estimated. We observed a significant difference ($p \leq 0.05$) in physical, chemical, and biological parameters between epigean soil and cave sediments by Tukey's test. We recovered a total of 20 isolates comprising the genera *Aspergillus* (50.0%), *Penicillium* (25.0%), *Talaromyces* (10.0%), *Trichoderma* (5.0%), *Purpureocillium* (5.0%) and *Scopulariopsis* (5.0%). The majority of the isolates (90%) showed cellulolytic activity, which is a higher percentage compared to that normally reported in the literature for sediments. Thus there is a high probability that the filamentous fungi act in nutrient cycling, thereby contributing to the quality and maintenance of the cave ecosystem. These results indicate that parameters such as total organic carbon, biomass, and relative humidity that tend to differ between caves and epigean environments, provide selective pressures for microorganisms that use alternative sources of energy and nutrients.

Introduction

Caves are subterranean environments that are generally considered oligotrophic environments with permanent darkness and a tendency towards environmental constancy in the deep zone (Culver and Pipan, 2009). These conditions provide highly specialized ecological niches for their inhabitants (Culver and Pipan, 2009; Engel et.al., 2001). Little is known about the distribution, population dynamics, and biochemistry of microorganisms in caves (Northup and Lavoie, 2004; Barton and Jurado, 2007), and for Brazil this knowledge is still incipient, according to Vanderwolf et al. (2013), who emphasized a large knowledge gap concerning the diversity of the fungal community in caves worldwide, including Brazilian caves, and highlighted the importance of studies on the distribution of fungal communities and their relationship to subterranean environments. The usage and exploration of subterranean environments for tourism, mining, hydropower, and other purposes often reaches excessive levels, leading to the destruction of habitats. Unfortunately, microbiological studies regarding subterranean microorganisms are neglected and are not considered when establishing parameters for cave exploration. In 2008, the National Center of Study and Conservation of Caves (Centro Nacional de Pesquisa e Conservação de Cavernas, CECAV) published a "term of reference" concerning cave exploration in Brazil that requested studies in several areas, but said little about microbiological studies (CECAV/IBAMA, 2008).

Studies worldwide have reported the extent to which tourism affects the microbial communities in caves (Pulido-Bosch et al., 1997). Lavoie and Northup (2006) confirmed that the input of organic matter by tourist visits supported the growth of exogenous bacteria and fungi in the subterranean environment. Sensitive indicators that can be used to monitor changes in subterranean environments include enzymatic activity, density, and the biomass of soil bacteria (Kennedy and Papendick, 1995; Matsuoka et al., 2003), because the soil microbiota are mainly responsible for the decomposition of organic wastes and nutrient cycling.

Fungi are dominant among cave microorganisms due to their high rate of dispersion, spore survival, and better capacity for colonization (Wang et al., 2010). Fungi act mainly as decomposers or parasites in caves (Santamaria and Faille, 2007; Yoder et al., 2009) and provide nutrients in

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assimilable forms for other organisms. Together with bacteria, fungi are also the main food sources for protozoans and terrestrial invertebrates such as *Isopoda* crustaceans and *Collembolan* entognathans (Bichuette and Trajano, 2006). The distribution of fungi along the cave is influenced by the availability of organic matter and microclimatic conditions such as water availability, temperature, and pH (Vanderwolf et al., 2013).

Although cave-food recycling completely depends on the catabolic and anabolic microbial processes that occur in the absence of light, the input of energy is considered to be insufficient to support the subterranean ecosystem. Some studies that assessed organic matter decomposition in aquatic and marine sediments in caves showed high microbial activity in the early stages of decomposition that correlated with increased microbial respiration (Fishez, 1991; Galas et al., 1996). Other researchers emphasized the importance of the microbial community as key organisms at the base of the subterranean environment food web and in decomposition processes (Pohlman et al., 1997; Graening and Brown, 2003). However, few researchers have studied the involvement of cellulolytic microorganisms in the decomposition process in cave environments (Semikolennykh et al., 1975; KoilRaj et al., 2012). Furthermore, researchers have focused on the cellulose degradation rate or cellulolytic potential of bacterial strains, with no interest in evaluating the role of the fungal community in cellulose decomposition.

The karst area of São Desidério (state of Bahia, northeastern Brazil) has unique characteristics, including a high diversity of troglomorphic organisms showing reduced pigmentation and eye or ocellus, with a wide range of new species to be described (Trajano and Bichuette, 2010; Fernandes et al., 2016). This biodiversity makes knowledge of the subterranean mycobiota highly relevant both for better understanding of specific food webs and for preservation purposes. In an application context, microbiological studies in subterranean environments might lead to the identification of new species (Nováková et al., 2012) that could be of biotechnological interest (Reynolds and Barton, 2014), contribute to knowledge about pathogenic microorganisms, and be useful for the development of protective actions in tourist caves (Porca et al., 2011) and the development of management plans for cave exploration (Nieves-Rivera, 2003; Nieves-Rivera et al., 2009).

This study is the first to focus on the cave mycobiota in the region of São Desidério. We evaluated the involvement of filamentous fungi in cellulose degradation from a subterranean environment to elucidate their role in food webs and ecosystem maintenance, thereby enabling better management of tropical cave environments.

Materials and Methods

The studied locality was the Gruta do Catão (12° 22′ 6″ S, 44° 52′ 3″ W) located in the Parque Municipal da Lagoa Azul approximately 15 kilometers from São Desidério, Bahia state, Brazil (Fig. 1). The cave is in a 19 hectare part of the João Rodrigues karst system, which is one of most important hydrogeological systems in South America. The cave receives approximately 150 tourists per week. It does not have graffiti and trash, but in the past, the cave was used as a football field. The cave is formed in pure dark-gray limestone from the Proterozoic eon, formed from shallow marine deposits. The weather of São Desidério is tropical, with summer rain. The annual temperature varies between 17.0 °C and 37.0 °C, and the rainfall reaches 1,700 mm per year, with well-defined rainy season from November to March (Santos et al., 2008).

