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Front cover: The Gallery in Magura Cave, Bulgaria.

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FOOD RESOURCE AVAILABILITY IN A QUARTZITE CAVE IN THE BRAZILIAN MONTANE ATLANTIC FOREST

Marconi Souza-Silva[1], Afonso Sálvio Júnior[2], and Rodrigo Lopes Ferreira[3]

Abstract: The litter produced in the exokarst and imported into a quartzite cave were measured, as well as the latter's availability and consumption in aquatic and terrestrial cave habitats. In the exokarst, the litter production increases during the rainy season, although the actual importation of this coarse particulate organic matter into the cave is mainly due to the wind. The sandstone soil and the percolation of water through the epikarst have a filtering effect, and the CPOM input into deeper zones in the cave is limited, despite the presence of a stream. In the terrestrial environment of the cave, percolating water increases plant detritus consumption somewhat during the rainy season, although it is still low, due to the generally dry soil conditions in the cave. The cave can be characterized as an oligotrophic system, especially in the deeper zones, with most invertebrate species occurring near the cave entrances, where the CPOM is more abundant. The forest promotes the presence of trogloxene taxa (opiliones: *Goniossoma* sp. and Araneae: *Enoploctenus* sp). Guano in the deeper zones of the cave is extremely scarce and old, so its importance as a basic resource for the cave fauna is quite limited. Conversely, termite colonies and scarce roots recruit trophic resources for the troglophile and troglobite species in the deeper zones. Several cave species were observed in these areas, apparently feeding on roots and the debris left by termites.

Introduction

Like soils below the litter zone, lakes, and estuaries, caves are primarily heterotrophic systems (Boling Jr. et al., 1975). The absence of light excludes primary photosynthetic producers, although bacterial chemotrophism is the basis of the primary production in some caves (Sarbu et al., 1996; Hose et al., 2000; Chivian et al., 2008). Food resources are thus generally secondary; they are brought into the subterranean environment by rivers or other water sources, whether by slow percolation through fissures in the ceiling or walls or by racing torrents arising during periodic flooding of streams (Simon et al., 2007). Roots can grow through fissures into the conduits of the cave, and animals in transit in the caves, especially bats, can also transport food resources, thus making them available for other inhabitants (Jasinska et al., 1996; Ferreira, 2005, Souza-Silva et al 2011). Above the caves, the availability of organic matter can be influenced by seasonal variation in production rates, hydrological events, or both. The activity of the fauna and the speed of detritus consumption are also dependent on abiotic factors, especially temperature and humidity (Jordan, 1985).

Food webs based on living plants constitute a high proportion of global biodiversity (Hooper et al., 2000), whereas the invertebrate cave fauna is assumed to be less complex, with a smaller number of species exploring the generally limited resources. These trophic webs are thus considered to be simpler than those on the surface (Culver, 1982). However, in caves where food is abundant, the system can be more complex (Jasinska et al., 1996; Ferreira and Martins, 1998; Ferreira and Martins, 1999; Ferreira et al, 2000, 2007, Souza-Silva et al 2011).

For decades ecologists have focused their studies on interactions between and among elements in the same system, with less emphasis given to elements found in border environments (Boling Jr. et al., 1975). Recent studies, however, have focused on the detritus transfer between ecological systems, as well as the consequences to the community structure on different spatial and temporal scales (Graening, 2000; Rodriguez, 2000; Simon et al., 2007; Hills et al., 2008; Schneider et al., 2011; Souza-Silva et al., 2011; Souza-Silva et al., 2012; Venarsky et al., 2012).

Almost all the information available concerning Brazilian cave fauna is directly related to carbonatic systems. It is thus extremely important to understand the primary relations between exokarstic and endokarstic compartments in non-carbonatic systems, especially the trophic processes involved in the dynamics of the exokarst and the caves.

This study was designed to evaluate the trophic ecology in a quartzite cave and its vicinity, including the surface production, transport to the cave, and consumption of coarse particulate organic matter. The invertebrate community present in these caves was also evaluated.

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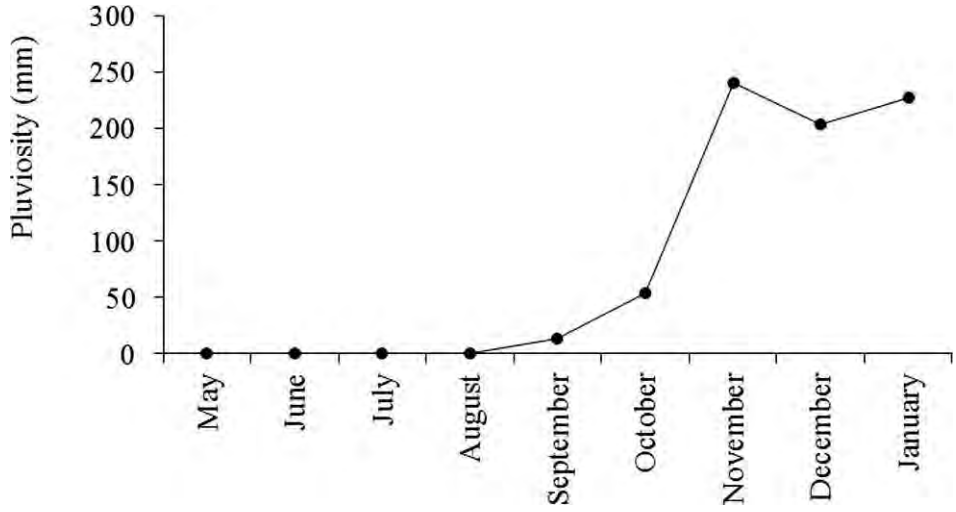

Figure 1. Average rainfall in the vicinity of Casa Cave, Parque Estadual de Ibitipoca, Lima Duarte, Minus Gerais, Brazil.

METHODS AND MATERIALS

STUDY AREA

This research was conducted in the Parque Estadual do Ibitipoca, a conservation area located in the district of Conceição de Ibitipoca in Lima Duarte, in the southeastern part of the state of Minas Gerais in Brazil (23K-615471.15 E/7599906.93 S). This park, which is open to tourism, has an area of 1488 ha, varying in altitude from 1300 to 1784 m. The climate is a typical high-altitude tropical climate, with pleasant summers and well-defined wet and dry periods. The study was conducted from May 2004 to January 2005, with the dry season stretching from May to August and the rainy season from August to January (Fig. 1).

The present study was developed in an undeveloped cave in the park, Casas Cave (21°42′39″S 43°53′41″W), which has 600 meters of linear development and a depth of 39 meters. It has wide passages and ample rooms formed by falling blocks of varying sizes (Fig. 2). The perennial stream in the cave is of allogenic origin. It emerges from a side passage, flows through the main passage, and disappears under breakdown. In addition, seasonal streams arise in different areas inside the cave during the rainy seasons. These are formed by allogenic and autogenic waters and disappear during dry seasons. One of those streams appears near the entrances and flows into the cave in the direction of the main stream. The other two streams rise in one internal chamber upstream; they join the perennial stream in different locations (Fig. 2). A wide variety of sedimentary deposits are found inside the cave, from meter-sized blocks to fine sand of fluvial origin (Silva, 2004). The surrounding vegetation is dense woodlands (Dias et al., 2002).

LITTER PRODUCTION AND TRANSPORT

To evaluate litter production and wind transport of coarse particulate organic matter from the exokarst system into the cave, litter traps were installed, both in the cave entrance and in the external environment just outside the cave. Four collectors (basket traps) consisting of 46 cm diameter metallic hoops covered with nylon net to form 60 cm cones (Pozo, 2005) were installed in the external zone just outside the two cave entrances, and another four were located 5, 10, or 15 meters just inside the cave entrances (Fig. 2).

In the stream inside the cave, two discrete contentions nets were installed (Fig. 2). The contention nets and the collectors were inspected monthly, and all litter collected was removed. In the laboratory, this litter was separated according to category (leaves, fruit, stems, and animal parts), dried in an oven at 70 °C for 72 hours, and then weighed. The air temperature and humidity were measured monthly with a manual thermo-hygrometer. Measures of pH and water temperature were made with a manual thermo–pH meter. Current speed and discharge volume at two points in the cave stream were determined monthly.

DETRITUS PROCESSING

The rate of processing and fragmenting of the organic plant material was determined using plant disks (64 mm^2 each) cut from leaves of *Philodendrum* sp. (Araceae) and inserted into 100 cm^2 bags made from 9 mm nylon net. This plant is typical of quartzite outcrops and is likely to be a source of resources for cave fauna; it can be transported into the interior of the caves by animals, wind, or water. The leaves were collected from the plant prior to abscission to eliminate the possibility that the experimental material had started decomposing on the ground. Each bag was filled with forty disks previously weighed. These bags were then distributed at random points outside the cave, as well as at three points on the ground inside the cave and two in the stream inside the cave (the litterbags of Fig. 2). At the locations where the bags were placed in the water, the speed of the current (m s^{-1}), the temperature (°C), the pH,

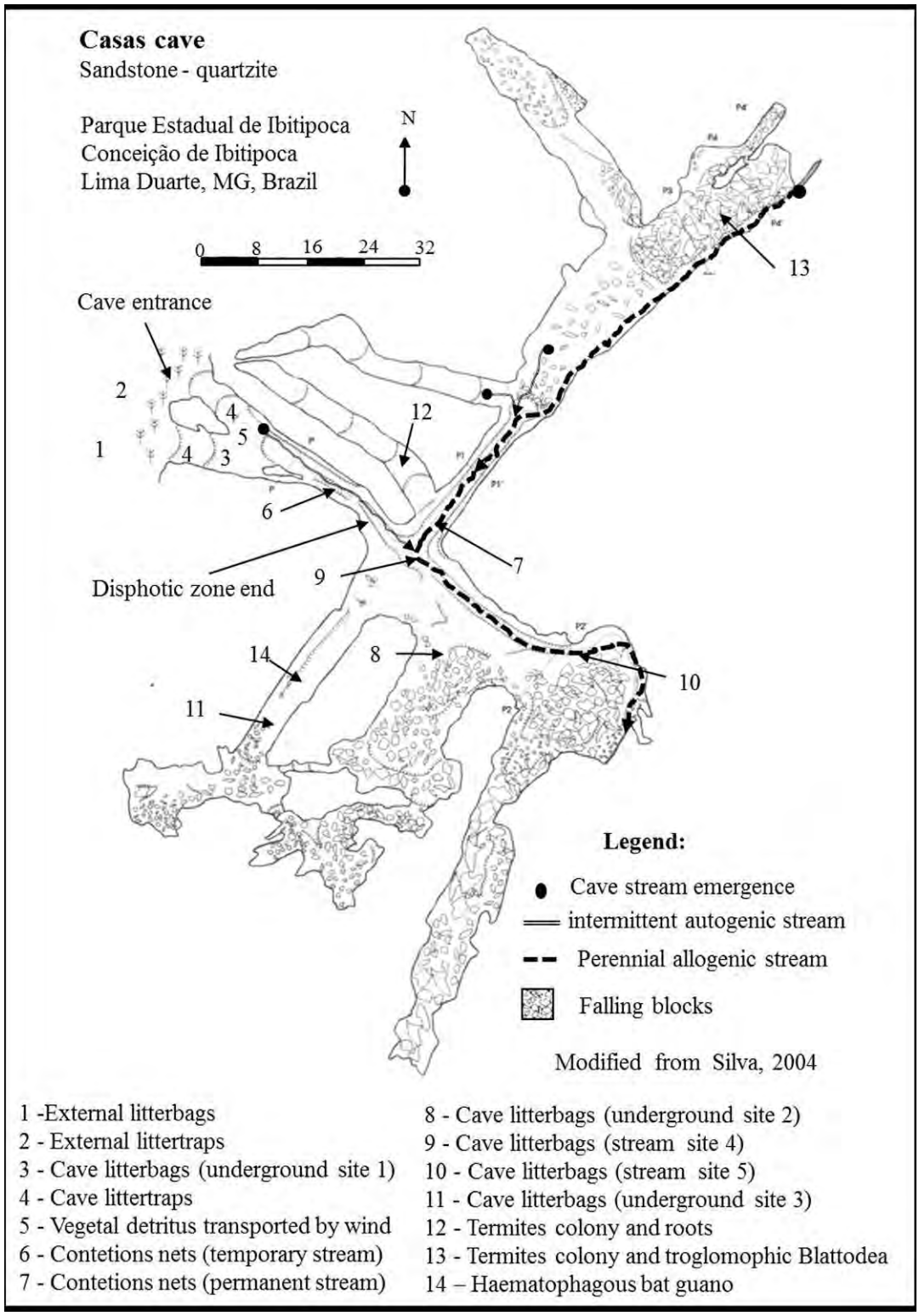

Figure 2. Sample sites of the experimental study of the resource dynamics in Casas Cave, Parque Estadual do Ibitipoca, Brazil.

and the discharge ($m^3 s^{-1}$) were measured. At the points on land inside and outside of the cave, the air temperature (°C) and humidity (%) were measured. Every two months, three of the bags were removed for depletion analysis that was performed by drying the remains of the disks and then weighing them. The difference in weight was used to determine the processing rate (Gallas et al., 1996, Barlocher, 2005).

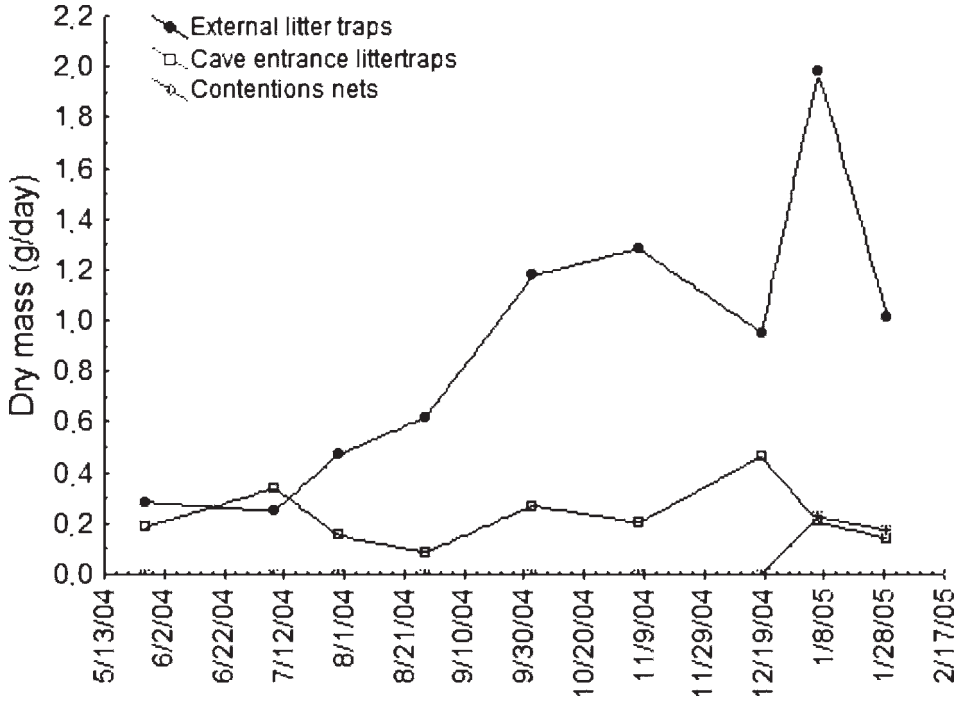

Figure 3. Rate of introduction of organic debris on the surface, in the entrance zone of Casas Cave, Parque Estadual do Ibitipoca, Brazil, and in the cave stream.

BIOLOGICAL INVENTORY/SURVEY

The terrestrial invertebrates in the Casas Cave were collected manually towards the end of the experiment in a visual inspection of the microhabitats under rocks, in organic deposits, and along the walls, floor, and ceiling of the cave, as well as in standing pools and running water. To evaluate the distribution and abundance of these invertebrates, the individuals found in the cave were plotted on a cave map (level BCRA-4C) using the methods proposed by Ferreira (2004). All of the organisms collected were identified to the lowest taxonomic level accessible and separated into morphospecies (Souza-Silva et al., 2011a). All of the visible coarse organic matter present was plotted and described in relation to state of deterioration, means of importation into the cave, and associated invertebrates (Fig. 2).

STATISTICAL ANALYSIS

Linear regressions were used to evaluate the relation between richness and abundance of invertebrates and the distance from the cave entrance (Zar, 1996). The detritus-processing constant (k^{-day}) was determined by fitting the exponential equation $M_t = M_0 e^{-kt}$ (Oslon, 1963; Weider and Lang, 1982; Allan, 1995), where M_t is the weight at time t, M_0 the initial weight, and t the exposure time of the sample. The invertebrate trophic relationships were observed during field study.

RESULTS

In the external environment near the Casas Cave, the average temperature was 18 °C (±3 SD) and the average humidity was 88% (±6 SD). At the entrance of the cave, the average temperature was 18 °C (±3 SD), and the average humidity 85% (±6 SD); inside the cave the average temperature was 21 °C (±2 SD) and the average humidity, 87% (±6 SD).

At both of the aquatic points inside the cave, the water temperature varied between 14 and 17 °C, and the water was slightly acidic, with a pH varying from 3 to 5. At stream site 4, the current varied from 0.08 m s^{-1} to 0.164 m s^{-1}, with a discharge of between 0.0052 and 0.008 m^3 s^{-1}. At the other point in the water (site 5), the current was somewhat faster, varying between 1.44 m s^{-1} and 0.325 m s^{-1}, with a discharge varying from 0.0080 to 0.238 m^3 s^{-1}.