Gruta do Catão has two entrance, each about 50 m wide and 7 m high, and the length of the cave is approximately 160 m; the area is approximately 15,000 m^2. The map of the cave was drawn by the Bambuí Speleological Research Group (Fig. 2). The cave floor is formed by sandy-clay fluvial deposits (dry sediment) that are believed to be the first resurgence of the João Rodrigues subterranean river. The cave does not have an aphotic region, but the brightness of the entrance zone, 100 to 200 lux, and the twilight zone, a maximum of 50 lux, differ from the 500 to 700 lux of the epigean area.

Sampling

The sampling was conducted in October 2012 (Chico Mendes Institute for Biodiversity Conservation (ICMBio/SISBIO license no. 10215). One quadrant of approximately 0.25 m^2 was sampled in three areas (Fig. 2): one in an epigean area (sample 3, outside the cave), one in the entrance zone (sample 1), and one in the twilight zone (sample 2) of the cave. Approximately 300 g of soil and cave sediment were collected at two different sites in each quadrant. Soil and cave sediment were collected from a depth of 0 to 10 cm with the aid of a gardening shovel and stored in sterile plastic jars. The samples were transported to the laboratory in coolers, homogenized and sieved (2 mm mesh), and stored in the refrigerator at 4 °C. The shovel and sieve had been disinfected by 2 min in 70% alcohol at the site before collecting the substrate. In the laboratory, each sample gave rise to four subsamples, for a total of eight subsamples at each sampled point. The air temperature (°C) and relative humidity (%) were measured at each sampled area with a minimum time interval of 1 minute between measurements (Thermo-hygrometer Instruntherm THAL-300, 0.1 resolution and ± 5.0% accuracy).

Chemical and Biological Parameters

Total organic carbon (TOC), microbial carbon and nitrogen biomass (MBC and MBN, respectively), and counts of colony-forming unit (CFU) were determined for the soil and cave-sediment samples. TOC concentrations were obtained by the method of wet digestion with the oxidation of organic C with 10 mL of 0.167 mol L^{-1} $K_2Cr_2O_7$ (potassium dichromate) and 20 mL of H_2SO_4 (sulfuric acid) (Walkey and Black, 1934).

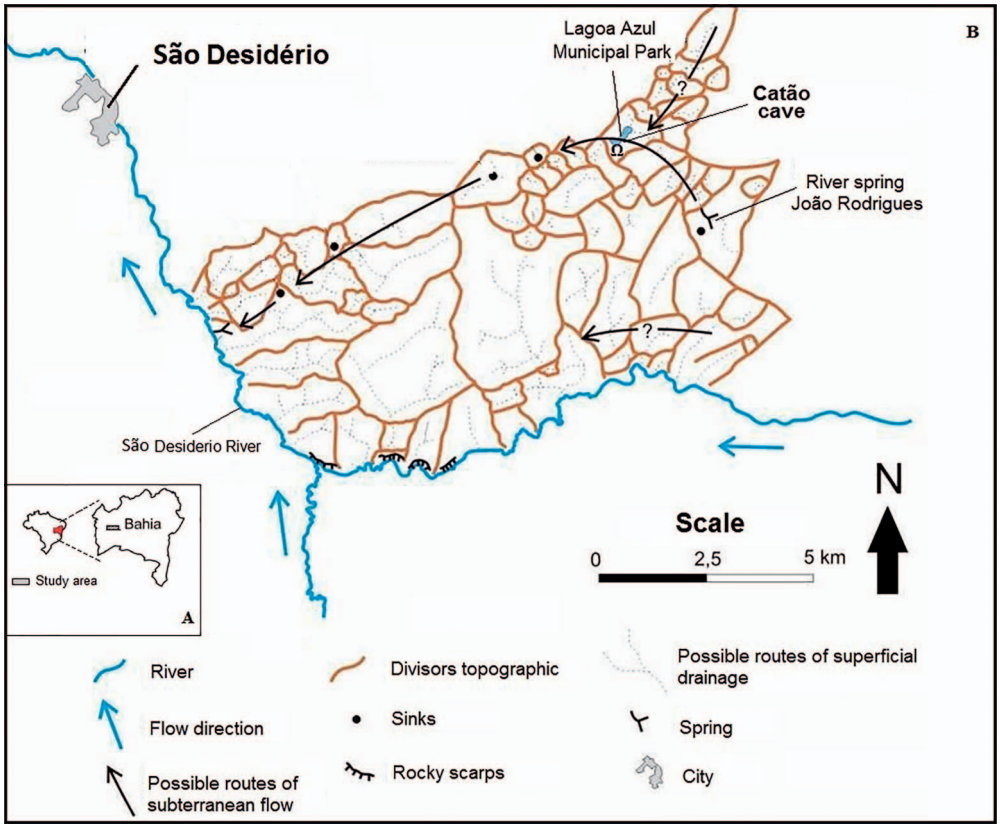

Figure 1. A (inset): Geographical location of São Desidério county. B: João Rodrigues karstic system and the possible route of the subterranean river João Rodrigues (modified from Sá Pereira and Godinho, 2013).

The MBC and MBN were evaluated in 10 g substrate samples previously adjusted to 40% moisture content. The MBC was evaluated by the fumigation-extraction method (Vance et al. 1987), and the MBN was evaluated according to the method of Brookes et al. (1985). After this process, a substrate extraction was performed with 0.5 M K_2SO_4. The moisture in the soil and cave sediment samples was estimated by the gravimetric method, drying at 105 °C for 20 h to 7 days after sampling, and the results were expressed as dry weight. The MBC contents were estimated with a spectrophotometer using the correction factor (K_{CE}) of 0.41 as recommended for tropical soils to avoid overestimating the results (Barbujia et al., 2010). The MBN of the substrate was determined by the addition of 1.5 mL of H_2SO_4 and 50 mg of a catalyst mixture ($K_2SO_4 + CuSO_4$, 10:1) to 20 mL of substrate extract. The N concentrations were determined by spectrophotometry using the K_{NE} correction factor of 0.54 according to Brookes et al. (1985).

The terrestrial culturable bacteria and fungi were determined by the serial dilution method using specific culture medium; the results were expressed as colony-forming units (CFU) per g of soil. A substrate sample (5.0 g in 50 mL of 0.85% saline solution) was suspended by shaking at 150 rpm for 30 min and serially diluted up to 1/100,000 prior to inoculation by spreading onto the solid media. All culture media were prepared in the laboratory by dilution the specific reagents in distilled water: Nutrient agar (5.0 g L^{-1} bacteriological peptone, 3.0 g L^{-1} beef extract, and 15.0 g L^{-1} agar, pH 6.8) was used for total bacteria, and Martin medium (1.0 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4 \cdot 7H_20$, 5.0 g L^{-1} soy peptone, 10.0 g L^{-1} glucose, 0.06 g L^{-1} Rose Bengal, and 15.0 g L^{-1} agar, pH 6.0) was used for total fungi (Wollum II, 1982). The inoculated plates were incubated in the dark at 27 °C and analyzed after 3 days (bacteria) and 7 days (fungi).

ISOLATION AND CHARACTERIZATION OF THE FUNGI

Fungi were isolated from the plates used for counting. Colonies were inoculated by streaking on Malt agar 3% (30.0 g L^{-1} malt extract, 3.0 g L^{-1} soy peptone, 0.05 g L^{-1} Rose Bengal, and 20.0 g L^{-1} agar, pH 5.5 to 6.0) with three replicates per dilution. The samples were incubated for 15 days at 25 °C. On the seventh and fifteenth days, the colonies obtained were isolated by streaking to obtain pure cultures. Each isolated strain was designated SDC x.y, for the study area (São Desidério) and the cave (Gruta do Catão); x is the collection point and y is to the number of the strain.

To ensure the genetic stability of the strains, they were preserved in sterile distilled water and refrigerated at 4 °C (Castellani, 1939). The characterization of the strains was based on their morphotypes, and the genus identification was

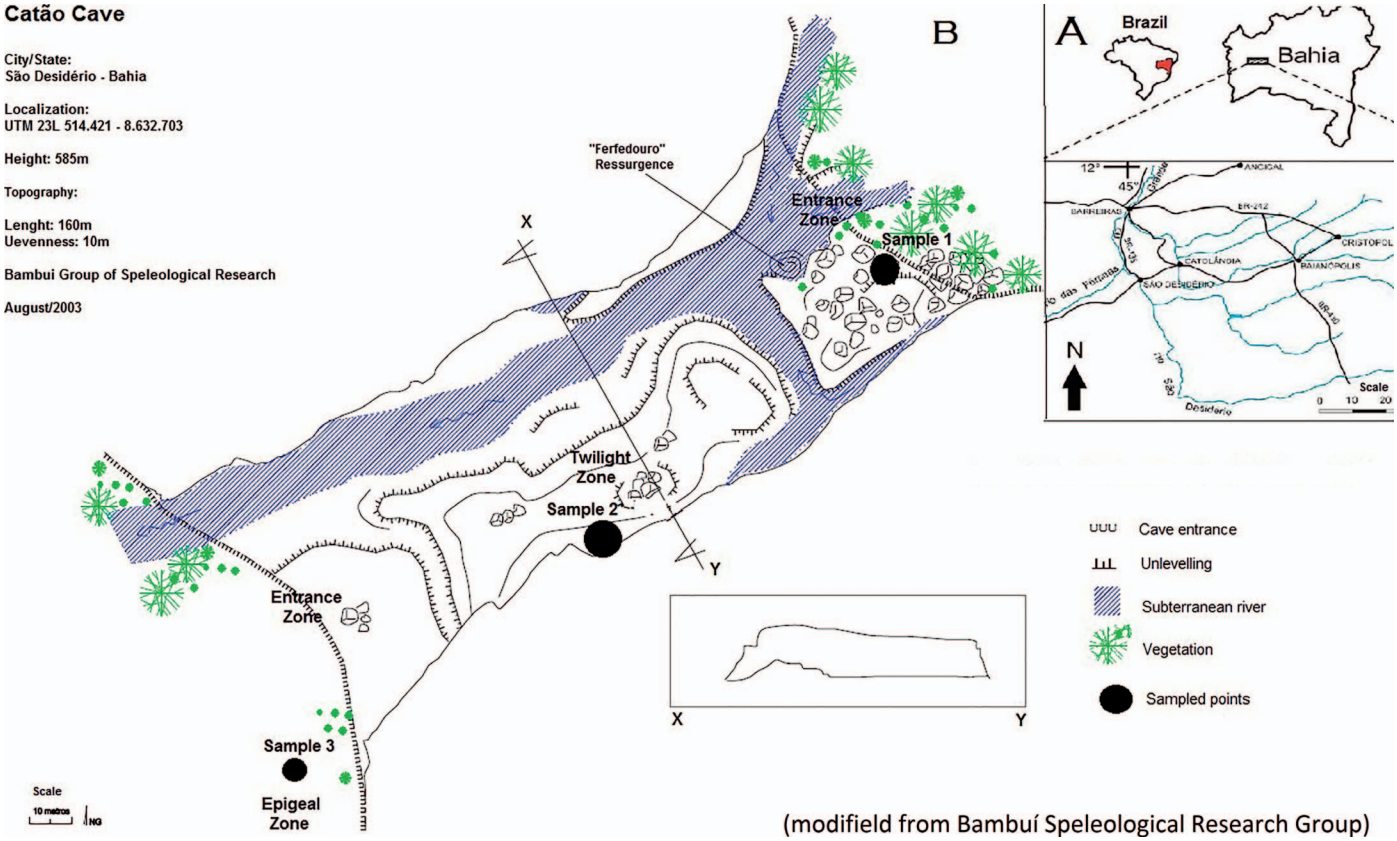

Figure 2. Plan of Gruta de Catão highlighting the entrances and the twilight zone. The three sampling sites are shown.

based on morphological characteristics such as reproductive structures, hyphae, and spores observed by optical stereoscopic and microscopy. Microscopic analyses were made using the microcultive technique with the aid of specialized literature (Barnett and Hunter, 1998).