LITTER PRODUCTION AND TRANSPORT

More litter mass was collected in the external collectors (0.89 g d^{-1}) than in those in the entrance (0.20 g d^{-1}) and aphotic cave zone (0.00 g d^{-1}) (Fig. 3). The litter collected in May weighed approximately 0.283 g d^{-1} of dry matter and in January 2 g d^{-1} of dry matter. Of the four cave collectors located near the entrance, only that 5 m from the entrance captured any litter (0.22 g d^{-1}). Most of the debris being transported to the interior of the cave by streams and caught in the contention nets consisted of parts of plants, especially leaves and stems, and such debris was encountered only during January, in the rainy season (Figs. 3 and 4).

The outside collectors retained 228 grams of dry organic matter during the course of the experiment, with a predominance of leaves (144 grams); 1.97 grams of animal

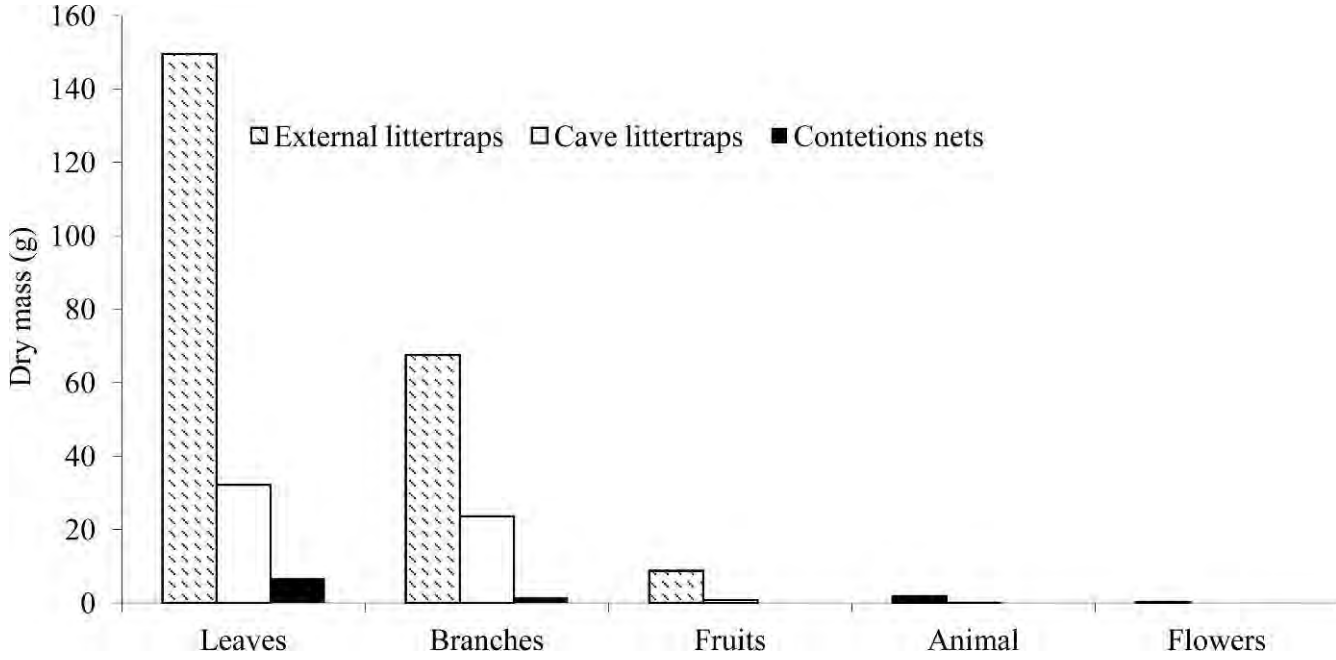

Figure 4. Types of plant detritus collected during the period of observation outside the entrance, in the entrance area of the cave, and in the stream nets.

carcasses were also retained (insects and arachnids). The cave-entrance collectors also captured a greater percentage of leaves (33 grams) than other types, although stems (23.58 grams) and dry fruits (1.11 grams) were also found. In the contention nets, only plant parts were retained: leaves and stems, with 6.75 and 1.54 g, respectively (Fig. 4).

The contention nets installed in the secondary autogenic drainage near the cave entrance collected CPOM being carried by the torrent only in January (Fig. 3). The other two contention nets in the aphotic cave stream contained only sandy material.

Detritus Processing in Cave

At both points in the water where mesh bags of leaf samples were distributed, there was a loss of 65% of the mass during the first 25 days (Fig. 5). At stream site 4, the remaining weight decreased to less than 20% in 186 days of exposure, and after the entire 269 days, only 12% remained (Fig. 7). At stream site 5, after 119 days of exposure, only 9% of the plant material remained. After this date, consumption diminished, and only 20% of the 9% remaining after 119 days had been consumed by the end of the experiment (Figs. 5 and 7). The processing rate of the plant debris (k^{-day}) in the aquatic environment was moderate to fast. The invertebrates observed in the bags in the stream were Diptera (Chironomidae) and Coleoptera (Elmidae).

During the first 25 days of exposure of the plant debris, there was a rapid drop in the weight at all four sites (50 to 60%). In the underground station 1 only 20% of the mass remained after 155 days. At stations 2 and 3 an average of 10% of the mass remained after 250 days. After the 269 days of the experiment, at least some of the plant material remained in all of the mesh bags left in terrestrial environments (Figs. 6 and 7).

The processing rate of the plant debris (k^{-day}) was quite slow in the epigean environment and cave entrance, whereas in the aphotic cave terrestrial zone it was somewhat moderate. Only *Pseudonannolene* sp. (Diplopoda: Pseudonannolenidae) were found in the hypogean terrestrial bags. Differences in initial and final plant disk masses in terrestrial and aquatic habitats of the Casas Cave are shown in Figure 7.

Invertebrates' Structure and Distribution within Cave

In Casas Cave, sixty-eight species of invertebrates were found. The richest orders were Araneae (18 spp.), Diptera (8 ssp.), Coleoptera (7 spp.), Acari (7 ssp.), and Hymenoptera (6 spp.) (Table 1). Species evidencing troglomorphic traits were from the Pselaphidae (1 sp.), Blattodea (1 sp.), and Projapygidae (1 sp.). There was a significant and negative relationship between richness ($\log_{10}$) and the distance from the cave entrance ($\beta = -0.61$; $p < 0.002$; $R^2 = 0.37$), as well as between abundance ($\log_{10}$) and the distance from the cave entrance ($\beta = -0.52$; $R^2 = 0.26$; $p < 0.001$) (Fig. 8).

Invertebrate Community and Trophic System

In the epigean and hypogean environments of Casas Cave, it was possible to delimit three distinct zones with distinct trophic networks: the exokarst (eutrophic), the

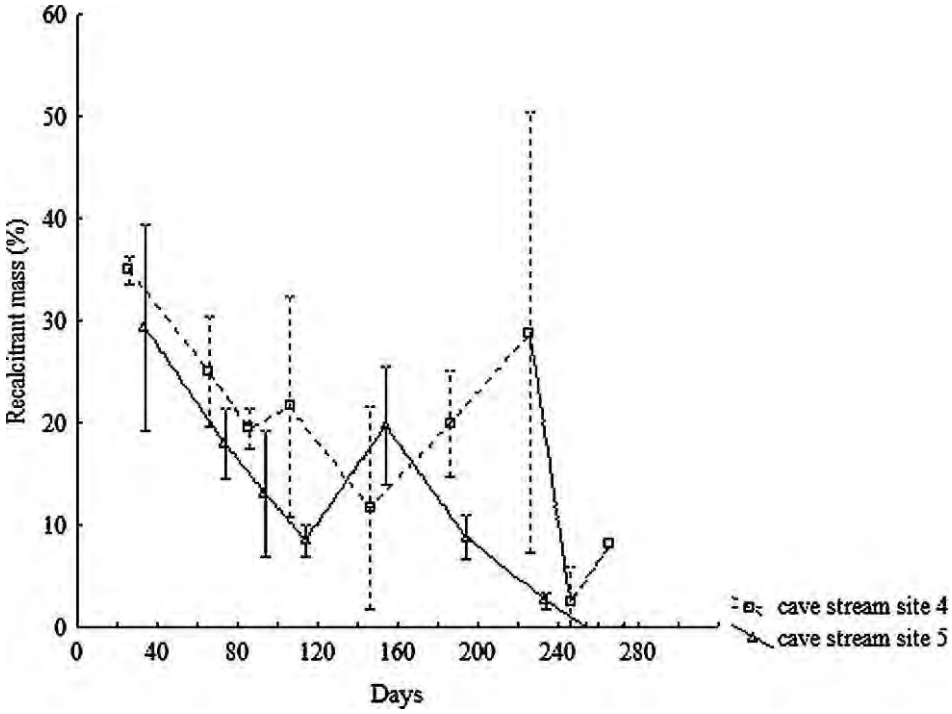

Figure 5. Decay of plant-detritus samples in the stream of Casas Cave, Parque Estadual do Ibitipoca, Brazil (mean and ± SD).

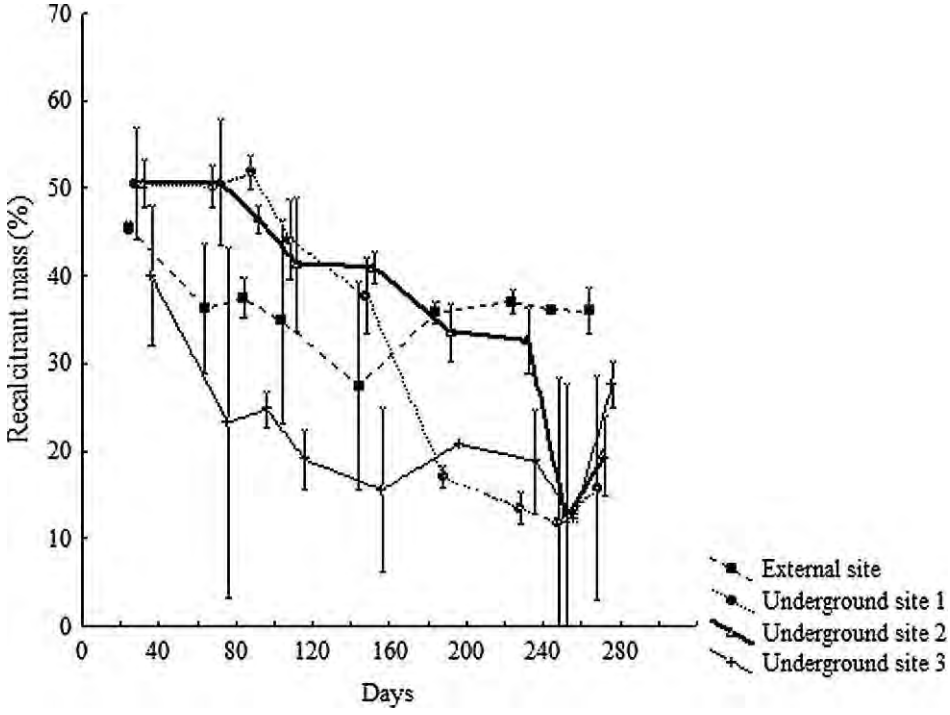

Figure 6. Decay of plant-detritus samples in terrestrial sites of Casas Cave and its exokarst, Parque Estadual do Ibitipoca, Brazil (mean and ± SD).

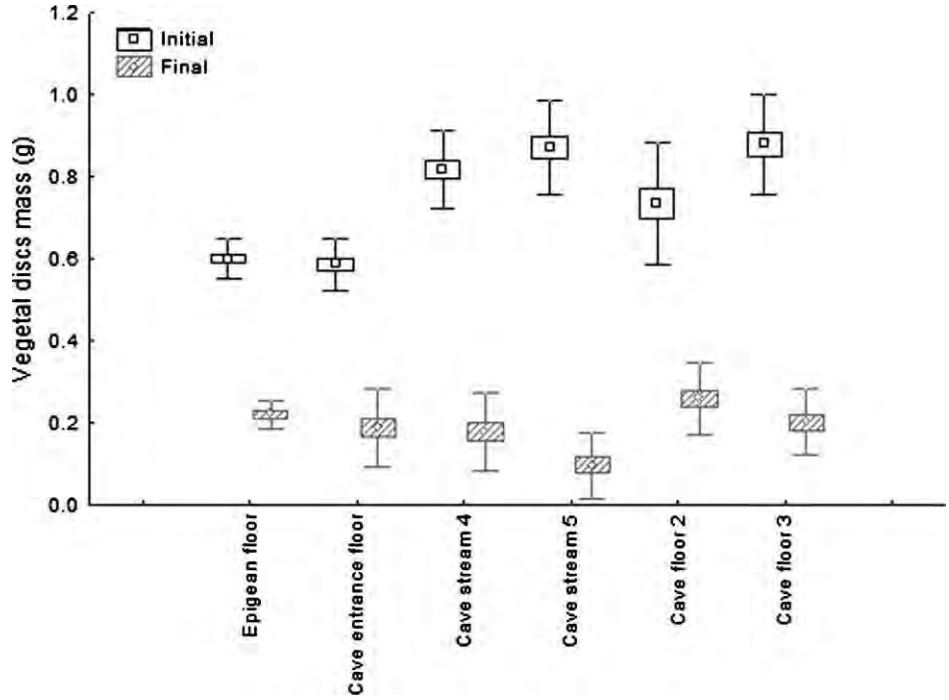

Figure 7. Initial and final plant detritus decay in different places (terrestrial and aquatic) in the quartzite Casas Cave and its exokarst, Parque Estadual do Ibitipoca, Brazil.

cave entrance (mesotrophic), and deepest portion of the cave (oligotrophic).

In the exokarst environment surrounding the entrance of Casas Cave, the dense rain forest produced more leaf litter during the rainy months of the year than during the dry months (Fig. 3). The slow processing rate of this plant debris and the limited transport of the coarse particulate organic matter (CPOM) resulted in the accumulation of a large amount, and in this environment, food resources were plentiful for invertebrates.

In the disphotic region of the cave, near to the entrance, the main food resources were ferns, mosses and scarce litter (leaves and branches from the surrounding trees). More CPOM was blown into Casas Cave during the rainy season than during the dry season, although this accumulated near the entrance of the cave. The downward-sloping floor is composed of highly permeable sand strewn with boulders, so it functions as a filter for the debris, with water passing easily, while the particulate material is retained. In this environment, processing is slow, although the low food-resource transport rate does promote accumulation. Given the slow processing rate, the system was classified as mesotrophic. The scavenger invertebrates found there were Turbellaria, Acari, Phalangopsidae, Tenebrionidae,

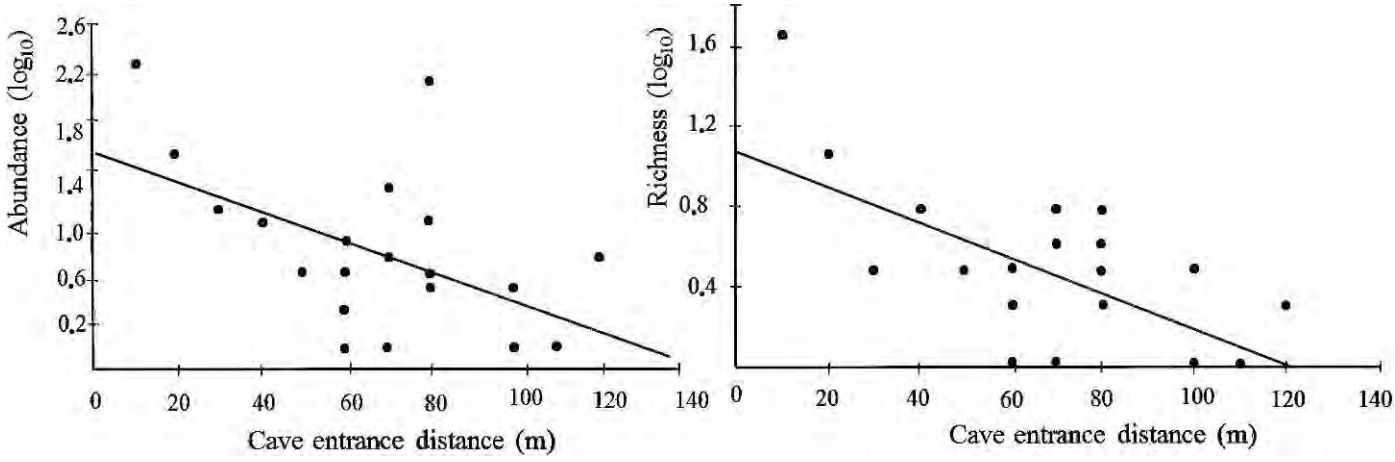

Figure 8. Relationship between invertebrate abundance and richness (log_{10}) with entrance distance in Casas Cave.

Table 1. Invertebrate communities structure in Casas Cave, Parque Estadual do Ibitipoca, Brazil.