Production of Cellulolytic Enzymes

To evaluate the production of cellulolytic enzymes by the isolated strains, the strains were cultivated in synthetic media with carboxymethylcellulose as the only carbon source (10.0 g L^{-1} CMC, 10.0 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 0.5 g L^{-1} KCl, 1.0 g L^{-1} K_2HPO_4, 0.5 g L^{-1} $MgSO_4$, 3.0 g L^{-1} $NaNO_3$, and 20.0 g L^{-1} agar, pH 5.6 to 6.0). The media were inoculated with the fungal strains using a platinum needle inserted at the center of the Petri dish and incubated for 14 days at 25 °C. On the seventh and fourteenth cultivation days, the formation of a degradation zone was revealed by the addition of 10 mL of Congo red solution (2.5 g L^{-1}) in 0.1 M Tris HCl buffer (pH 8.0). The solution was discarded after 30 min, and the cultures were washed with 5 mL of 0.5 M NaCl in the same buffer. The halos (D_H) and colony diameters (D_C) were measured, and the ratios between them were used to estimate the enzyme index $EI = D_H/D_C$. The enzymatic index is a measure of the enzyme activity by radial diffusion in solid medium (Nogueira and Cavalcanti, 1996).

Statistical Analysis

The chemical and biological variables were submitted for basic descriptive statistics (Shapiro-Wilks). Analysis of variance and Tukey's test with 5% probability threshold were also applied to verify the significance of the differences among the results. To analyze the influence of the variables on the data, we used principal component analysis in which the main components were greater than or equal to 1 eigenvalue following the Kaiser criterion (McCune et al., 2002). The program PAST version 8.2 (Hammer, 1999) was used for statistical analysis and graphical production.

Results

Table 1 shows the mean values of the temperature and relative humidity of the air and the moisture content of the soil or dry sediment for the different sampling sites. The mean temperature in the twilight zone of the cavern was 25.1 °C, and the relative humidity of the air was 70.7% to 75.2% at the cave sites. The epigean area showed a higher temperature (27.4 °C) and lower relative humidity (64.3%). The soil moistures in the epigean area and the sediment in the entrance zone of the cave was higher (14.5% and 10.3%, respectively) compared to the twilight zone (5.71%).

Table 1. Mean values and standard deviations of abiotic parameters (air temperature and relative humidity of air and soil) in the sampling sites.

Abiotic Parameters	Epigean Zone	Entrance Zone	Twilight Zone
Air temperature (°C)	27.4 ± 0.20	25.8 ± 0.32	25.1 ± 0.75
Relative humidity of the air (%)	64.3 ± 0.42	70.7 ± 0.55	75.2 ± 1.85
Moisture content (%)	14.5 ± 0.27	10.3 ± 0.28	5.71 ± 0.21

The total organic carbon values differed among the sampling sites ($p \leq 0.0028$; Table 2). The epigean area had 2.41 g g^{-1} of carbon in the soil on average, whereas the entrance and twilight Zones showed mean values of 1.3 g g^{-1} and 0.49 g g^{-1} of sediment, respectively. The highest C and N microbial biomasses were found in the epigean area and the lowest in the twilight zone. The MBC ranged from 59.9 mg g^{-1} of sediment in the twilight zone to 1428.1 mg g^{-1} of soil in the epigean are, with significant differences between all three sampled sites ($p \leq 0.0001$). The amount of the N microbial biomass was also significantly different among the three sampled sites ($p \leq 0.0020$), with values ranging between 0.51 mg g^{-1} of sediment in the twilight zone and 6.5 mg g^{-1} of soil in the epigean area.

The epigean environment had higher amounts of culturable bacteria compared to the other sites in the cave ($p \leq 0.0010$; Table 2). However, there was no significant difference between the values found for the sites inside the cave ($p = 0.6080$). There was no significant difference in culturable fungi among the three sampled sites ($p \geq 0.8070$), but the epigean area showed a greater coefficient of variation of their averages compared with the subterranean environment ($CV_{Epg} = 66.9$; $CV_{EZ} = 35.2$; $CV_{TZ} = 37.4$).

The principal-component analysis aims to summarize a large number of variables in two dimensions. The principle components represent the eigenvalues, with the component 1 representing the largest variance of the data, and the component 2 representing the second largest variance of the data. The principal-component analysis of the variables (Fig. 3) revealed that the culturable fungi explained the majority of the data variance (76.2%). Figure 3 shows that the points for the subsamples in the epigean area (Epg) were more dispersed compared with the other sites, indicating that the heterogeneity of the soil at this location was higher compared to the other sites. The points representing the data from the entrance zone (EZ) and twilight zone (TZ) were closer to one another and the TZ points were the most clustered, demonstrating greater uniformity of the data.

Only the isolates from the entrance and twilight-zone samples were identified and evaluated for production of cellulolytic enzymes because the aim of the study was to evaluate the role of the fungal community of the subterranean environment on cellulose degradation. A total of twenty fungal morphotypes were isolated, including seven from the entrance zone and 13 from the twilight zone (Table 3). The strains were identified in the following genera: *Aspergillus* (50.0%), *Penicillium* (25.0%), *Talaromyces* (10.0%), *Trichoderma* (5.0%), *Purpureocillium* (5.0%) and *Scopulariopsis* (5.0%).

The strains isolated from the cave entrance and twilight zones were evaluated for the production of cellulolytic enzymes. Figure 4 shows the enzyme indices obtained after fungal cultivation on synthetic media. The halo indicating the degradation of carboxymethylcellulose was observed in 18 of the examined strains (90%). The highest enzyme indices were found for the following strains: *Penicillium* SDC 2.13, IE 2.40, *Scopulariopsis* SDC 2.1, IE 2.46, and *Talaromyces* SDC 2.9, IE 2.48. All of the strains, with the exception of *Aspergillus* sp 4 (section *Nigri*) and *Trichoderma*, which did not produce degradation halos, showed a higher enzyme index on the seventh cultivation day compared to the fourteenth.

DISCUSSION

Analyses of the physical abiotic variables revealed that they were significantly different between sampling locations. The largest amount of total organic carbon was observed in the epigean area, as expected, because this location had a greater richness of carbon sources. The input of organic carbon into the subterranean environment occurs through two main pathways: small openings, cracks, and sinks that allow the entry of leaves, wood, and debris from streams and epikarstic environments (Simon et al., 2007), or carbon

Table 2. Total organic carbon (TOC), microbial biomass carbon (MBC), microbial biomass nitrogen (MBN), culturable bacteria and fungi estimated for the soil and sediment of the Gruta do Catão in the Epigean area, Entrance Zone and Twilight Zone.

Sampled Sites	TOC (g g^{-1} soil)	MBC (mg g^{-1} soil)	MBN (mg g^{-1} soil)	Bacteria CFU g^{-1} soil	Fungi CFU g^{-1} soil
Epigean area	2.41*	1428.1*	6.64*	1.8 10^6*	3.0 10^5*
Entrance Zone	1.30**	146.18**	1.74**	1.02 10^6**	2.57 10^5*
Twilight Zone	0.49***	59.95***	0.52***	9.0 10^5**	2.7 10^5*

Note: Values with different numbers of asterisks in a column differ between sites with significance ($p < 0.05$).

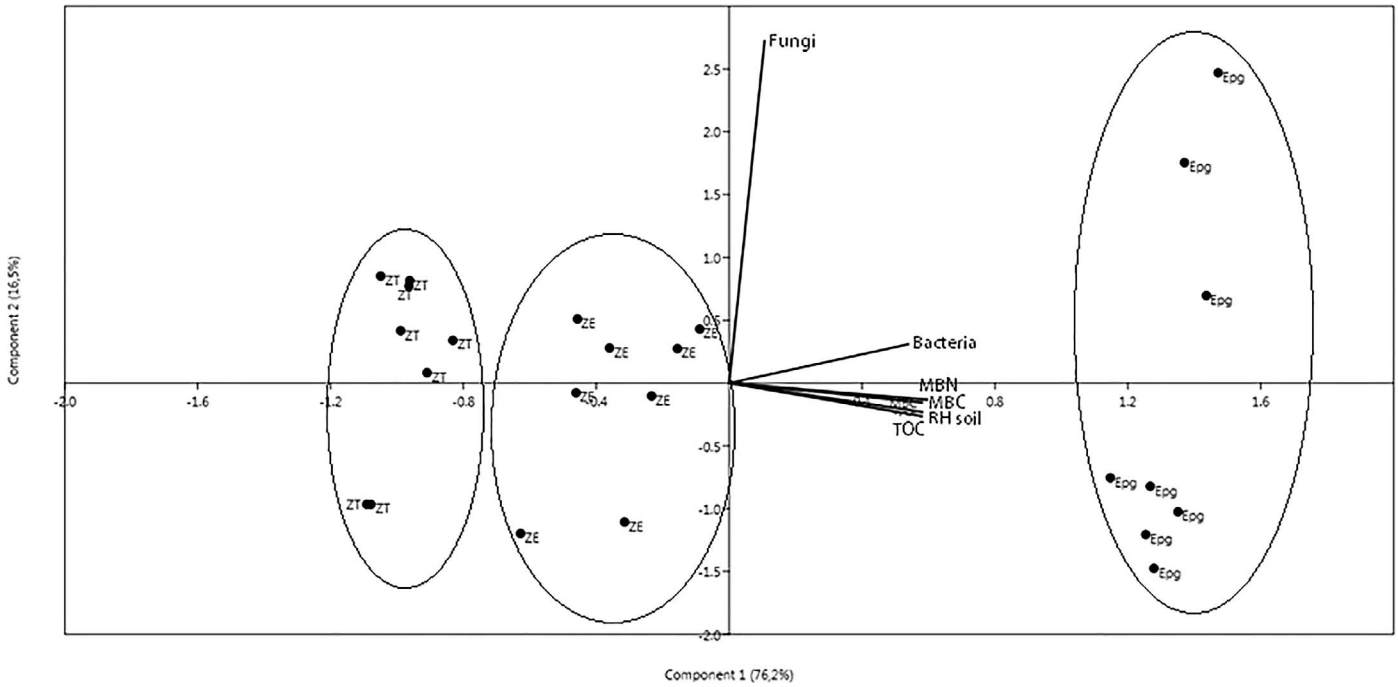

Figure 3. Principal component analysis highlighting the variables (total organic carbon (TOC), microbial biomass carbon (MBC), microbial biomass nitrogen (MBN), total organic carbon, and culturable bacteria and fungi) that had greatest effect on the distributions of the data from the subsamples of each sample site (Epg = epigean area; EZ = entrance zone; TZ = twilight zone). The vectors represent the weight of each variable on the variance of the data.

particles brought by animals that enter and leave the caves (Graening and Brown, 2003) or visiting sightseers.