Taxons	Taxons	Morpho-Species	
		Cave Floor	Cave Stream
Platyhelminthes	Dugesiidae	2	
Oligochaeta	NI		1
Acari	NI	6	1
Araneae	Ctenidae (*Ctenus* sp. *Enoploctenus* sp.)	2	
	Pholcidae (*Mesabolivar* sp.)	1	
	Dipluridae	3	
	Theraphosidae	1	
	Orthognatha	1	
	Theridiidae	1	
	Theridiosomatidae (*Plato* sp.)	1	
	Ochyroceratidae	1	
Opiliones	Gonyleptidae (*Goniosoma* sp.)	1	
Palpigradi	Eukoeneniidae (*Eukoenenia* sp.)	1	
Pseudoscorpionida	Chernetidae	1	
Diplopoda	Pseudonannolenidae (*Pseudonannolene* sp.)	1	
Chilopoda	Lithobiomorpha	2	
	Geophilomorpha	1	
Collembola	Entomobryidae	3	
Coleoptera	Tenebrionidae	1	
	Curculionidae	1	
	Pselaphidae	2	
	Scarabaeidae	1	
	Elmidae		2
Diplura	Projapygidae	1	
Blattaria	NI	1	
	NI	1	
Diptera	Chironomidae		2
	Tipulidae		1
	Culicidae	1	
	Phoridae (*Conicera* sp)	1	
	Mycetophilidae	1	
Ensifera	Phalangopsidae (*Endecous* sp.)	2	
Ephemeroptera	NI		3
Heteroptera	NI	1	
	Reduviidae (*Zelurus* sp.)	1	
	Ploiaridae	1	
Hymenoptera	Formicidade	5	
	NI	1	
Homoptera	NI	1	
Isoptera	Nasutitermitinae	1	
Plecoptera	Perlidae		1
	Gripopterygidae		1
Psocoptera	Psyllipsocidae	1	
Trichoptera	Leptoceridae		1
Richness		55	13

Scarabaeidae, Curculionidae, Pselaphidae, Formicidae, Leptoceridae and Psocoptera; the predators were limited to Reduviidae, Ploiaridae (Emesiinae), Ctenidae, Pholcidae, Dipluridae, Ctenizidae, Theridiidae, and Ochyroceratidae. Culicidae and Gonyleptidae used the cave entrance for shelter, although they have to leave temporarily in search of food in the surrounding area (Fig. 9).

In the deeper portions of the cave, plant debris was scarce, resulting in an oligotrophic environment. Most of the food resources available were limited to mineralized

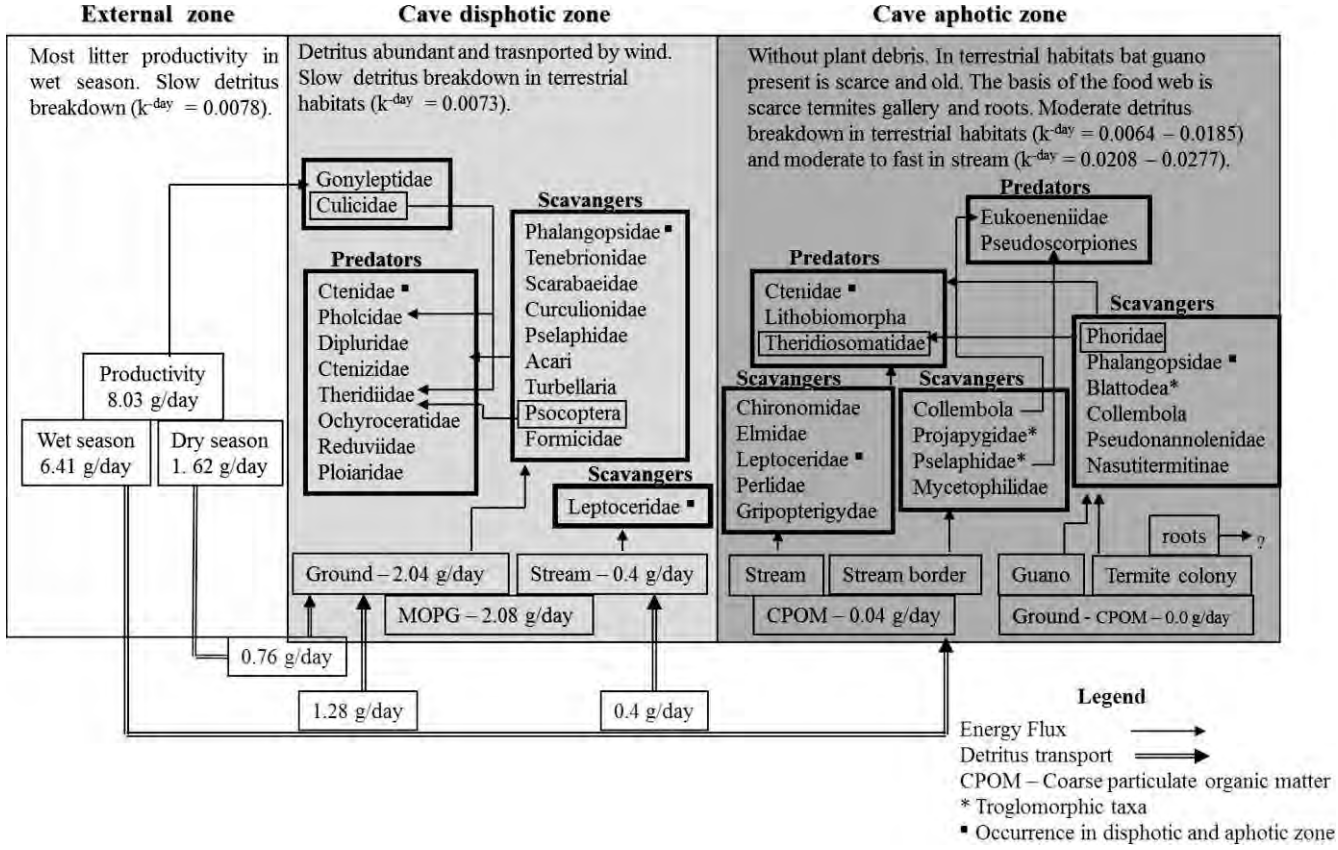

Figure 9. Trophic dynamics in Casas Cave and its exokarst, Parque Estadual de Ibitipoca, Brazil.

guano, as well as the detritus left by termite colonies established at isolated locations around the cave; few roots from external plants had penetrated the ceiling. In this deeper environment of the cave, the scavengers were Chironomidae, Elmidae, Leptoceridae, Perlidae, Gripopterigydae, Projapygidae, Pselaphidae, Mycetophilidae, Phoridae, Phalangopsidae, Blattodea, Collembola, Pseudonannolenidae and Nasutitermitinae found in guano and the termite nests. The predators were Ctenidae, Theridiosomatidae, Pseudoscorpiones, Palpigradi, and Lithobiomorpha (Fig. 9).

Discussion

The invertebrate fauna composition in Casas Cave, especially in relation to families, is similar to that registered in other quartzite caves in the state of Minas Gerais (Ferreira, 2004; Souza-Silva et al., 2011b). The invertebrates collected in Casas Cave were located largely near the cave entrance, becoming progressively less rich and abundant as one penetrates farther into the cave. This is due to the scarcity of organic resources in the more internal portions of the cave. This picture of intense oligotrophy in the more internal portions may be what is determining the relatively greater richness of species associated with the entrance areas (Prous et al., 2004; Li, 2006).

The depth of light penetration in a cave entrance depends on the position, general direction, and shape of the conduit, as well as the size of the cave entrance. The light gradually loses its intensity and changes in spectral content with increasing distance from the entrance (Sket, 2004). Phototrophic organisms are present, but gradually disappear towards deeper zones. Flowering plants are the first group to disappear, followed by ferns, mosses, green algae, and finally cyanobacteria (Sket, 2004; Pentecost, 2004). These habitats are considered as ecotones between the surface and dark cave habitats (Sket, 2004; Prous et al., 2004).

The absence of fresh guano deposits in the interior of the cave suggests that bats no longer inhabit the cave, though in the past various colonies were established and regularly deposited guano in the interior rooms. The colonization of caves by bats is important for the constant production of guano and the maintenance of a well-developed invertebrate cave community's structure, especially in permanently dry cave environments (Souza-Silva et al., 2011; Ferreira and Martins, 1999). The abandonment of this cave by the bats may have been a consequence of the intense flow of tourists along the trail to the park's administration center, located some 200 m from the cave entrance. Moreover, the trail remains illuminated at night, and this may also have influenced bats' habits, leading

them to seek refuge in other caves of the region. Abandonment by bats has probably contributed to the intense trophic poverty encountered in many of the caves in the park in the middle and long term.

The relationship of litter fall to rainfall can depend on vegetation type, as well as weather conditions. Outside Casas Cave debris accumulates on the ground in greater quantities during the rainy season, although there is higher humidity of the air and greater availability of water in the hydromorphic sandy soil. The rain-forest environment here may lead to less leaf loss from water stress than is typical elsewhere due to greater amounts of sunshine during drier periods.

Since the sandy soil at the entrance of the cave is very permeable, rain water rapidly filters through it. This hinders the formation of floods that could transport coarse particulate organic matter into the cave and also carries any dissolved organic matter to deeper layers of the soil. The CPOM in the cave is mainly blown in by wind as it falls from the trees. The force of this wind, however, is insufficient to transport the CPOM very deeply into the cave. Limestone caves also tend to experience greater transport of debris into the hypogean environment during the rainy season (Souza-Silva et al., 2011; Souza-Silva et al., 2007). However, in contrast to what happens in the quartzite surroundings of Casas Cave, forests in limestone areas have well-defined phenophases and produce somewhat more leaf litter during dry periods (Brina, 1998). The limited production of leaf litter in the exokarst around Casas Cave during the dry period may be related to the dense fog that frequently covers the higher areas of the Parque Estadual do Ibitipoca all year round.

Surface streams in riparian forests may receive large amounts of CPOM, transporting it into caves when passing through the swallets connected to the hypogean environments. Leaves and branches that have fallen to the ground are also moved more easily during periods of rapid water flow resulting from intense rains (Webster et al., 1999). But for Casas Cave, the low rate of CPOM transport is at least partially determined by the epikarstic hydraulic dynamics above the cave. Some streams arise from the water percolating through the ceiling or flow for long stretches through and around fallen rocks; these conditions filter out all CPOM. This means that the resulting trophic webs will have limited food resources and be generally less complex, based mainly on biofilms, with invertebrate communities largely consisting of filtering organisms (Simon et al., 2003). Litter exclusion in stream influences local food webs (Wallace et al., 1997). The exclusion led to accelerated breakdown by reducing nutrient limitation (Tank and Webster, 1998). Limestone cave streams without openings to the surface are fed by water percolated through the soil and bedrock. Such disconnected streams receive only fine particulate organic matter and dissolved organic matter transported through small fractures in the rock (Simon and Benfield, 2001).

The occurrence of additional resources derived from termite dens and the garbage dumps of ants is rare in cave environments, so their utilization by invertebrates is limited. Such resources rarely function as the trophic basis for more complex food webs in caves (Ferreira, 2000). However, in Casas Cave, the presence of such resources, coupled with the limited availability of other options, has changed the situation, and here one finds an abundance of organisms associated with and close to the termite nest.

Leaves in streams, disconnected from the surface, break down at slow rates, generally similar to those found in lakes and low-order streams (Simon and Benfield, 2001). However, the processing of plant debris in the hypogean stream in Casas Cave is similar to that found in hypogean streams connected to the surface (Simon and Benfield, 2001). Leaves in hypogean streams disconnected to the surface lose mass slowly, and at roughly the same rate as wood. Leaf breakdown is relatively fast in hypogean streams connected to the surface (Simon and Benfield, 2001). Initial rates of nutrient loss are fast due to leaching by the abrasive force of the water. Moreover, the moderate processing can be under influence of recalcitrant *Philodendrum* leaves and little richness of shredding invertebrates.

The difference in the processing rates observed for the two sample sites in the stream inside the cave is probably related to the greater discharge and current speed at site 5, where small neighboring streams flow into the main stream. The scarcity of shredding invertebrates also suggests that the main agent for processing organic plant material in the aquatic environment of this cave is the physical and chemical action of the water.

In the terrestrial environment, high temperature and humidity are fundamental for rapid decomposition, since these regulate the metabolism of the decomposing organisms; these conditions can also cause differential liberation of phenolic compounds and the leaching of leaves during decomposition (Nicolai, 1988). According to Souza-Silva et al. (2011), unfavorable environmental conditions such as low humidity and low soil temperature may inhibit colonization by animals and decrease the rate of plant-debris processing in terrestrial environments in caves. The relatively low rates of plant debris processing found in the terrestrial environments inside Casas Cave may be due, at least partially, to the lack of favorable soil humidity, as this may have caused the low richness and abundance of fragmenting invertebrates. The somewhat larger processing rate at site 3 may reflect the presence of a more humid soil, which does permit the activity of the fragmenting invertebrates present (Diplopoda), especially since percolating water dripped directly onto the litterbags (Humphreys, 1991).

There are no studies at the moment concerning organic matter dynamics and its relation to invertebrate communities in quartzite caves. However, at least for some Brazilian limestone caves, there is some available information concerning trophic relations in guano invertebrate

communities (Ferreira and Martins, 1998; Ferreira and Martins, 1999; Ferreira et al., 2000, 2007). Since the guano is an ephemeral resource, the associated communities can frequently exhibit a dynamic succession related to the changing condition of the guano. The presence of resident bat colonies, however, can provide constant renewal of the organic matter (Ferreira et al., 2000).

The food web (as well as the invertebrate communities that form it) found at Casas Cave is similar to that described for other Brazilian caves, based on anthill garbage, roots, dead animals, guano, and detritus (Ferreira and Martins, 1999; Ferreira, 2000). The main scavenger species found in Casas Cave, as well as their predators, belong to families commonly found in other Brazilian cave organic substrates.

Considerable differences can be observed when comparing the communities associated with the euphotic/disphotic and aphotic zones of the cave (Fig. 9). Such differences are more evident considering the taxa substitution and low similarity between these communities. These two zones represent distinct compartments located in a same cave. Most of the trophic resources brought into the disphotic/euphotic zone by water or wind remain there and provide favorable conditions for the establishment of many invertebrate species. These facts make us question some important issues. How interactive are the para-epigean and hypogean communities? What are the actual contributions of ecological, environmental, and evolutionary conditions in determining the distribution of species in a cave?

Since food resources are limited in deep zones of the cave, most species might ''prefer'' areas near the entrance (Machado et al., 2001; Prous et al., 2004). However environmental conditions such as temperature and humidity are much more variable than in the deep zones. On the other hand, the environmental stability found in the deep zones is almost always combined with a scarcity of food resources. Because of these differences, the communities become clearly distinct, possibly due to competitive exclusion. According to the literature, species that are more specialized are well adapted to the scarcity of food in the deep zones. These organisms also have lower water-retention capacity due to their thin cuticle and greater surface in relation to their body mass (Culver, 1982).

Culver and Poulson (1970) previously demonstrated that the fauna in the temperate entrance zone of Cathedral Cave are more similar to the fauna inside than outside the cave. Contrary to that, Prous and colleagues (2004) have demonstrated a higher invertebrate similarity between the epigean and ecotone region in two tropical caves, suggesting that the external environment in tropical systems is more important as a source of potential species to colonize the ecotone.

The ecotone, at least for the studied cave, may function as a filter between the two adjacent environments, allowing only pre-adapted organisms to cross it and thus to colonize the cave. The low richness observed in deep zones of the cave could reflect not only food scarcity, but also a strong ''filter effect'' imposed by the rich community established near the cave entrances.

Conclusions

This study has investigated the food-resource availability in a quartzite caves at high elevation in the Atlantic Rain Forest region of Brazil and its influence on the invertebrate fauna distribution and abundance. In such environments, the transfer of allochthonous resources to interior regions of the cave is critical to foster invertebrate diversity. The amount of debris transferred into any cave depends on litter fall during the rainy season and consequent transport of coarse particulate organic matter into the cave.

The presence of tourism in and around the caves and pseudokarst of the state park of Ibitipoca investigated here seems to have disturbed the historic bat population and fostered their abandonment of the cave studied, thus contributing to a decrease in the availability of food resources for the invertebrate fauna.

The trophic relationships identified here sustain only a fragile community of invertebrates, and these results suggest that similar issues should be considered for other caves, since the maintenance of invertebrate and even vertebrate communities often depends on the integrity of the encompassing exokarst.

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S.K. Carmichael, M.J. Carmichael, A. Strom, K.W. Johnson, L.A. Roble, Y. Gao, and S.L. Bräuer – Sustained anthropogenic impact in Carter Saltpeter Cave, Carter County, Tennessee and the potential effects on manganese cycling. *Journal of Cave and Karst Studies,* v. 75, no. 3, p. 189–204. DOI: 10.4311/2012MB0267

SUSTAINED ANTHROPOGENIC IMPACT IN CARTER SALTPETER CAVE, CARTER COUNTY, TENNESSEE AND THE POTENTIAL EFFECTS ON MANGANESE CYCLING

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Abstract: Anthropogenic impact is a pervasive problem in heavily trafficked cave systems and fecal contamination is equally problematic in many cave and karst waters worldwide. Carter Saltpeter Cave in Carter County, Tennessee exhibits Mn(III/IV) oxide coatings associated with groundwater seeps, as well as manganese oxide growth on litter. Culturing results revealed that Mn(III/IV) oxide production on litter was associated with Mn(II)-oxidizing fungi. Immediately prior to this study, a massive Mn(II)-oxidizing biofilm bloomed at a cave seep. During the course of this study from 2009–2011, the seep exhibited a dramatic visual reduction in Mn(III/IV) oxide production, which was hypothesized to correlate with a decrease in fecal nutrient input. Molecular methods (16S rRNA gene sequencing) confirmed the presence of *Bacteroides-Prevotella* human fecal indicators in this seep, and most probable number assays and ion chromatography of the associated seep water confirmed nutrient loading at the site. Further, phylogenetic analysis from clone sequences suggested a strong initial human-specific fecal signature (50% of the sequences clustering with human feces sequences) in July 2009, and a weaker human signature (20% clustering) by June 2011. Most Probable Number (MPN) analyses of heterotrophic bacteria at this site suggested that Mn(II) oxidation was correlated with heterotrophic activity, due to point source exogenous nutrient loading.