The epigean area also had higher microbial biomass values compared to the internal sites in the cave. According to Matsuoka et al. (2003), the diversity of vegetation and its presence all year in the Epigean areas influence the yield and quality of leaf litter, thereby contributing to higher levels of microbial biomass. According to Lavoie and Northup (2006), D.J. Feldhake observed that the organic matter and microbial biomass content were lower in sediments from North American caves compared to forest soils. Moreover, recent research using molecular methods showed higher values for microbial biomass on the surface than inside Kartchner Caverns, USA (Ortiz et al., 2013; Ortiz et al., 2014).

The significant difference in the concentration of bacteria, based on colony-forming units, between the epigean zone and the cave sites can be explained by the smaller organic content available inside the cave. Similar studies have shown this

Table 3. Terrestrial fungi isolated from the Entrance and Twilight Zone of Gruta do Catão.

Entrance Zone		Twilight Zone	
Code	Identification	Code	Identification
SDC 1.1	*Aspergillus* sp.3	SDC 2.1	*Scopulariopsis*
SDS 1.2	*Aspergillus* sp.5	SDC 2.2	*Talaromyces*
SDC 1.3	*Penicillium* sp.1	SDC 2.3	*Trichoderma*
SDC 1.4	*Aspergillus* sp.6	SDC 2.4	*Aspergillus* sp.1
SDC 1.5	*Penicillium* sp.2	SDC 2.5	*Purpureocillium*
SDC 1.6	*Aspergillus* sp.4 (*section Nigri*)	SDC 2.6	*Aspergillus* sp.5
SDC 1.7	*Penicillium* sp.3	SDC 2.7	*Penicillium* sp.2
		SDC 2.8	*Aspergillus* sp.8
		SDC 2.9	*Penicillium* sp.3
		SDC 2.10	*Aspergillus* sp.9 (section *Flavi*)
		SDC 2.11	*Aspergillus* sp.2
		SDC 2.12	*Aspergillus* sp.7
		SDC 2.13	*Penicillium* sp.4

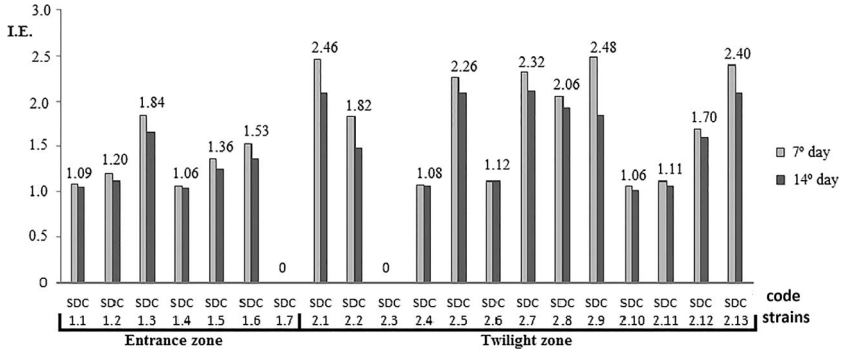

Figure 4. Enzymatic index (IE) of the filamentous fungi strains isolated from the sediment in Gruta do Catão and cultivated in synthetic media with carboxymethylcellulose for 7 and 14 days at 25 °C (IE= D_H/D_C, where D_H is the halo diameter and D_C is the colony diameter). The horizontal labels are the strain numbers in Table 3.

same pattern in caves, but KoilRaj et al. (2012) found a greater range in bacterial density in soils (2.1×10 to 3.7×10^5 CFU g^{-1} soil) in four Indian caves. There were no significant differences in the culturable fungi among the sampling sites, although the culturable fungi in the epigean area showed greater variation compared to the sampled sites inside the cave. The culturable fungi reported in a study conducted by KoilRaj et al. (2012) were lower compared to our study, ranging between 1×10^4 and 1.9×10^5 CFU g^{-1} soil.

According to the principal component analysis of the variables (Fig. 3), the sites in the epigean area and the entrance and twilight zones fall in different and well-defined clusters. The points representing data from the epigean were more dispersed in comparison with the other sites; the clusters for the cave zones were closer to one another, and the points of twilight zone were the most tightly clustered. Soil is a heterogeneous environment consisting of a mosaic of microhabitats, and in an epigean area there is well-conserved native vegetation. Therefore, the complexity and the nutrient resources in epigean soil produce a greater heterogeneity of microhabitats relative to the sediment inside the cave that directly influences a greater variability in fungal density in our subsamples. The entrance zone of the cave is a transition area between two distinct ecosystems and contains a greater variety of niches and substrates compared to the twilight zone.

The number of fungal isolates was relatively low compared with the data obtained by other authors studying Atlantic Forest and Tropical Forest soil ecosystems, who isolated 80 to 110 strains (Ruegger and Tauk-Tornisiselo, 2004; Delabona et al., 2012). Our low numbers of strains were probably due to selection favoring only some strains. Some species of fungi require light to induce sporulation and metabolic activity (Tisch and Schmoll, 2010), and these species will have more difficulty surviving in the cave environment. The input of microorganisms by dripping water and periodic flooding of the river may carry organisms that cannot survive the cave conditions and are used by opportunistic fungi as a source of nutrients. Use of a nutrient-rich medium for the isolation of fungi may have restricted the growth of strains that are better adapted to oligotrophic conditions (Tomova et al., 2013). We observed that the number of isolates was higher in the samples deeper inside the cave compared to the entrance zone. Periods of flooding in caves can be considered a natural control of the diversity and distribution of fungi in the soil, and the subterranean environment takes two to three months to recover a community close to the original composition (Shacklette and Hasenclever, 1968; Vanderwolf et al., 2013). Thus the lower number of isolates in the entrance zone at Gruta do Catão can be explained by the fact that this site is more susceptible to flood pulses, being closer to the stream.