Introduction

Karst systems are vital sources of drinking water and support some of the most fragile and diverse ecosystems on Earth (van Beynen and Townsend, 2005). However, a combination of bedrock porosity and high hydrologic conductivity allow contaminants to penetrate limestone bedrock and move through karst conduit systems quickly, especially during periods of high velocity flow (Vesper et al., 2001). For this reason, karst hydrologic systems are often associated with channeled non-point source groundwater pollution (Green et al., 2006; Worthington, 2011).

It is not always possible to visually assess biological contamination in a karst or cave system. As a result, indirect methods of water monitoring for microbial contaminants are frequently employed by researchers: culture-based studies of water to detect coliform bacteria (Mikell et al., 1996; Rusterholtz and Mallory, 1994), molecular techniques to identify the presence/absence of fecal indicators and endemic species in karst systems (Ahmed et al., 2008; Johnson et al., 2011; Johnston et al., 2012; Porter, 2007; Roslev and Bukh, 2011), and the use of fluorescent dye-tracers and microspheres to model water flow and pathogen dispersal in conduit systems (Goeppert and Goldscheider, 2011). Direct study of karst environments is restricted to open conduits that are large enough to allow for human movement and environmental manipulation. Thus, cave research has become a focal point in delineating the effects of anthropogenic impact on karst terrain.

As is the case with karst systems in general, anthropogenic impact in cave systems is a phenomenon that has been documented worldwide (Gillieson, 2011). Human-induced alterations in the cave environment have been shown to destroy microhabitats (Northup, 2011), alter cave biogeochemical cycles, and impact sensitive cave fauna such as bats (Blehert et al., 2011). Any type of impact within caves is primarily manifested at the lower trophic levels, particularly in cave microbial communities that have the potential to exert powerful bottom-up controls on ecosystem health and stability (Horner-Devine et al., 2003). Due to the constancy of the cave environment, impact is quickly detrimental, and hard to reverse.

In 2009, a study was initiated to characterize the geomicrobiology of ferromanganese deposits in Carter Saltpeter Cave, Carter County, Tennessee (Carmichael et al., 2013). Human impact within the cave system was evident throughout the 2009–2011 study period and researchers often noted: 1) an abundance of graffiti

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covering cave rocks and walls, 2) a prevalence of litter throughout the cave system, and 3) a distinct sewage odor present in some portions of the cave, some of which have active water flow. One of the water sources for the cave is known to be contaminated by a variety of inputs, including fecal coliforms (Gao et al., 2006), and many streams in this region are listed as impaired bodies of water (Johnson, 2002). At one of these sites, a thick, dark black, microbial biofilm was present in the water seeping from the cave wall and flowing onto the cave floor (Carmichael et al., 2013). The dark color of the seep was due to microcrystalline Mn(III/IV) oxide minerals, as detected with a Leucoberbelin Blue field test developed by Krumbein and Altmann (1973). Members of the Mountain Empire Grotto in Johnson City, Tennessee, suggested that the appearance of the biofilm coincided with a time in which a local septic tank company had been seen dumping raw sewage into a sinkhole that is hydrologically linked to the cave (John Matthews, personal communication). Additionally, early molecular microbiological analyses documented the presence of several dominant Operational Taxonomic Units (OTUs) in clone libraries constructed from biofilm material that were closely related (≥97% identical) to environmental clones isolated from fecal contaminated water and/or activated sludge (Carmichael et al., 2013), providing further circumstantial evidence of nutrient loading/sewage contamination at this site.

From 2009–2011, the appearance of the biofilm changed drastically, losing its dark black color, and exhibiting a dramatic visual reduction in Mn(III/IV) oxide production, though estimated cells/g wet weight biofilm material remained relatively constant (Carmichael et al., 2013) and field tests continued to demonstrate the presence of microcrystalline Mn oxides, as detected using Leucoberbelin Blue (Krumbein and Altmann, 1973). Given the dramatic change in the appearance of the biofilm over the duration of the study and the molecular evidence of potential contamination, we hypothesized that the appearance and bloom of the biofilm was linked to an acute, point source nutrient loading event in an area hydrologically connected to this shallow cave system. Thus, we initiated this study to document the extent of human impact within the cave system, and to either validate or alleviate concern over potential fecal contamination at the site.

Methods

Field Sites and Sample Collection

Carter Saltpeter Cave (Fig. 1) is located in the Ordovician Knox Dolomite in Carter County, Tennessee. At a depth of approximately 30 m, Carter Saltpeter Cave (CSPC) represents a relatively shallow epigenic cave system typical of those found within the southern Appalachian region. Environmental conditions within the dark zone of the cave are consistent with those found in other cave systems (Northup and Lavoie, 2001). A variety of carbonate speleothem formations occur throughout the cave system (e.g. flowstone, dripstone, soda straws, corrosion residue), and the cave is particularly enriched in ferromanganese deposits. The cave is located in close proximity to both agricultural land and residential areas, and during the time of this study the cave entrance was neither gated nor protected from human traffic. Daniel Boone Caverns (Fig. 1), located 53 km north of CSPC near Ft. Blackmore, Virginia, is also located in the Knox Dolomite, but is on an isolated and forested ridge and the entrance is gated and locked. While there are also ferromanganese deposits in Daniel Boone Caverns, there is no agricultural runoff infiltrating into the cave, nor is there evidence of significant human impact. For the purposes of this study, Daniel Boone Caverns is considered a geologic analogue of CSPC, as it shares the same host rock and has a similar climate and rainfall patterns; and therefore, is used as an intact control system to compare with the heavily impacted CSPC.

From 2009–2012, deposits from seeps and litter scattered throughout CSPC were screened for the presence of Mn oxides using 0.04% Leucoberbelin Blue (LBB), a redox indicator that is oxidized by Mn(III) or Mn(IV) to produce a bright blue color change (Krumbein and Altmann, 1973). Samples from seeps were collected aseptically by scraping the deposit coating down to the solid rock base using a sterile falcon tube. Care was taken to sample at locations within a deposit that tested LBB-positive for Mn(II) oxidation and to maximize the sampling of black/chocolate brown coatings in these locations. Samples were stored on ice, transported to the lab, and immediately processed for DNA extraction, electron microscopy, and/or culturing (Table 1).

DNA samples in this study were obtained from July 2009 to June 2011 in roughly three-six month intervals from two sites containing Mn(III/IV) oxides in CSPC (Fig. 1). The first site, Mn Falls, experienced a dramatic change in appearance over the course of one year (Fig. 2a and 2b). The water coming from the Mn Falls seep is hypothesized to be hydrologically connected to a sinkhole where there was an alleged sewage release from a septic tanker truck in 2008 (John Matthews, personal communication). Mud Trap Falls (Fig. 2c) is a second Mn(II)-oxidizing community located approximately 30 m away from the Mn Falls seep. However, Mud Trap Falls is not hydrologically connected to Mn Falls and did not exhibit massive biofilm streamers, or other similar changes in appearance during this time. Therefore, Mud Trap Falls was selected as a comparison site. Water samples were also obtained from the Upper and Lower Shipwreck drip pools in Daniel Boone Caverns (Fig. 1) for comparison, as these water sources did not appear to have ferromanganese deposits associated with them. The temperature, pH, and conductivity of the water at the Mn Falls seep and in the drip pools in Daniel Boone Caverns was measured using either a VWR SymPHony, Fisher Scientific accumet AP85

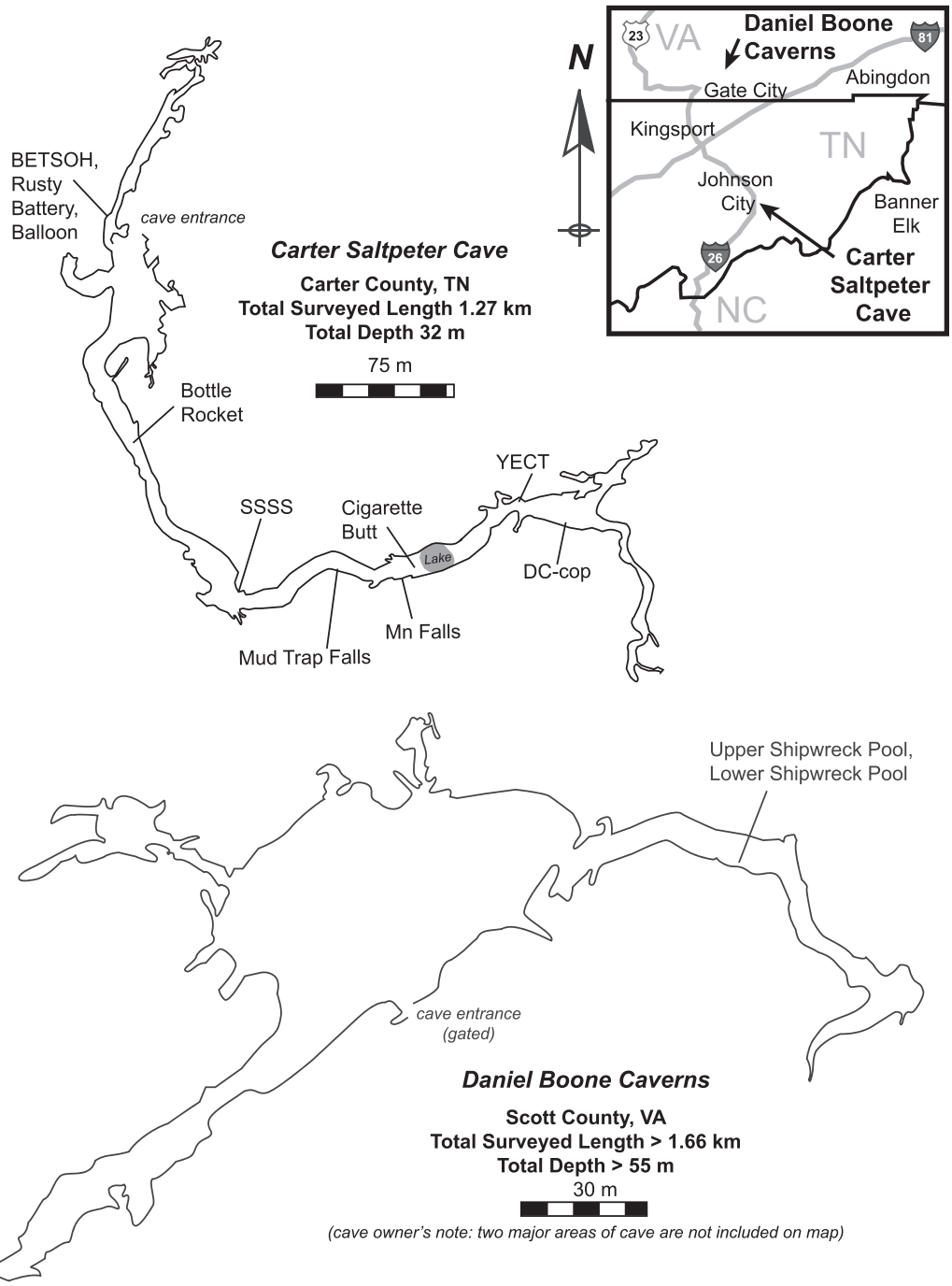

Figure 1. Maps of the Carter Saltpeter Cave (CSPC) and Daniel Boone Caverns, modified with arrows to indicate sampling locations. Regional map inset shows the relative location of the two cave systems within the upper Tennessee River Basin. Cave survey of CSPC conducted on February 8, 1981 by L. Adams, R. Knight, R. Page, and T. Wilson. Cave map drafted by L. Adams and adapted by S. Carmichael. Initial cave survey of Daniel Boone Caverns conducted in 1969 by M. Starnes, B. Lucas, D. Breeding, C. Stowers, and B. Balfour, and an additional survey was conducted from July-November 1996. Two substantial passages in the cave have not yet been surveyed. Cave map adapted by S. K. Carmichael.

Portable Waterproof pH/Conductivity Meter, or a YSI 556 MPS (Multiprobe System), then sampled via syringes and filtered through a 0.45 µm micropore filter into sterilized Nalgene bottles and refrigerated.

Geochemical and Mineralogical Analyses

The anion concentrations in water from the Mn Falls seep and from drip pools in Daniel Boone Caverns were measured using a DionexICS-1600 Ion Chromatograph at

Table 1. Descriptive summary of samples obtained from cave Mn(III/IV) oxide deposits in this study. Cave names are abbreviated as follows: Carter Saltpeter Cave (CSPC), Daniel Boone Caverns (DBC). See Figure 1 for locations of the individual samples. Analyses conducted are abbreviated as follows: Most probable number assays (MPNs), polymerase chain reaction (PCR), powder X-ray diffraction (XRD), single crystal micro X-ray diffraction (μ-XRD), Fourier Transform Infrared Spectroscopy (FT-IR), ion chromatography (IC), and electron microscopy (EM).

Sample (Location) and Sample Type	Date Collected (mm/dd/yyyy)	Analyses Conducted
Mn Falls (CSPC)	09/01/2009	μ-XRD (biofilm)
	05/05/2010, 06/30/2010	MPNs
Mn(III/IV) oxide biofilm	07/04/2009, 07/31/2009, 01/15/2010, 05/05/2010 09/14/2010, 10/05/2010 11/09/2010, 05/16/2011, 06/16/2011	PCR with *Bacteroides* primers
water	03/24/2010, 05/05/2010, 06/30/2010, 08/18/2010 10/05/2010, 04/21/2011 09/23/2011, 09/06/2012, 11/15/2012	IC
flowstone, mud substrate	09/01/2009	XRD, EM
Mud Trap Falls (CSPC)		
Mn(III/IV) oxide coating	07/04/2009, 07/31/2009, 01/15/2010, 05/05/2010 06/30/2010, 09/14/2010 10/05/2010, 11/09/2010, 05/16/2011, 06/16/2011	PCR with *Bacteroides* primers
	01/152010, 06/30/2010	MPNs
	09/10/2009	μ-XRD (coating material)
flowstone, mud substrate	09/10/2009	XRD, EM
DC-cop (CSPC)		
animal feces	05/10/2010	Fungal cultures, XRD (substrate)
SSSS (CSPC)		
cotton sock	10/05/2010	Fungal cultures, EM
YECT (CSPC)		
black electrical tape	06/30/2010	Fungal cultures, μ-XRD (coating material, cultures), EM, FT-IR (cultures)
BETSOH (CSPC)		
black electrical tape	09/06/2012	Fungal cultures
Bottle Rocket (CSPC)		
fireworks	09/06/2012	Fungal cultures
Balloon (CSPC)		
balloon	09/06/2012	Fungal cultures
Rusty Battery (CSPC)		
rusty battery	09/06/2012	Fungal cultures
Upper Shipwreck Pool (DBC)		
water	07/18/2010, 09/22/2011, 10/14/2012	IC
Lower Shipwreck Pool (DBC)		
water	07/18/2010, 09/22/2011, 10/14/2012	IC

Appalachian State University. Characterization of the sampled substrate was performed on a Shimadzu 6000 X-ray Diffractometer (XRD) with ICDD PDF-4 Minerals software, and on a FEI Quanta 200 Environmental Scanning Electron Microscope with an EDAX Si-Li energy dispersive spectrometer (ESEM-EDS). Identification of Mn oxide species was performed on a Rigaku D/Max Rapid micro-X-ray Diffractometer at the Smithsonian

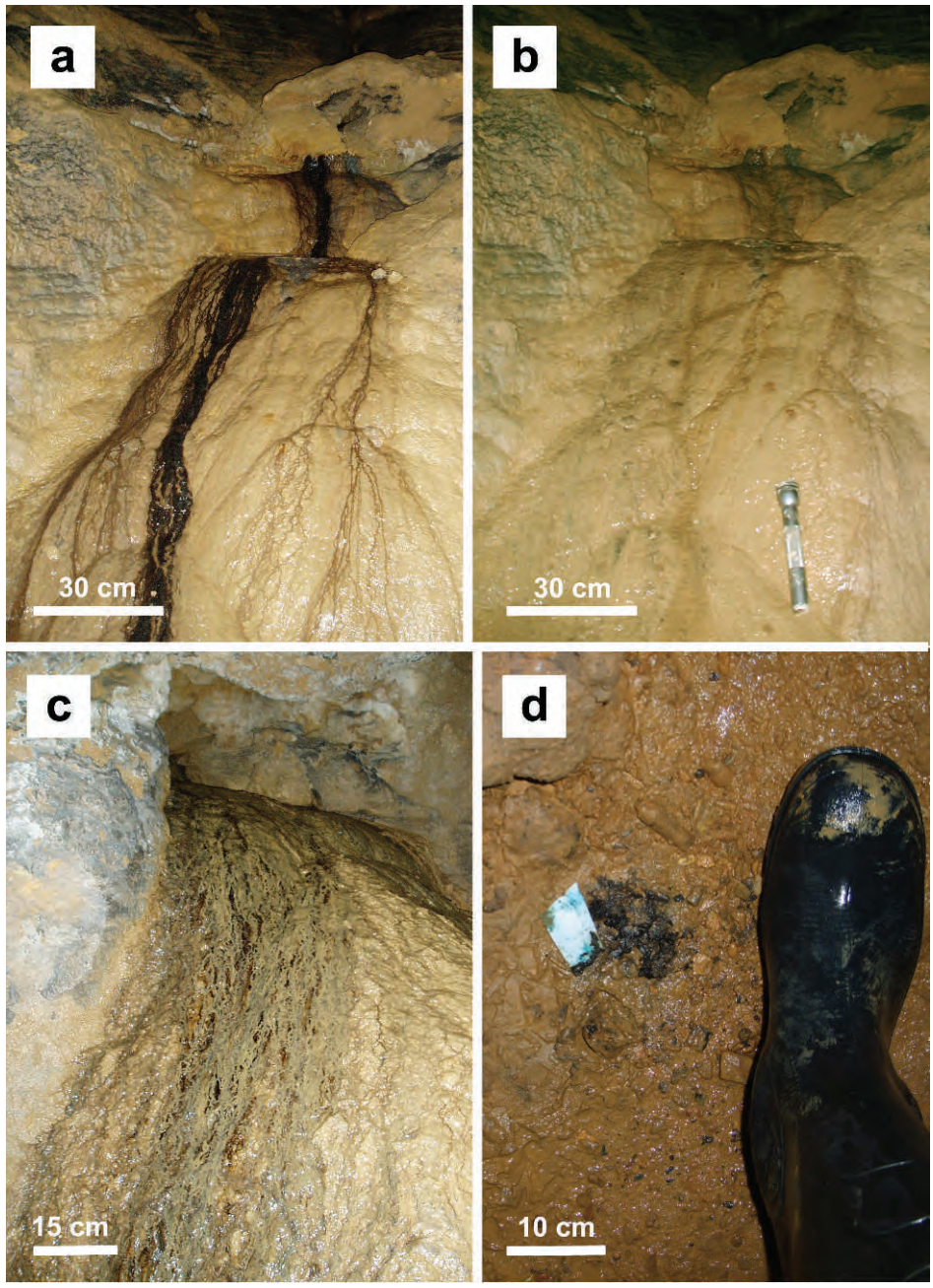

Figure 2. (a) Carter Saltpeter Cave (CSPC), Mn Falls site, July 2008, photo taken by Mountain Empire Grotto member John Matthews several months after a septic tanker truck may have released sewage into a local sinkhole. Image is published with permission. (b) CSPC, Mn Falls site, June 2010, Mn oxides reduced. Mn oxides have steadily decreased with time at the site since sampling began in July 2009. (c) CSPC, Mud Trap Falls site, July 2009. (d) LBB positive test (in bright blue on the filter paper) demonstrating the presence of Mn oxides on feces. Boot for scale. The surrounding clay tested LBB negative.

National Museum of Natural History, and via Fourier Transform Infrared Spectroscopy (FT-IR) using a KBr pellet press method with a Thermo Nicolet Magna 550 IR Spectrometer at Appalachian State University. FT-IR spectra were compared to Mn oxide reference spectra from Potter and Rossman (1979).

Detection of Human-Specific *Bacteroides-Prevotella* 16S rRNA Gene Sequences

DNA was extracted from cave samples using a bead beating protocol with the Fast DNA Spink Kit for Soil (MP Biomedicals, Solon, OH). The concentration of extracted DNA was determined using a NanoDrop ND-

1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Human-specific *Bacteroides-Prevotella* 16S rRNA gene sequences were amplified from Mn Falls biofilm material using a primers designed by Bernhard and Field (2000a) with a demonstrated detection limit of 1.4×10^{-6} g dry feces/liter. A nested PCR approach was utilized in an attempt to amplify the region of interest in DNA extracted from samples collected at Mud Trap Falls and Mn Falls between July 2009 and June 2011. Only two samples from Mn Falls amplified: July 2009 (*F*) and June 2011 (*4*). Approximately 3 ng of environmental DNA was used as a template for the first round of PCR amplification using the universal *Bacteroides-Prevotella* primers 32F (5′-AACGCTAGCTACAGGCTT-3′) and 708R (5′-CAATCGGAGTTCTTCGTG-3′) (Bernhard and Field, 2000b). Each 50 μL reaction contained 1.25 U AmpliTaq Gold (Applied Biosystems, Carlsbad, CA), 50 μM each primer, 1X PCR Gold Buffer (Applied Biosystems, Carlsbad, CA), 2 mM $MgCl_2$ Solution (Applied Biosystems, Carlsbad, CA), 200 μM each dNTP, and 2X BSA (New England Biolabs, Ipswich, MA). An MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, CA) was used for all PCR-amplification reactions. The amplification protocol for the first round of PCR is as follows: an initial denaturation of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 2 min, followed by a final extension of 72 °C for 6 min. PCR amplifications were conducted in triplicate to reduce individual PCR bias and visualized on a 1% agarose gel stained with GelRed Nucleic Acid Stain (Phenix Research, Candler, NC). A single positive band of approximately 700 bp was visualized in DNA samples *F* (July 2009) and *4* (June 2011) obtained from the Mn Falls biofilm. Replicate amplifications of each DNA template were pooled for downstream use in the next round of PCR.

One μL of PCR product from the pooled amplifications of either *F* (July 2009) or *4* (June 2011) DNA was used as a template for PCR amplification of human-specific *Bacteroides-Prevotella*16S rRNA gene sequences in the second round of the nested protocol. PCR amplification was conducted using the *Bacteroides-Prevotella* human-specific forward primer HF183 (5′- ATCATGAGTTCACATGTCCG-3′) paired with the *Bacteroides-Prevotella* universal reverse primer 708R (5′-CAATCGGAGTTCTTCGTG-3′) (Bernhard and Field, 2000a). Reaction conditions mimicked those given for the first round of the nested protocol, with the following adjustment in the amplification protocol: an initial denaturation of 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 2 min, followed by a final extension of 72 °C for 6 min. Amplifications were conducted in triplicate to reduce individual PCR bias, and 5 μL of PCR product for each template was visualized on a 1.5% agarose gel stained with GelRed Nucleic Acid Stain (Phenix Research, Candler, NC) to verify the presence of a ca. 600 bp band. Amplifications of each PCR template were pooled and concentrated by rotary evaporation to a volume of approximately 10 μL. The concentrated PCR product for each template was run on a 1.5% agarose gel stained with GelRed Nucleic Acid Stain (Phenix Research, Candler, NC). A ca. 600 bp band for each template was manually excised from the gel and purified using an UltraCleanGel-Spin DNA Extraction Kit (Mo-Bio Laboratories, Carlsbad, California). Purified PCR products of each DNA template were cloned into TOPO TA *pcr*®2.1 vectors (Invitrogen, Carlsbad, CA), and plasmid DNA extracted from transformants using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) was screened using the M13F(−20) primer. Glycerol stocks were sequenced using the M13F (−20) primer. Sequencing was conducted at Beckman-Coulter Genomics (Danvers, MA). OTUs were determined by DOTUR analysis (Schloss and Handelsman, 2005), and representative sequences for each OTU were chosen based on sequence length and quality. For phylogenetic analysis, additional sequences of interest were selected using ARB (Ludwig et al., 2004) and the NCBI taxonomic database (Johnson et al., 2008). OTU and additional sequences of interest were aligned using the on-line SILVA aligner (Pruesse et al., 2007). A phylogenetic tree (Figure 3) was constructed using the PHYLIP software package (Felsenstein, 2004) by conducting both neighbor-joining and maximum likelihood analysis. Clone sequences were deposited in GenBank under the accession numbers JN820135-JN820146.

Most Probable Number Assays

Most probable number (MPN) assays were employed to estimate the number of culturable heterotrophic microorganisms and heterotrophic Mn(II)-oxidizing microorganisms in CSPC (Table 3), as described previously (Bräuer et al., 2011; Johnson et al., 2012), with some modifications. Samples were collected from Mn Falls and Mud Trap Falls in January, May, and June of 2010 and stored overnight at 4°C. A new media that we designed, FMO2 growth medium (Carmichael et al., 2013), was used for MPN assays. Samples were centrifuged to concentrate the wet biomass and the supernatant was removed. Samples were weighed and diluted 1:10 with medium. Serial dilutions of 1:10 diluted samples were made, ranging from 10^{-2} to 10^{-11} and inoculated in Cellstar 96 well culture plates (Greiner Bio-One, Monroe, NC). Each well contained 250 μL sterile media and was inoculated with 25 μL of either sample material, a positive control (*Leptothrix sp.*), or a negative control in eight replicates per sample. A control plate for each sample was inoculated and tested immediately for Mn(II) oxidation and heterotrophic metabolism using 50 μL 0.04% LBB (rows 1–4) and 50 μL 0.3% iodonitrotetrazolium chloride (INT) (Sigma Aldrich) (rows 5–8), respectively. Background level colorimetric results from the control plate test were recorded and used for comparison with the incubated plates. Plates were incubated in the dark at 10°C for 4 weeks (to mimic cave

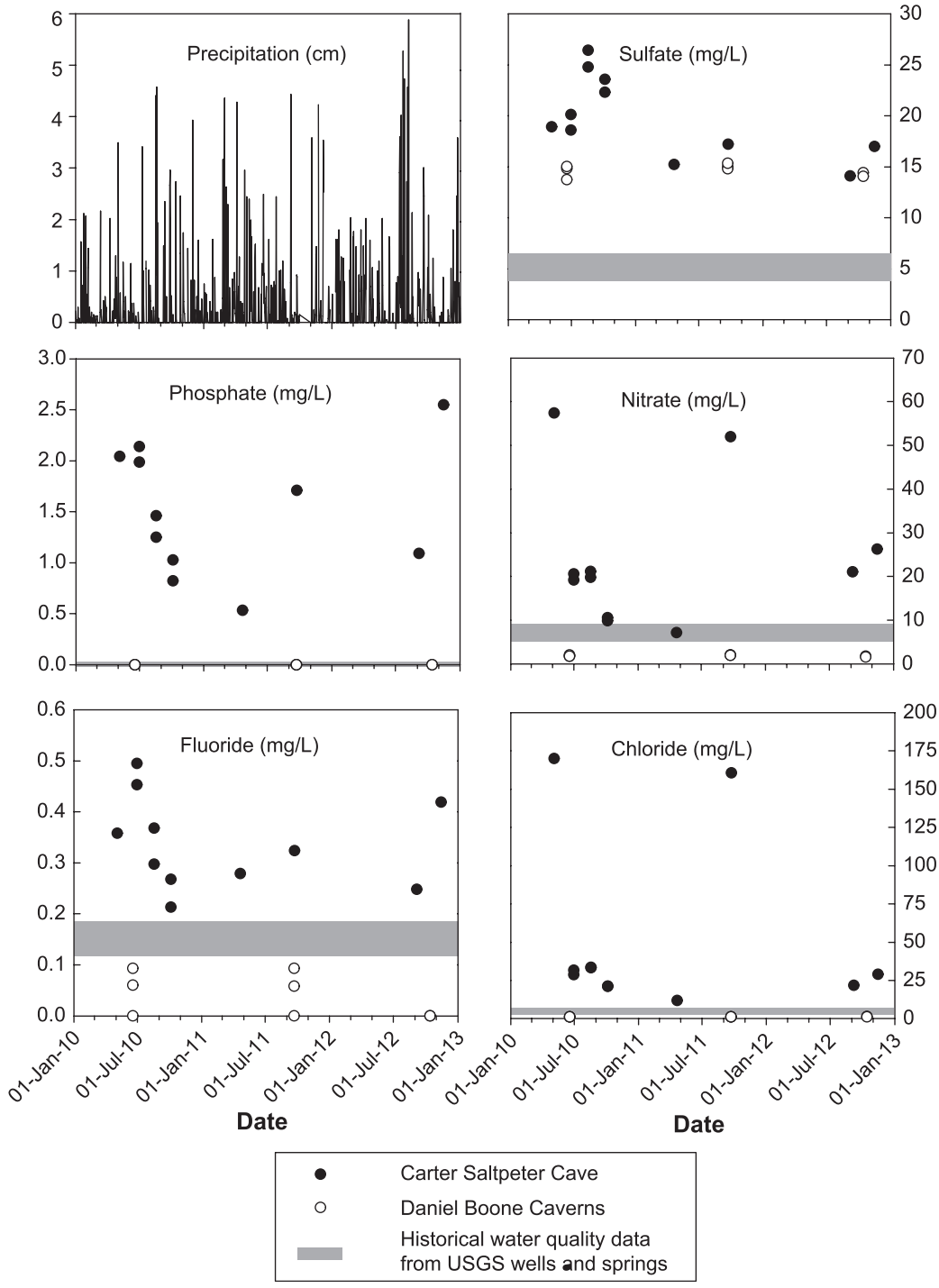

Figure 3. Ion chromatography results for water collected from Carter Saltpeter Cave (CPSC) Mn Falls seep, and from the upper and lower drip pools of Daniel Boone Caverns from May 2010–December 2012. Historical water quality data was collected by the United States Geological Survey in 1997–1998 from wells and springs in the Knox Dolomite located within 25 km of CSPC (data from USGS National Water Information System, http://nwis.waterdata.usgs.gov/usa/nwis/qwdata, accessed August 29, 2012) using sites USGS 03465770, USGS 03486175, and USGS 361726082181801. Precipitation data for Johnson City for the time covering the study were obtained from the National Climatic Data Center (http://www1.ncdc.noaa.gov/pub/orders/118435.csv, accessed January 8, 2013) using gauge GHCND:USC00404666.

Table 2. Field measurements of temperature, pH, conductivity, and dissolved oxygen for Mn Falls in Carter Saltpeter Cave (CSPC) and drip networks in Daniel Boone Caverns (DBC) at various dates from May 2010 to December 2013.

Date	Cave	Temp., °C	pH	Conductivity, μS cm^{-1}	DO, ppm	Field Observations
05/05/2010[a]	CSPC	13.1	7.00	436	8.2	water running clear, sampled from water flowing into pool at base of seep
05/05/2010[a]	CSPC	13.1	6.96	460	7.2	water running clear, sampled from water running across flowstone in middle of seep
06/30/2010[b]	CSPC	13.9	7.44	[d]	…	sampled from water flowing into pool at base of seep; cave floor damp to dry
06/30/2010[b]	CSPC	15.7	7.47	[d]	…	sampled from stagnant pool at base of seep; cave floor damp to dry
04/21/2011[b]	CSPC	13.4	8.51	523	…	sampled from middle of seep, water running over flowstone; cave floor very wet
09/23/2011[b]	CSPC	13.1	8.03	737	…	sampled from water flowing into pool at base of seep
09/06/2012[b]	CSPC	13.4	8.44	677	…	sampled from middle of seep, cave floor damp
11/15/2012[b]	CSPC	13.1	7.64	[d]	…	sampled from top of seep, cave floor dry
07/28/2011[b]	DBC	[d]	8.07	483	…	upper pool
07/28/2011[b]	DBC	[d]	8.06	428	…	lower pool
09/22/2011[b]	DBC	12.2	8.02	354	…	upper pool
09/22/2011[b]	DBC	11.8	8.34	352	…	lower pool
10/14/2012[c]	DBC	11.8	7.87	327	11.5	upper pool
10/14/2012[c]	DBC	11.7	7.97	321	10.2	lower pool

[a] Measurements collected with a Fisher Scientific accumet AP85 Portable Waterproof pH/Conductivity Meter Kit and Hanna Instruments HI 9146 Portable Microprocessor Dissolved Oxygen Meter.
[b] Measurements collected with a VWR SymPHony.
[c] Measurements collected with a YSI 556 MPS (Multiprobe System).
[d] Meter error.

conditions) and scored immediately using LBB and INT as described above. Plates were then returned to the dark at 10°C and allowed to incubate overnight to note additional color change, if any. MPN assays were scored again after 24 hours, with no notable change in results being observed. Results were applied to Curiale's freeware MPN calculator (accessed at http://www.i2workout.com/mcuriale/mpn/index.html) to estimate the total number of culturable heterotrophic microorganisms and culturable heterotrophic Mn(II)-oxidizing microorganisms in cave samples.

Culturing of Fungi From Litter

Fungal culture enrichments were obtained using litter that tested LBB-positive in the cave. Litter was swiped on plates containing an AY (Santelli et al., 2011) agar-solidified media. The plated cultures were tested for Mn(II)-oxidation via LBB colorimetric assay. To discourage contamination by other fungi and bacteria, all Mn(II)-oxidizing fungal samples were recultured on fresh media at least three times using a stab and swipe method. Several samples (YECT Stab 1 and SSSS) were maintained on media containing Ampicillin to prevent bacterial contamination. Samples were incubated in the dark at room temperature for 2–6 weeks.

Electron and Light Microscopy of Fungal Cultures

Light microscopy of fungal cultures grown on AY media was performed using an Olympus SZX12 Zoom Stereo Microscope with a Sony HDR-HC7 HDV video camcorder. For transmission electron microscopy with energy dispersive X-ray spectroscopy (TEM-EDS) and for element mapping via scanning transmission electron microscopy (STEM), samples were mounted on Carbon Type B formvar-coated copper 200 mesh grids (Ted Pella, Inc.), air dried, carbon coated, and analyzed using a JEOL JEM-1400 TEM with a G-135 Pentafet Sealed Window X-ray detector at 80 kV and/or 120 kV in the Dewel Microscopy Facility at Appalachian State University.

Environmental scanning electron microscopy with energy dispersive X-ray spectroscopy (ESEM-EDS) was performed on a FEI Quanta 200 Environmental SEM with an EDAX Si-Li EDS detector in the Dewel Microscopy Facility at Appalachian State University. Portions of

Table 3. Total culturable heterotrophic bacteria and culturable heterotrophic Mn(II)-oxidizing bacteria present in Carter Saltpeter Cave biofilms.