The genera obtained in this work are most commonly isolated in cave environments, both in Brazil (Casirillión et al., 1976, Taylor et al., 2013) and worldwide (Nieves-Rivera, 2003; Nieves-Rivera et al., 2009; Nováková et al., 2012; Vanderwolf et al., 2013). Taylor et al. (2013) obtained the same genera as the most abundant and in similar proportions at Gruta Lapa Nova, Brazil. Contrary to the observations in our study, other researchers obtained a greater diversity of genera in studies of subterranean environments (Nieves-Rivera et al., 2009). A comparison with other studies on cave microbiota is difficult, because these studies are scarce and use different approaches, such as focusing on identifying Basidiomycetes (Pedro and Bononi, 2007) and fungi associated with invertebrates (McCarthy et al., 2011).

Despite the genera being common, the analysis of their cellulolytic enzyme production revealed that 90% of the isolates produced cellulases (18 strains). In the study by Ruegger and Tauk-Tornisiselo (2004), only 45% of the strains isolated from the soil of the Atlantic Forest exhibited a degradation halo. Therefore, the percentage of isolated strains producing cellulases was higher in comparison with other studies. Because subterranean rivers contain dissolved organic carbon, particulate organic matter, and biopolymers such as cellulose introduced from the surface soil by percolating water (Culver and Pipan, 2009), we may conclude that the periodic flooding of the subterranean Rio João Rodrigues in the Gruta do Catão causes a discharge of nutrients and carbon into the

sediment of this cave. Moreover, during rainy periods a large amount of organic matter, mainly plant residues, from the surface is washed down by flooding into the subterranean cavities.

Fungi are the main agents involved in cellulose decomposition at the soil surface (Griffin, 1985; Jennings, 1987), although copiotrophic bacteria dominate the early stages by consuming simple carbohydrate chains such as glucose. Cellulolytic enzymes in fungi are activated following the decrease in bacterial biomass caused by the depletion of these carbohydrates, thereby enabling the fungi to dominate the remainder of the cellulose-waste decomposition process (Hu and van Bruggen, 1997). Other factors also enable fungi to be more successful than other microorganisms in cellulose decomposition, such as their growth in the hyphal and mycelium forms. This characteristic enables them to cross soil with poor nutrients in the search of resources that are distributed unevenly in the environment and penetrate plant tissues to release cellulolytic enzyme complexes (de Boer et al., 2005).

The results of this study indicate that the majority of filamentous fungi isolated from this subterranean environment are able to produce enzymes that hydrolyze cellulose. Thus, based on the high percentage of cellulolytic fungi found in this study, we can infer that they may play a fundamental role in the subterranean environment. The fungi may function in the decomposition of the organic waste and nutrients and make them available to other organisms in assimilable forms, in addition to serving as an important source of food for some organisms at the base of the food chain (Cubbon, 1976, Poulson and Lavoie, 2000). Therefore, we can infer that cellulolytic hydrolysis may be an important pathway for energy flow and carbon in subterranean ecosystems.

Studies monitoring the fungal community composition and distribution during a hydrological cycle in a karst area would contribute to our understanding of the mycological and functional diversity of terrestrial mycobiota communities and their distribution patterns in the subterranean environment. With our information and knowing the times of higher or lower abundances, we could predict changes in the abundance of the fungal community during periods with increased tourist flow and reduce the negative impact of this activity on cave biodiversity. Moreover, research is needed in subsurface environments with different lithologies in Brazil to enable better assessment of the ecological processes and to preserve these ecosystems, thereby allowing better comparisons among the environments and a more robust assessment of the importance of each cavity.

Conclusion

The dry sediment of the subterranean environment in this study is different from the epigean soil in most of the examined chemical and biological parameters. These differences provide unique niches in the subterranean environment for organisms and the fungal community. A diversity of fungal genera commonly isolated from similar environments were found in the cave sediment. Ninety percent of the strains exhibited cellulase production, indicating that this environment favored the development and propagation of fungi that possess alternative and more complex mechanisms to obtain energy and nutrients. This high proportion of cellulolytic fungal isolates suggests that they are important organisms for nutrient cycling, thereby contributing to the quality and maintenance of the cave ecosystem. The flood periods can be considered as a natural phenomenon that control the diversity and distribution of the fungi in the soil, so the monitoring of their density, richness, and enzymatic activity along the cave is important before and after these floods. Future management plans should include more detailed microbiological studies to better understand the relationship between the microbiota and Gruta do Catão's ecosystem and to allow better management of parks and protected areas.

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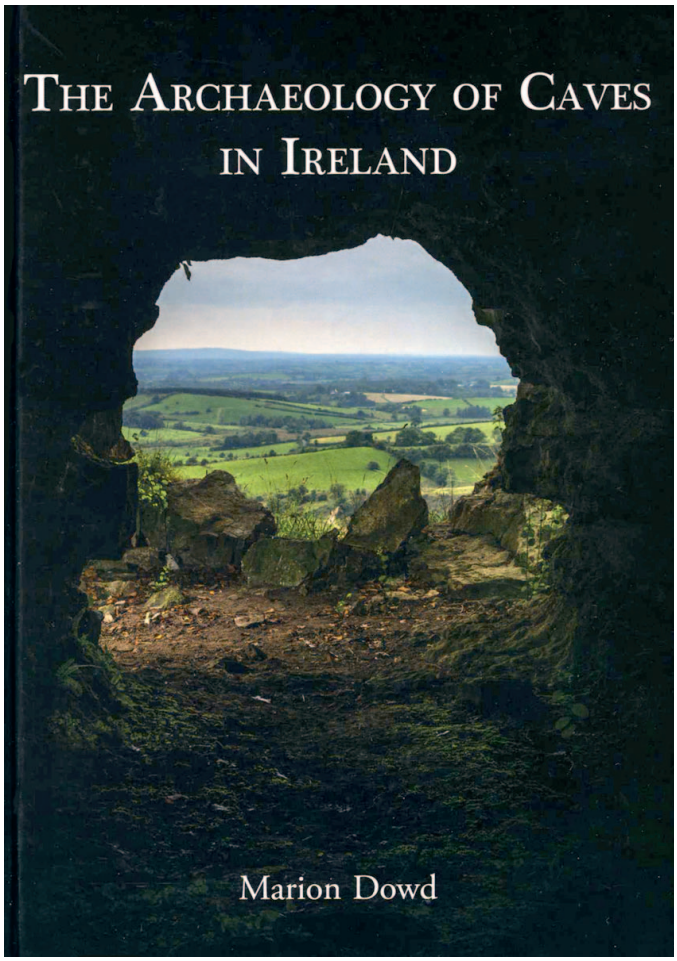
BOOK REVIEW



The Archaeology of Caves in Ireland

Marion Dowd. Oxford & Philadelphia, Oxbow Books, 2015. ISBN 978-1-78297-813-8 (hardcover); 24.7 × 17.5 cm, 314 p., US $85; digital edition 978-1-78297-814-5.