Sample (Sample Date)	Total Heterotrophic Bacteria			Heterotrophic Mn(II)-oxidizing Bacteria					CulturableMn(II)-oxidizing Bacteria (%)
	95% CI lower	MPN	95% CI (upper)	95% CI (lower)	MPN	95% CI (upper)	Total Bacteria[a] (cells mL^{-1})	Culturable Bacteria (%)	
Mud Trap Falls (01/15/2010)	3.6×10^7	1.1×10^8	3.2×10^8	4.5×10^7	1.4×10^8	4.6×10^8	2.6×10^9	4.2	5.4
Mn Falls (05/05/2010)	6.9×10^9	2.5×10^{10}	8.9×10^{10}	9.3×10^7	3.2×10^8	1.1×10^9	3.6×10^9	≥100.0[b]	8.9
Mud Trap Falls (06/30/2010)	3.2×10^6	9.6×10^6	2.9×10^7	…	…	…	…	0.4[c]	…
Mn Falls (06/30/2010)	3.2×10^8	9.6×10^9	2.9×10^{10}	…	…	…	9.8×10^9	98	…

[a] Total bacterial cell number was estimated in a previous study by real-time quantitative PCR (Carmichael et al., 2013).
[b] The 95% CI for heterotrophic bacteria lies within the error range of total bacterial cell number as estimated by real-time quantitative PCR (Carmichael et al., 2013).
[c] Percentage is based on the total bacterial cell number estimated from the qPCR analysis from the winter collection (2.6×10^9; Carmichael et al., 2013).

fungal growth from AY stab cultures were transferred onto a piece of carbon tape and mounted on a 13 mm aluminum stub and run under low vacuum conditions between 15 and 20 kV, with a 5 μm beam spot size.

Results

Geochemistry and Mineralogy

Measurements of temperature, pH, dissolved oxygen, and conductivity were conducted in the field (Table 2). Temperatures ranged from 13–15 °C, pH from 7.4–8.0, and conductivity from 436–737 μS/cm. Higher conductivities were associated with a shallow (5 cm deep) pool at the base of the Mn Falls seep, which contained significant fine sediment. IC analyses of phosphate, nitrate, sulfate, fluoride, and chloride for Mn Falls from 2010–2011 all demonstrated variable but significantly higher values of these anions in comparison to water from Daniel Boone Caverns and water from nearby wells and springs, and these variations did not appear to be correlated with precipitation patterns (Fig. 3).

SEM-EDS and XRD measurements indicate that the substrate for both Mn Falls and Mud Trap Falls consists of a mixture of nontronite clay and calcite. Bacterial Mn(III/IV) oxides at both Mud Trap Falls and Mn Falls were tentatively identified via single crystal micro-XRD as poorly crystalline buserite, a Na-rich layered phase in the birnessite group (Post, 1999) with broad peaks indicating the presence of 9.5-7.3 Å sheets. FT-IR analysis of cultured fungi from electrical tape contained a broad absorbance peak from 3700–3200 cm^{-1}, indicating O-H bonds, and additional sharper peaks associated with Mn oxide octahedral bonds at approximately 1631, 727, 530, 470 cm^{-1}. Peaks associated with amines and polysaccharides were also present. This pattern indicates that disordered todorokite, a Ca-rich mineral with a tunnel structure, may be the dominant Mn(III/IV) oxide present, although poorly crystalline todorokite and birnessite group minerals do frequently coexist in biologically produced Mn(III/IV) oxides, as the biological formation of either layer or tunnel structures is dependent on local solution chemistry (Zhu et al., 2010). Regardless, the Mn(III/IV) oxides described in this study are nm scale and poorly crystalline; and therefore, cannot be definitively determined using traditional powder X-ray and electron diffraction techniques. Our findings are consistent with observations of biologically produced Mn(III/IV) oxides in previous studies (Spiro et al., 2010, and references therein).

Detection of Human-Specific *Bacteroides-Prevotella* 16S rRNA Gene Sequences

A molecular-based survey of the Mn Falls site was initiated to detect the presence or absence of human fecal indicators in DNA extracted from the biofilm from 2009–2011. A primer set designed by Bernhard and Field (2000a; 2000b) targeting human-specific *Bacteroides-Prevotella* was

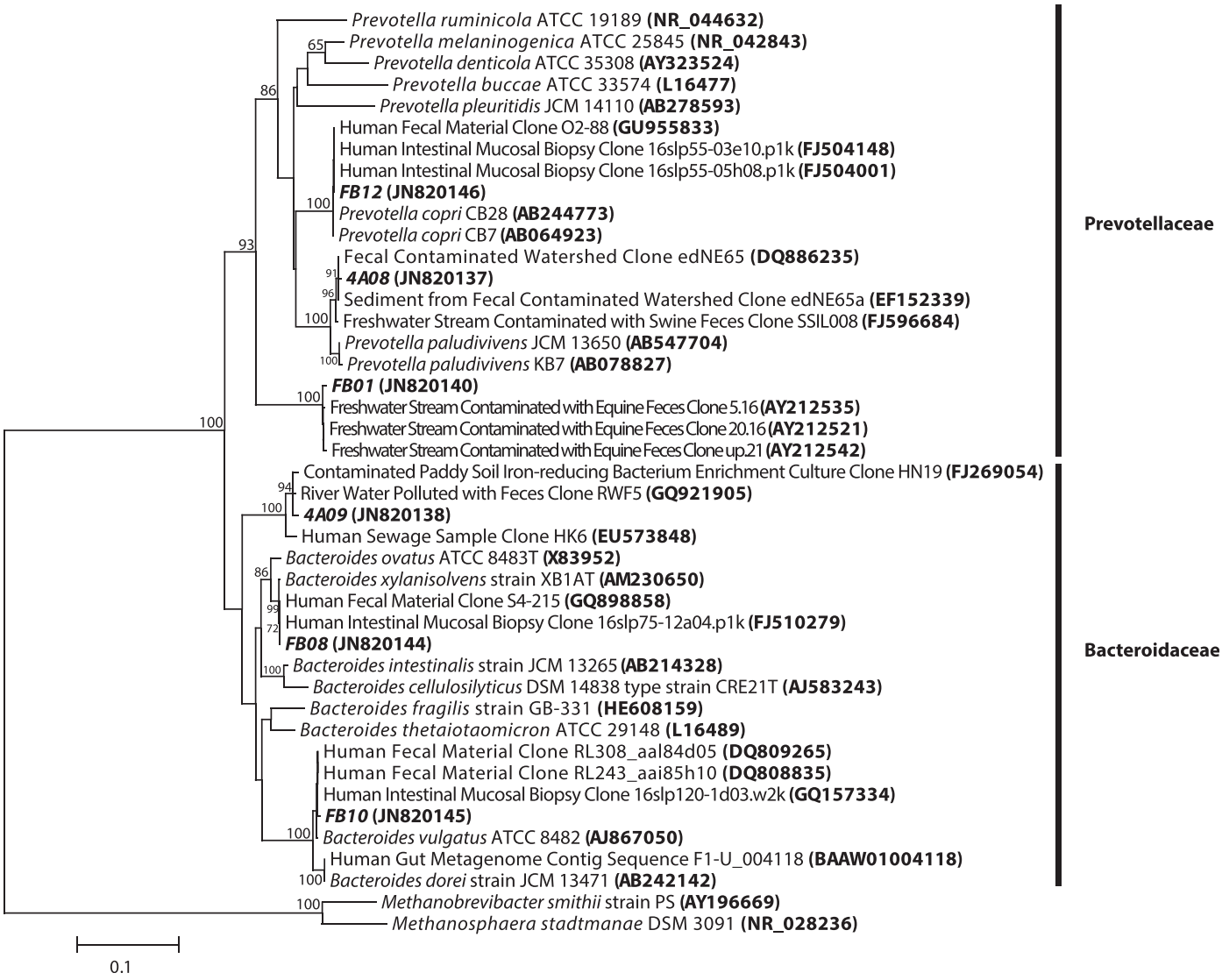

Figure 4. Neighbor-joining tree inferring the phylogenetic placement of SSU rRNA gene sequences obtained from the Mn Falls biofilm found in Carter Saltpeter Cave (CSPC) in this study in either July 2009 (sequences beginning with *F*) or June 2011 (sequences beginning with *4*). Alignments were created using the on-line SILVA aligner. Dendogram was created using PHYLIP. Bootstrapping values are shown for nodes that were supported >50% of the time with maximum-likelihood analysis (data not shown). *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* were used as outgroups. Branch lengths indicate the expected number of changes per sequence position (see scale bar).

chosen due to its sensitivity and reliability according to previous reports in the literature (Ahmed et al., 2009a; 2009b). Multiple attempts at amplification of DNA extracted from both Mn Falls and Mud Trap Falls samples across multiple collection dates approximately every 3–6 months from 2009–2011 (Table 1) resulted in only two positive amplifications. Both were from Mn Falls: sample *F*, extracted in July 2009 during an initial sampling trip to the cave when the biofilm was in bloom (OTUs from this extraction are prefaced by an *F* in Figure 4), and sample *4*, extracted in June 2011 during a more recent sampling trip to the cave when the biofilm was drastically reduced in appearance (OTUs from this extraction are prefaced by a *4* in Figure 4). Multiple attempts at amplification of DNA extracted from Mud Trap Falls were unsuccessful. Clones were binned into OTUs for phylogenetic analysis using a 99% cutoff. Results revealed 6 unique OTUs (Fig. 4) out of ca. 15 total sequences.

Three OTUs (*FB12*, *4A08*, and *FB01*) clustered within the *Prevotella* spp. (Fig. 4), a genus that is commonly isolated from the oral cavity, upper respiratory tract, and urogenital tract of humans (Shah and Collins, 1990). Clone *FB12* shared 100% identity over a ca. 700 bp read to a clone isolated from a study of the microbiota of the human intestine (Walker et al., 2011), and 99% identity to clones isolated from the human gut (Hayashi et al., 2002). *FB12*'s

closest cultured relative was *Prevotella copri*, the type strain of which was isolated from human feces (Hayashi et al., 2007). Clone *4A08* shared 99% identity to environmental clones isolated from fecal contaminated watersheds (Lamendella et al., 2007; 2009), and 97% identity to its closest cultured relative, *Prevotella paludivivens*, a species isolated from rice-plant residue (Ueki et al., 2007). Clone *FB01* shared 99% identity to environmental clones isolated from equine-fecal contaminated water (Simpson et al., 2004).

The three remaining OTUs (Fig. 4), *4A09*, *FB08*, and *FB10*, represented sequences that are members of the *Bacteroides* spp., a genus commonly isolated from the mammalian gastrointestinal tract (Shah and Collins, 1990). Clone *4A09* shared 98% identity over a ca. 700 bp read to environmental clones isolated from river water polluted with feces (Ju-Yong et al., 2010) and human sewage samples (Dorai-Raj et al., 2009). Clone *FB08* shared 100% identity to clones isolated from the human intestine (Walker et al., 2011) and human feces. Clone *FB10* shared 99% identity to clones isolated from the human intestine and human feces in a study investigating the association of human gut microbial ecology with obesity (Ley et al., 2005).

Molecular evidence from this study indicates the presence of a *Bacteroides-Prevotella* fecal signature in DNA extracted from the Mn Falls biofilm in July 2009 and June 2011. Phylogenetic analysis from clone sequences suggests a stronger human-specific signature in July 2009, with 50% of the sequences clustering with sequences from human feces, a conclusion that is consistent with the hypothesis of sewage contamination localized at this site within the cave. A weaker human signature, as defined by a lower percentage of sequences (20%) clustering with sequences from human feces, was detected in the June 2011 sample of Mn Falls.

Most Probable Number Assays

Most probable number (MPN) assays of total culturable heterotrophic bacteria and total culturable heterotrophic Mn(II)-oxidizers at Mn Falls and Mud Trap Falls were conducted on samples obtained on three different occasions, January 2010 (Mud Trap Falls), May 2010 (Mn Falls), and June 2010 (Mn Falls and Mud Trap Falls) (Table 3). Cultivation-based enumeration of heterotrophic Mn(II)-oxidizing bacteria revealed no significant differences between population numbers at Mud Trap and Mn Falls.

Results from cultivation-based enumeration of *total* heterotrophic bacteria revealed an interesting trend (Table 3), however. Data from the January 2010 sampling of Mud Trap Falls indicated an average count of 1.1×10^8 cells/g wet weight; data from the May 2010 sampling of Mn Falls indicated an average count of 2.5×10^{10} cells/g wet weight. No overlap in 95% confidence intervals was observed between these two samples, which is indicative of a significant difference in total culturable heterotrophic bacteria between these two sites. June 2010 data from both sites reflects the same pattern, with an average count of 9.6×10^6 cells/g wet weight at Mud Trap Falls and 9.6×10^9 cells/g wet weight at Mn Falls. The difference between the two sites in June 2010 data is more pronounced, as total cultivable heterotrophic bacteria at Mn Falls outnumbered Mud Trap falls by three orders of magnitude, with no overlap observed in 95% confidence intervals. An observed increase in the total culturable heterotrophic bacteria at the Mn Falls site is suggestive of greater nutrient loading at this site. A slight overlap was observed between the 95% upper confidence interval at Mud Trap Falls in January 2010 and the 95% lower confidence interval at Mn Falls in June 2010. However, the percent of culturable heterotrophic bacteria, within the total population estimated using qPCR, was consistently higher (approaching 100%) at Mn Falls (Table 3), a finding that is supportive of nutrient loading at the Mn Falls site.

Mn(II) Oxidation Associated With Litter

Field observations indicated distinct increases in microbial Mn(II) oxidation in response to nutrient input. Field samples unintentionally amended with litter or feces became encased in a slimy Mn(III/IV) oxide coating, examples of which included an abandoned sock, a corroding battery, cigarette remains, electrical tape, fireworks, balloons, and feces from a small herbivorous mammal (Fig. 2d). In an effort to better characterize the responsible organisms, culturing experiments on the litter produced a variety of Mn(II)-oxidizing fungal species (Fig. 5), many of which have not yet been described in the literature and whose phylogenetic associations are still uncertain. Scanning and transmission electron microscopy of these fungi have shown that Mn is sequestered in spores, and/or along hyphal junctions and septa (Fig. 5e, 5f).

Discussion

Karst aquifers and deep cave systems with minimal human impact are considered to be oligotrophic environments, defined by less than 2 mg total organic carbon per liter (Barton and Jurado, 2007), and several nutrients such as nitrogen, sulfur, phosphorous, and iron are considered to be additional limiting factors in these systems (Goldscheider et al., 2006). Microbial species within pristine caves (those that have little or minimal human impact) are adapted to leading an oligotrophic lifestyle (Northup et al., 2003). However, gut-related microbiota, which are generally not endogenous members of the cave microflora, are adapted to environments containing high levels of carbon and other nutrients (Ley et al. 2006). Therefore, it is reasonable to hypothesize that sites within cave systems that experience impact from sewage/nutrient loading would demonstrate higher culturable heterotrophic cell counts than areas without impact. There was no significant difference between the total bacterial number estimated

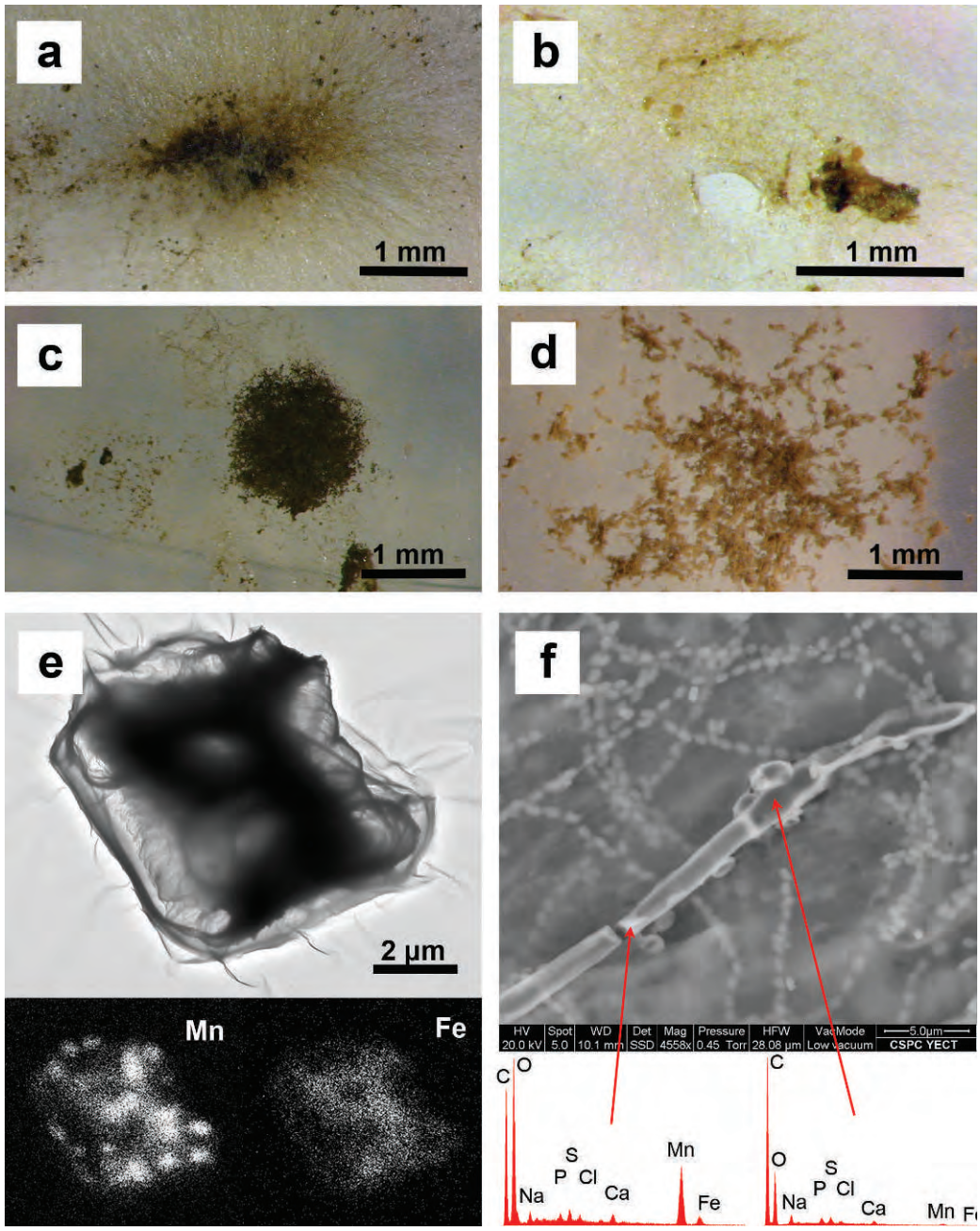

Figure 5. Light microscopy of Mn (II)-oxidizing fungi from Carter Saltpeter Cave on AY agar-solidified media, cultured from electrical tape (a), fireworks (b), an abandoned sock (c), and a rusty battery (d). Scanning transmission electron microscopy of fungi cultured from the abandoned sock indicates the presence of Mn and Fe within fungal spores (e). Backscattered scanning electron microscopy imaging of fungi cultured from electrical tape indicates elevated levels of Mn within hyphal junctions, but minimal Mn content within the hyphal interiors (f).

using qPCR in a separate study (Carmichael et al., 2013) and total culturable heterotrophic bacteria estimated using MPN analyses at the Mn Falls site on the June 2010 sampling date, indicating that a large number of the bacteria present at the site were culturable.