This is a well-researched book that is comprehensive in its coverage. It is based on an impressive review of the literature and historical archives and the author's personal visits to more than one hundred caves in Ireland. It is well-illustrated with excellent color plates, drawings, and other illustrations. The research began as Dowd's master's degree topic and continued through her doctoral research. Ireland does not necessarily jump to the forefront when one thinks of karst regions of the world. However, approximately 40 percent of Ireland is underlain by limestone, and there are 980 recorded caves, 45 of them greater than 1 km in mapped length. The longest is the 16-km Poulnagollum–Poulelva cave system, but there is no known archaeology in this cave. Dowd indicates that 91 caves have recorded archaeological remains dating from the Mesolithic Period (beginning at 8000 BCE) up to the present day.

The book contains three background chapters, followed by seven chapters describing archaeological cave sites by chronological period, an endnote on the future of cave archaeology in Ireland, and an appendix listing significant cave sites with map-coordinate locations. American cavers and archaeologists might find the publication of cave coordinates to be either ill-advised because of problems with trespassing, landowner relations, and looting or refreshing because the Irish public has a much stronger sense of heritage preservation.

Chapter 1, titled "People in Caves, Caves and People," is a thoughtful discourse on the multiple meanings of caves as sacred or spiritual realms and the symbolic importance of caves in antiquity and in modern times. Dowd places the archaeology of Irish caves into a world context, demonstrating a very good command of archaeological cave literature from around the world. A summary of Dowd's research would have been a good contribution to the recent book edited by Holley Moyes, *Sacred Darkness: A Global Perspective on the Ritual use of Caves* (University Press of Colorado, 2012).

Chapter 2, "Excursions into Places of Fearful Darkness – 300 Years of Investigating Caves," is a history of Irish cave research from the antiquarian interest in artifacts and bone deposits through the founding of speleology as the science of caves. Important contributions to Irish speleology are noted, such as those of E. A. Martel, who visited Ireland in 1895 and inspired interest in cave research, and Jack Coleman, who is considered the father of Irish caving.

Chapter 3 provides a brief background on cave origin and karst geology in Ireland and a much longer discourse on the archaeology of caves. Of particular interest is the discussion of recent research projects to radiocarbon-date more cave sites using material from older collections and newer excavations. Dowd also provides an interesting meditation on why cave research in Ireland still remains largely ignored, despite a boom in archaeological excavations in that country during the first decade of the twenty-first century, corresponding to the so-called Celtic Tiger economic boom. While part of the explanation is practical most development during the economic boom did not involve karst regions—the other part is a familiar story. Despite the association between archaeology and caves in the public eye, most archaeologists are reluctant to work in caves because of the logistical difficulties and the complex geological contexts. Cave and rock-shelter sites present some of the most difficult depositional contexts to interpret that archaeologists will encounter.

The remaining chapters summarize what is known about cave archaeology for the major chronological periods, the Mesolithic (8000–3900 BCE), Neolithic (3900–2400 BCE), Bronze Age (2400–600 BCE), Iron Age (600 BCE–400 CE),

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Medieval Period (400–1550 CE), and Post-Medieval Period (1550 CE to present). There are some common themes that have parallels in other parts of the world. For example, during the Mesolithic caves were primarily used for human burial. This trend continued during the Neolithic, with an increase in ritual behavior and perhaps a greater emphasis on secrecy and isolation. Use of caves intensified during the Bronze Age, with burial ritual and votive offerings still an important component. However at that time, rather than being isolated, archaeological sites in caves were found in prominent locations in conjunction with other monuments. Although there is some evidence for domestic occupation in caves throughout the earlier periods, it was not until the Bronze Age that coastal caves acquired the most convincing evidence for longer-term domestic occupation.

In contrast, evidence of Iron Age use of caves is impoverished, and by the fifth century CE, when Christianity reached Ireland, caves appear to have lost their ritual and spiritual significance. Instead we see evidence that caves were used for more mundane purposes such as storage and habitation. As Dowd suggests, "Adoption of Christianity may have led to somewhat of a general 'demystification' of caves" (p. 174). In the Post-Medieval Period caves became important places of refuge and stashing of arms during the years of Irish rebellion (1641 and 1798), War of Independence (1919–1921), Irish Civil War (1922–1923), and, in northern Ireland, up to the more recent "Troubles" of 1969–1994. Another use of caves in this period was the clandestine distilling of *poitín*, an Irish version of moonshine, which was outlawed by King Charles in 1661.

In summary, Dowd's book is rich in detail. It is an enjoyable read and I learned much about a cave region about which I had little previous knowledge. However, it is a book about archaeology first and caves second. As Dowd states, "This book is the first dedicated examination of the relationship between people and caves on the island of Ireland spanning a period of 10,000 years" (p. 262). There are relatively few cave maps or description of specific caves beyond their archaeological remains. I don't mean this as a criticism, but the general cave enthusiast should be aware that the theme that ties this book together is the archaeological remains in caves. It is primarily a book about the human perception of caves and evolution of that perception through time.

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GUIDE TO AUTHORS

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***Journal of Cave and Karst Studies* Distribution Changes**

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