Significantly higher average cultivable cell counts and consistently higher percentages of culturable bacteria at the Mn Falls site relative to Mud Trap Falls support the hypothesis of localized nutrient loading/sewage contamination at the Mn Falls site in CSPC. This hypothesis is bolstered by several field studies investigating nutrient loading in caves via tourism (Ikner et al., 2007), septic effluent (Simon and Buikema, 1997), and input of rich carbon sources such as guano, feces, and human traffic (Mulec et al., 2012) where, in all three studies, cultivable counts of bacteria (either total CFUs, total aerobic

bacteria, or total fecal coliform bacteria), were at least two orders of magnitude higher than similar counts in low impact zones within the caves. Experimental manipulations of soil plots have also revealed an increase in bacterial biomass within plots fertilized by feces or a combination of lint and feces relative to plots fertilized with lint alone or control plots (Chelius et al., 2009), findings which support our hypothesis that the bloom of biofilm material and increase in culturable numbers of bacteria at Mn Falls were likely due to nutrient-loading at this site.

It is important to note that cultivation-based experiments are inherently biased by media design and inoculation/incubation techniques (Rusterholtz and Mallory, 1994), and that dilution of fecal matter, which would occur within karst conduit systems, does affect the ability to culture and detect fecal indicator bacteria in environmental samples (Ahmed et al., 2008b). The present study addresses this bias via molecular-based characterization of the Mn Falls microbial community. Molecular surveys demonstrated the presence of a *Bacteroides-Prevotella* fecal signature in DNA extracted from the Mn Falls biofilm in both July 2009 and June 2011, with a stronger human signature detected in the July 2009 sample. *Bacteroides* spp. represents a prominent new alternative indicator for the detection of fecal pollution in environmental samples due to an inability to survive in a non-host environment for lengthy periods of time, a strict association with warm-blooded animals, and a relative abundance of members of this genus in fecal samples as compared to traditional indicators (Ahmed et al., 2008a). Molecular-based methods have also been successfully employed in other studies to detect the presence of human fecal indicators in karst aquifers (Johnson et al., 2011; Reischer et al., 2007) and human impact in caves (Johnston et al., 2012).

Water chemistry from Mn Falls also points to point source nutrient loading via septic effluent. Field tests (pH, conductivity, etc.) revealed values for CSPC that were consistent with those reported from other caves and local springs contaminated with fecal indicators (Johnson et al., 2011; Simon and Buikema, 1997). Elevations in septic-associated chemicals including chloride, fluoride, and phosphate strongly suggest the presence of septic input from 2010 to 2011 (Fig. 3). Chloride and phosphate are commonly associated with septic effluent, and chloride is often used as a tracer for septic plumes (Denver, 1989; Minnesota Pollution Control Agency, 1999; Robertson et al., 1998). Fluoride concentrations, while highly variable, may also be elevated quite significantly (Minnesota Pollution Control Agency, 1999). Naturally occurring elevated fluoride levels are unlikely, as fluorite is not abundant in the surrounding bedrock (Hoagland et al., 1965) and is not soluble at pH values <8 in the presence of calcite (Miller and Hiskey, 1972). In addition, Johnson City (located less than four miles to the northwest) fluoridates their municipal water to attain values 1 mg/L (Washington County Water and Sewer Department, personal communication), which also points to septic effluent as a likely source of high fluorine levels in the seep. Further, CSPC is located in an active conduit system, which would be consistent with relatively rapid changes in water chemistry. Sulfate and nitrate are also variable yet elevated with respect to pristine cave waters and local well and spring waters. The proximity of the USGS wells and springs to CSPC make these elevations unlikely to be the result of land use differences. The elevated levels of nutrients (particularly fluoride) that remain in the water may be due to continued flushing of the conduit leading to the seep rather than continuous sewage input, which would have been detected via MPN analyses and gene sequencing.

Although it is difficult to visually assess changes in microbial diversity in response to eutrophication or other disturbances directly in the field, changes in function are easily seen among Mn and Fe oxidizing microbial communities, where such changes are visible as a significant alteration in the abundance of metal oxide produced. The Mn Falls bloom, with an associated distinct fecal odor, appeared in 2008, and both have slowly disappeared over the course of the study (Fig. 2). Several mechanisms of heterotrophic Mn(II) oxidation have been proposed. Bacteria such as *Pseudomonas putida* GB-1 (Geszvain et al. 2013), various species of *Bacilli* (Francis and Tebo, 2002), and *Leptothrix discophora* SS-1 (Corstjens et al. 1997) are all thought to use multicopper oxidases for Mn(II) oxidation. Whereas the bacteria *Aurantimonas maganoxydans* SI85-9A1 and *Erythrobacter* sp. strain SD-21 are thought to use a manganese-oxidizing peroxidase (Anderson et al. 2009). Similarly, fungi of the *Basidiomycetes* are known to use a manganese peroxidase enzyme (Liers et al. 2011) to oxidize Mn (II). MPN analyses in this study, however, suggest that Mn(II)-oxidation at this site was most likely correlated with heterotrophic activity, an idea consistent with one that has recently emerged in the geomicrobiological and geochemical literature: that Mn (II)-oxidation can be affiliated with reactive oxygen species (ROS) production by both heterotrophic bacteria and fungi. For example, Learman et al. (2011) reported that *Roseobacter* oxidizes Mn(II) indirectly via ROS that are most likely generated as a normal part of electron transport during aerobic, heterotrophic respiration. In addition, Hansel et al. (2012) have shown that *Stilbella aciculosa*, an Ascomycete fungus, oxidizes Mn(II) via extracellular ROS produced during asexual reproduction. Heterotrophic Mn(II)-oxidation associated with ROS production also makes sense thermodynamically, since the first electron transfer in the oxidation of Mn(II) to Mn(III/IV) was demonstrated to be a rate limiting step, whereas Mn(II)-oxidation by superoxide, hydrogen peroxide or a hydroxyl radical were shown to be favored reactions (Luther, 2010).

Although previous studies have indicated that Mn(III/IV) oxide production in the CSPC groundwater seeps was primarily associated with Mn(II)-oxidizing bacteria

(Carmichael et al., 2013), culturing results in this study revealed that Mn(III/IV) oxide production on litter was associated with Mn(II)-oxidizing fungi (Fig. 5). Rapid fungal Mn(III/IV) oxidation of feces and litter (including a balloon, fireworks, a discarded sock, electrical tape, and a battery) over the course of several weeks also supported heterotrophic growth in a nutrient-limited cave environment.

Although there was a dramatic (albeit gradual) visual reduction in Mn oxide production during sampling trips from 2009–2011 (Fig. 2a, 2b), minor amounts of Mn oxides continued to be produced during this time, as shown by LBB testing. The reasons for this reduction are not entirely clear, but dissolution of birnessite group structures by siderophores (chelating agents) in microbial secretion and/or membranes, as demonstrated for *Shewanella oneidensis* (Duckworth and Sposito, 2007; Fischer et al., 2008), is a possible mechanism to reduce the presence of birnessite group Mn oxides. Poorly crystalline buserites (Na-rich birnessites) were identified in the biofilm at Mn Falls in this study, and Mn(II)-oxidizing bacteria are known to produce poorly crystalline birnessite group minerals (Spiro et al., 2010 and references therein), which is consistent with siderophore dissolution of Mn oxides in the Mn Falls biofilm. Other chemical factors such as pH, redox potential, or concentrations of nutrients or Mn(II) may also have played a role.

Conclusions

Multiple techniques including molecular, chemical, and culture-based analyses conducted over a three-year extended study have provided evidence of sustained anthropogenic impact within the Carter Saltpeter Cave system. Our study has provided compelling field data in support of the hypothesis that Mn(II)-oxidation can be associated with both bacterial and fungal heterotrophic activity, which in this case, was most likely stimulated in response to point source exogenous nutrient loading.

Due to the inherent stability of the cave environment and the highly adapted cave macro- and microfauna, this type of anthropogenic impact has the potential to disrupt the delicate balance of life within a cave and exert a strong negative effect on ecosystem function. Localized variations in geochemistry and nutrient availability have been shown to impact microbial community structure (Johnston et al., 2012; Barton and Jurado, 2007; Shabarova and Pernthaler, 2010) and niche diversification (Engel et al., 2010; Macalady et al., 2008). Further, the composition of cave microbial communities mediates and stabilizes biogeochemical cycling and mineralization processes within an environment (Portillo and Gonzalez, 2010; Portillo et al., 2009).

Legal protection for cave and karst systems exists at the Federal and State levels, although enacted legislation varies in the degree of protection and is often wrought with loopholes (van Beynen and Townsend, 2005). The effectiveness of legislation, guidelines, recommendations, and management plans is contingent on the accumulation of high quality baseline data that delineates the sources of contamination within a system and on the documentation of specific impacts within a cave or karst system (Northup, 2011). Clearly, there is a continued need for research, especially in regions such as the Appalachians, where cave density is high and research in cave systems is lacking.

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ODONATA OCCURRENCE IN CAVES: ACTIVE OR ACCIDENTALS? A NEW CASE STUDY

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Abstract: Caves are environments that host unique faunas and may be important for organisms not exclusively dependent on caves. The occurrence of epigean taxa in caves is often considered accidental, but their study can provide useful information on cave colonization. Records of Odonata underground are extremely scarce. We have identified larvae of *Cordulegaster bidentata* in two caves, one natural and one artificial, from Lombardy in northwestern Italy. They occurred in pools near the cave entrance that have 84 lux of maximum illuminance, reached in early spring. In both caves we found a high density of larvae, and some of them were at very advanced instars. They had an important role in the cave's trophic web, exerting a high predation pressure on larvae of the salamander *Salamandra salamandra*. The plasticity of some Odonata species may allow them to take advantage of underground springs.

Introduction

Caves are environments that host unique faunas. Cave communities include cave-dependent species, the so-called troglobites, with specific adaptations to subterranean environments (Pipan and Culver, 2012). Many species that are not cave-dependent, troglophiles, can also exploit subterranean environments during phases of their life cycles (Culver and Pipan, 2009). The study of typical epigean taxa that enter caves can be of great interest for understanding the mechanisms of cave colonization. Although in many cases epigean organisms that are more or less occasionally found in caves are considered accidentals and of limited interest for biospeleological studies, some authors emphasize that active exploitation of the cave environment is often mistaken for accidental occurrence (Romero, 2009).

Caves are generally stable environments with specific features, such as constant water availability and relatively constant temperatures that make them particularly suitable for certain species. For instance, in karstic landscapes, surface water is extremely limited, while water is more frequent underground. In these areas, underground water might be important for the reproduction of semi-aquatic insects. Several taxa of semi-aquatic insects have been observed in underground environments (reviewed in Romero, 2009). However, records of Odonata underground are extremely scarce, with most found in the grey literature. Thompson and Kiauta (1994) reviewed dragonfly observations from subterranean environments and reported 34 taxa from caves and other similar underground habitats, 17 of which are from Yucatan cenotes and constitute a peculiar habitat that is ecologically different from most caves. Most of the remaining records refer to individuals accidentally drifted into caves by floods or streams or that are listed without any additional information (Thompson and Kiauta, 1994). Records of Odonata from caves include *Calopterix* sp. larvae from the Lepini Mountains in Italy (Latella, 1992) and adults of *Hemicordulia australe* at the entrance of a cave during winter in New Zealand (Marinov, 2010). Even if the occurrence may be accidental, the larvae of several species are able to survive, and in some cases, successfully metamorphose in caves (Thompson and Kiauta, 1994). There is also evidence of species that may actively select caves for breeding, as in the case of *Gynacantha nourlangie* in Australia (Thompson and Kiauta, 1994) and *Somatochlora meridionalis* in Italy (Carchini, 1992). Adults of *G. nourlangie* are often associated with caves, while the latter has been reported for two caves in Italy where its ability to breed in underground habitats could be an advantage because surface waters often dry up (Thompson and Kiauta, 1994). Nevertheless, ecological information on Odonata in caves remains extremely limited, both spatially and taxonomically.

In this study, we performed an extensive survey of the benthic communities in 52 natural and artificial spring caves in northern Italy to assess Odonata occurrence. Here we report the first two findings of *Cordulegaster bidentata* larvae in Italian underground springs, highlighting their role in the trophic web of these sites.

Material and Methods

We surveyed natural and artificial caves in a karstic area between the Lecco and Como districts (Lombardy, northwestern Italy). See Manenti et al. (2009), Manenti et al. (2011), and Manenti and Ficetola (2013) for additional details on the study area. We chose to survey spring caves only, thus excluding caves entered by surface streams in which Odonata larvae could have passively drifted. We

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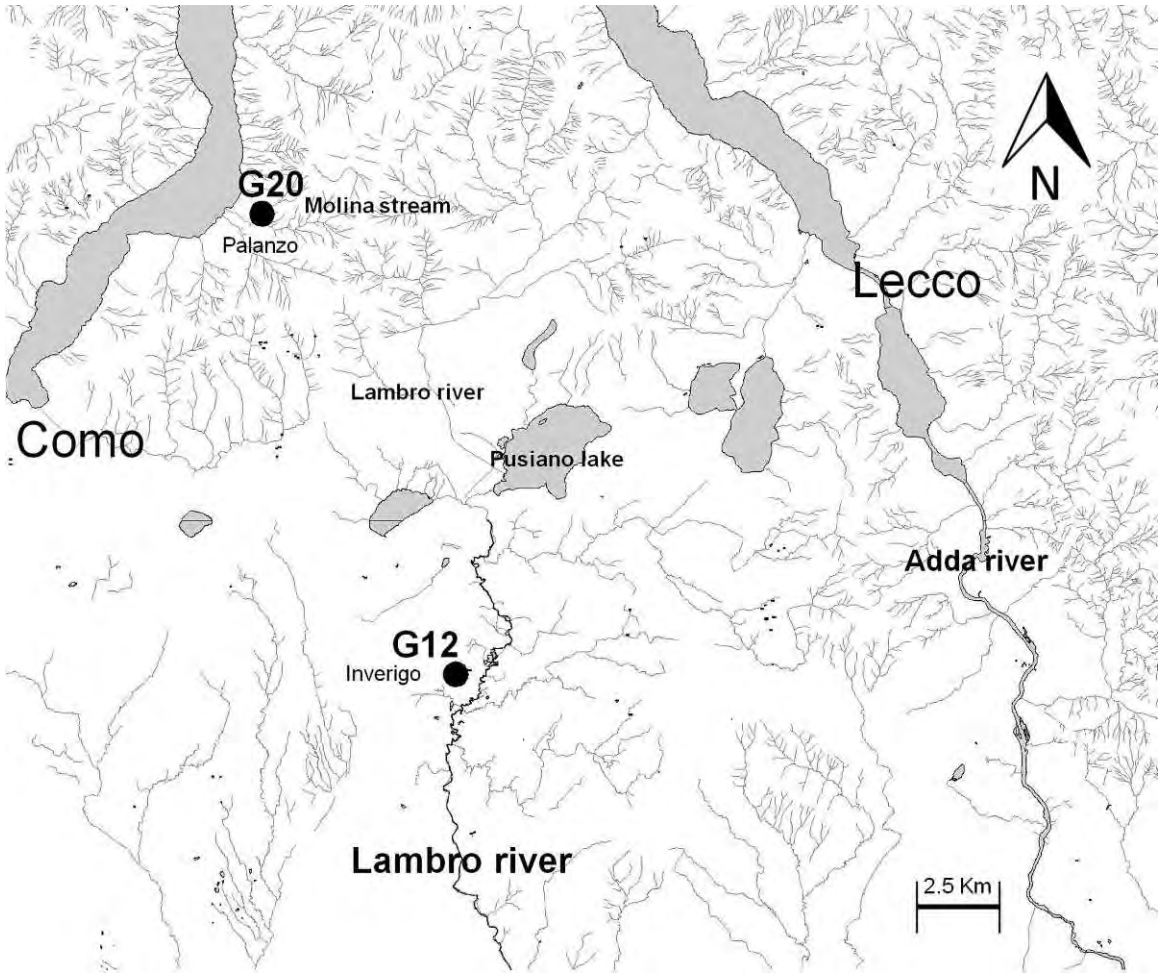

Figure 1. Study area and location (black circles) of the two caves with *Cordulegaster bidentata* larvae.

performed surveys between February 2009 and September 2012 in 26 natural and 26 artificial caves with underground water. The caves monitored constitute 80% of the natural spring caves and 100% of the known artificial underground springs in the study area. Each cave was monitored from 2 to 15 times (average: 4.1 surveys per cave). In at least 50% of the surveys we recorded three parameters describing micro-climate and the physical environment: water temperature (°C) and the maximum and minimum intensity of light incident on the water bodies, measured in lux. These parameters were measured using an EM882 multi-function thermo-hygrometer and a light meter (PCE Instruments). The minimum illuminance recordable by the light-meter was 0.01 lux. We used a fine-mesh net to collect the aquatic macroinvertebrates. The underground water bodies generally had limited surface area (average: 2.30 m^2, maximum: 16 m^2), and we were able to sample all their floors. For each larva of *Cordulegaster bidentata* found, we measured total length, head width (i.e., the maximum distance between the margins of the eyes) and, if present, wing sheaths' length. The latter characters allowed us to identify the final (F-0) or penultimate (F-1) instars as reported in Ferreras-Romero and Corbet (1999) for *C. boltoni*. Several study caves were also breeding sites for the fire salamander *Salamandra salamandra*, which can be a frequent prey of *Cordulegaster* in pre-alpine streams (RM pers. obs.). As a measure of predation pressure, we counted the percentage of salamander larvae showing injuries suggesting predation attempts, and we used an unequal-variance *t* test to compare the frequency of injured larvae between caves with and without *C. bidentata*.

RESULTS

We detected *Cordulegaster bidentata* in 2 caves out of the 52 sampled (Fig. 1). Water flows from both caves, and downstream of the caves there are no stable surface water bodies or streams because the water meets highly permeable sediments. Thus accidental occurrence of the larvae is unlikely.

The first cave is an artificial underground spring with a length of 5.3 m. Its distance from the nearest other water is 41 m. This cave (G12 in Fig. 1 and Table 1) was sampled monthly from September 2010 to February 2011 without

Table 1. Main features of the two caves inhabited by *C. bidentata*.

Cave	Town	Locality	Altitude, amsl	Iluminance, lux max	Iluminance, lux min	Area, m^2	Depth, cm	Other Taxa Present	Coordinates
G20	Faggeto Lario	Palanzo	530	84	3.5	2.5	25	Lumbricidae Chironomidae Lymnephilidae	45°44′30″ N 9°14′7″ E
G12	Inverigo	Orrido4	376	47	0.01	2.8	75	Tipulidae Limonidae Chironomidae Lymnephilidae Gordiidae	45°44′30″ N 3°17′21″ E

detecting any dragonfly larva. Sampling was repeated in April and September 2012; in these sampling events we detected eight and five larvae of *C. bidentata*, respectively (maximum observed density: 2.8 larvae/m^2). The cave houses at its entrance a pool of 2.8 m^2 with a maximum depth of 75 cm. The maximum recorded illuminance was 47 lux in April, but during all the sampling events part of the pool was in complete darkness (illuminance 0.01 lux). Larvae were found at the bottom of the pool or along the edges. In April, we found four larvae at the F-1 stage, while in September, we found two larvae at the F-0 stage and two at F-1 stage.

The second cave was sampled in March 2010 without any observation of dragonfly larvae. In April 2012 we found seven larvae of *C. bidentata*. It is a natural cave with a length of 11 m and a large entrance (3.5-m wide). The cave (G20 in Fig. 1 and Table 1) contains a small stream with a pool 0.5 m from the entrance that has an area of 2.5 m^2 and a maximum depth of 25 cm. Its distance from the next stream is 28 m. We recorded seven larvae inside the cave. Four larvae were at the F-0 stage and two at the F-1. Six larvae were found in the pool (larvae density 2.4/m^2), while one was 3 m above in the small stream. The substrate is muddy and sandy and offers an ideal shelter for *C. bidentata* larvae. The maximum and minimum illuminance reached during the April sampling were 84 and 3.5 lux.

Both caves are breeding sites of the fire salamander *Salamandra salamandra*, which successfully grows and metamorphoses in caves (Manenti et al., 2011). In the caves with *C. bidentata* the average percentage of injured fire salamander larvae was 36%, while it was 3.1% in the caves without *C. bidentata*. The frequency of injured tails was significantly higher in the caves with *C. bidentata* (t = 6.03, df = 2.57 p = 0.014) (Fig. 2).

Discussion

Cordulegaster bidentata is widespread in southern Europe (Carchini, 1983; Askew, 1988) and often occurs in the pre-alpine streams of Italy (Boano et al., 2007; Balestrazzi and Pavesi, 2008; Siesa, 2010), but to our knowledge, it has been reported in unusual habitats such as caves only once, in 1986 and 1987 from a cave in Switzerland (Thompson and Kiauta, 1994; Keim, 1996, p. 67–68). In that case, some exuviae and a dead female were reported in a subterranean lake.

Our survey provides new data for this species and presents interesting evidence for the ability of this species to actively use underground habitats. In particular, over 52 caves sampled, we found 2 sites, both with more than 2.5 *C. bidentata* larvae per square meter inside. The larvae of *Cordulegaster bidentata* usually develop in springs, small streams, or brooks with shallow water, low flow, and many pools (Heidemann and Seidenbusch, 2002). Both of our caves host underground springs with water bodies close to

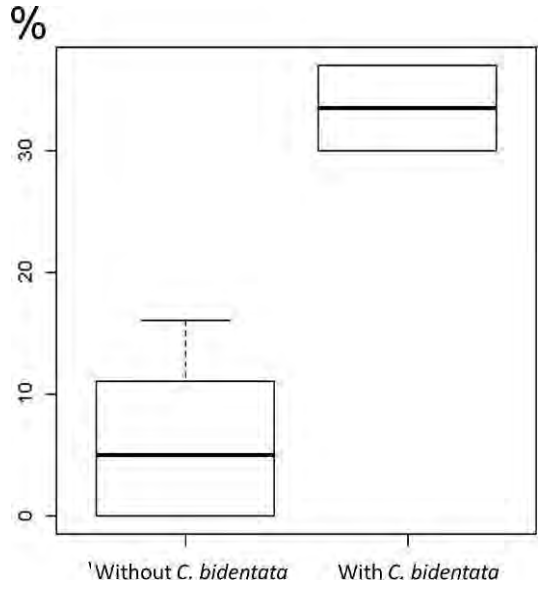

Figure 2. Boxplot of the percentage of fire salamander larvae with injured tails in caves without and with *C. bidentata*.

the entrance. They are caves without influent streams, therefore, the presence of larvae cannot be explained by drifting or trapping from epigeous environments. Downstream from the entrance, the occurrence of highly permeable detritus does not allow the existence of permanent water nearby. The sites' conformations and distances from other streams or springs allow us to hypothesize two explanations for *C. bidentata* occurrence, both involving some form of active use of the cave. First, during periods of heavy rain, the springs may overflow, becoming connected with downstream watercourses. In these periods, dragonfly larvae could move upstream and reach sites not yet exploited to access trophic resources (Grand and Boudot, 2006). Second, the underground springs are not far from cave entrances, and the sectors closest to the entrance may receive some light. It is therefore possible that adults entering into caves may have laid eggs there. The observation of larvae at the final stage of development suggests that larvae can successfully grow in these underground pools, indicating an interesting plasticity of the species. The sites used are small cavities with suitable pools near the entrance that receive some light from outside, but they receive much less light than the standard habitat of this species. For instance, in April, with cloud cover at 12:00 pm the average minimum illuminance of surface streams inhabited by *Cordulegaster* within woodlands in our study area is 800 lux.

The use of caves by *C. bidentata* is infrequent, as we found them in only two caves and only during some surveys. Larvae are probably confined to illuminated water bodies near to the cave entrance. Underground springs usually have limited richness of invertebrates (Manenti et al., 2009), especially when compared to a typical stream inhabited by *C. bidentata* larvae (Lang et al., 2001). The scarcity of food items, the cold temperature, and the lack of light can be disadvantageous to the developmental rate of the larvae. On the other hand, the larval life cycle of *C. bidentata* can be longer than two years (Ferreras-Romero and Corbet, 1999; Grand and Boudot, 2006). In karstic areas, surface streams are often temporary, and underground springs can provide more stable environments. Furthermore, caves with *C. bidentata* contained salamander larvae that may constitute major prey items for dragonfly larvae.

Actually, larvae of *C. bidentata* can have an important role in the food webs of these underground environments. Larvae of the *Cordulegaster* genus are opportunistic predators that are able to consume a wide range of prey items, and they are often the dominant predators in the benthic communities of small streams and brooks (Bo et al., 2011); in pre-alpine streams, they often prey on fire salamander larvae. Salamander larvae are rather frequent in underground springs because these environments typically are without predators, which enhances larval survival (Manenti et al. 2011). If dragonfly larvae can reach these environments, they may find prey abundant, and their role as top predators can have an important function in the food web of caves.

CONCLUSIONS

Records of dragonflies underground are scarce (Thompson and Kiauta, 1994), especially concerning non-accidental cases and reproduction. The latter seems to be favored in the cases of crepuscular species or in areas where stable surface water is lacking (Carchini, 1992; Thompson and Kiauta, 1994). Our findings of *Cordulegaster bidentata* larvae could be linked to the latter aspect, especially considering the dry springs and summers that have been recorded in our study areas during the last two years. These results provide a new interesting case study for Odonata. Underground environments are among the less-known habitats, but are an important compound of biodiversity.

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LUMINESCENCE OF SPELEOTHEMS: A COMPARISON OF SOURCES AND ENVIRONMENTS

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Abstract: Calcite speleothems are typically coarsely crystalline, colored various shades from brown through orange and white, and strongly luminescent. For most speleothems, the color is due to higher molecular weight humic substances incorporated in the calcite crystal structure, while the luminescence is mainly due to lower molecular weight fulvic acids. The fine scale banding of luminescence intensity has importance as a climatic indicator. A suite of eighteen speleothems spanning a range of geologic and climatic settings and a range of colors from deep brown to nearly white were selected for detailed characterization and spectroscopic measurements. Spectra were measured on solid samples and on solutions prepared by dissolving the speleothems in dilute HCl. The luminescent emission appears as a single broad band with peak wavelength varying from 390 to 450 nm. The excitation spectra are typically more complicated, with several maxima, and show more locality-to-locality variation. The emission bands shift to longer wavelengths as the excitation bands move to longer wavelengths, indicating that a mixture of molecular species is being selectively excited. The spectra of the solutions are similar but not identical to the spectra of the crystalline solids. The decay time of the luminescence (phosphorescence) is in the range of 0.5 to 0.7 second. Comparison of speleothem spectra from caves in different climatic settings and of speleothem spectra from the same cave indicate that each speleothem produces spectra characteristic of specific overlying soils and pathways through the epikarst and the vadose zone. No features were discovered that characterize regional scale geologic or climatic settings.

Introduction

Speleothems, especially stalagmites and flowstone, have become important paleoclimate archives that provide information about continental interiors (Fairchild and Baker, 2012). Along with the isotope and trace element profiles that compose the paleoclimate signal are the color and luminescence profiles along the growth directions of the speleothems.

Numerous investigations of the color and luminescence spectra of speleothems tie the color (White, 1981) and luminescence firmly to humic substances or their calcium salts, with the most intense luminescence arising from the low molecular weight fulvic acid fraction (White and Brennan, 1989; van Beynen et al., 2001). The humic substances originate from overlying soils and are flushed into the cave on a seasonal cycle (Toth, 1998; van Beynen et al, 2000; van Beynan et al., 2002; Tatár et al., 2004; Ban et al., 2008). Details of soil characteristics, flow path through the epikarst, and precipitation intensity and distribution all influence the pattern of color and luminescence banding for any particular speleothem.

The objective of the present investigation is to compare the luminescence spectra of speleothems collected from a variety of climatic settings. As such, this paper extends the excellent investigation of speleothem luminescence by van Beynan and his colleagues (van Beynan et al., 2001).

Experimental Methods

Sources of Speleothems

The speleothems examined are listed in Table 1 arranged by sample number. The sample numbers refer to a master cave mineral collection built up over many years. The locations from which the speleothems were collected are given, along with a short description and a semiquantitative measure of the color determined by comparison with the Munsell charts. All specimens discussed in this paper consist entirely of calcite. Aragonite- and gypsum-containing speleothems were excluded.

Luminescence Spectroscopy

UV-excited luminescence measurements were made on a Hitachi F-4010 spectrofluorophotometer. This instrument uses a xenon arc lamp as a UV source and has two 0.2-m grating monochromators allowing both excitation and emission scanning. For a few spectra, a filter with a sharp cut-off at 400 nm was placed over the emission spectrometer entrance slits to eliminate artifacts from higher orders of the diffraction grating. The instrument can accommodate both liquid and solid samples and so could be used to compare the spectra of solutions with the spectra of the original solid speleothem.

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Table 1. Description and sources of speleothems examined.

Number[a]	Color and Form[b]	Cave Source
12	Stalagmite	Weller's Cave, Pa.
61	Orange-pink crystal [7.5YR 8/4]	Tintic Mine Cave, Utah
155	Dark brown stalactite [5YR 2.5/1]	Lawrence Dome Pit Cave, W.Va.
166	Colorless clear crystal	Crystal Ball Cave, Ariz.
305	Gray stalagmite [10Y 7/1]	Butler Cave, Va.
669	Tan stalactite [10YR 8/2]	Overholt Blowing Cave, W.Va.
675	Tan stalactite [2.5Y 8/4]	Hesston Cave, Pa.
690	Yellow-brown stalactite [2.5Y 8/5]	Prah Cave, Pa.
691	Orange-brown stalactite [7.5YR 5/6]	Prah Cave, Pa.
706	Yellow-orange stalactite [2.5Y 8/6]	Cueva del Guacharo, Venezuela
743	Colorless clear crystal	Crystal Ball Cave, Ariz.
841	White flowstone	Timpanogos Cave, Utah
844	White stalactite	Timpanogos Cave, Utah
1013	White monocrystalline stalactite	Cueva del Guacharo, Venezuela
1014	Orange-brown stalactite [7.5YR 5/8]	Cueva del Guacharo, Venezuela
1067	Tan stalactite [2.5Y 8/4]	Coffee River Cave, Jamaica
82MM004	Dark brown stalactite [5YR 3/2]	Peacock Cave, W.Va.
83MM005	Dark brown crevice filling [5YR 3/2]	Rilda Canyon, Utah

[a] Sample numbers refer to a collection of cave materials maintained by the corresponding author.
[b] Colors are also indicated by the Munsell system (in brackets).

Sample Preparation

Calcite is an excellent phosphor host and, under the proper excitation, can produce luminescence from incorporated transition metal ions such as Mn^{2+} or from charges trapped in defects in the calcite structure (White, 1990), in addition to luminescence from included organic material. By dissolving the calcite crystals, luminescent features due to transition metal ions in the crystal field of the calcite structure or to defect centers in the crystal should be eliminated. Organic molecules, however, should survive in solution. Because the electronic transitions in aromatic molecules such as humic and fulvic acids take place between molecular orbital states that are strongly localized, these molecules should retain their luminescent properties in solution. However, possible changes in the details of the spectra must be investigated.

Spectra measured on solid chips of speleothem or on dissolved fragments integrate over the luminescent banding, so the spectra of the solutions will represent a superposition of whatever distribution of molecular species that might have been present within the bands of the original speleothem. However, the spectra of individual bands, measured using laser excitation, (Crowell and White, 2012) are very similar, suggesting that the banding is more a question of concentration and humic-to-fulvic ratio rather than speciation of the fulvic component.

Bulk solutions were prepared by dissolving 425 mg of powdered speleothem in 3.5 mL of 2 N HCl. The solutions were not filtered. After the powders had dissolved, any undissolved material was allowed to settle and samples of the clear liquids, which had pH values between 6 and 8, were drawn off with a pipette. These solutions were placed in silica-glass cuvettes for measurement in the spectrofluorophotometer.

Measurements of luminescence spectra of solid speleothems were on cleavage chips broken to a size that would fit the sample holder of the spectrofluorophotometer. Both sawed surfaces and packed powdered samples scattered so much light from the source that plasma lines from the xenon arc appeared as artifacts in the spectra. Use of cleavage chips also avoided any artifacts that might be introduced by sawing and grinding operations. Luminescence intensities were plotted as count rates, but no scale is given since the measured intensity depends sensitively on instrument slit widths and on the exact orientation of the specimen. For this reason, one cannot compare intensities from specimen to specimen.

Luminescence Spectra of Speleothems

Spectra of Speleothems in Solution

Samples of all eighteen speleothems were dissolved, and luminescence spectra of the solutions were measured. The spectrum of each solution was scanned first to identify the main emission peak. Then, with the emission monochromator set at the wavelength of the peak, the excitation spectrum was scanned. Finally, additional emission spectra were scanned with excitation wavelength set at the maxima in the excitation spectra. Figures 1 through 3 display the spectra.

With one exception (specimen 1013), the spectra have the same general appearance. The luminescence emission spectra consist of a single broad band with peak wavelengths ranging from 369 to 438 nm. The excitation

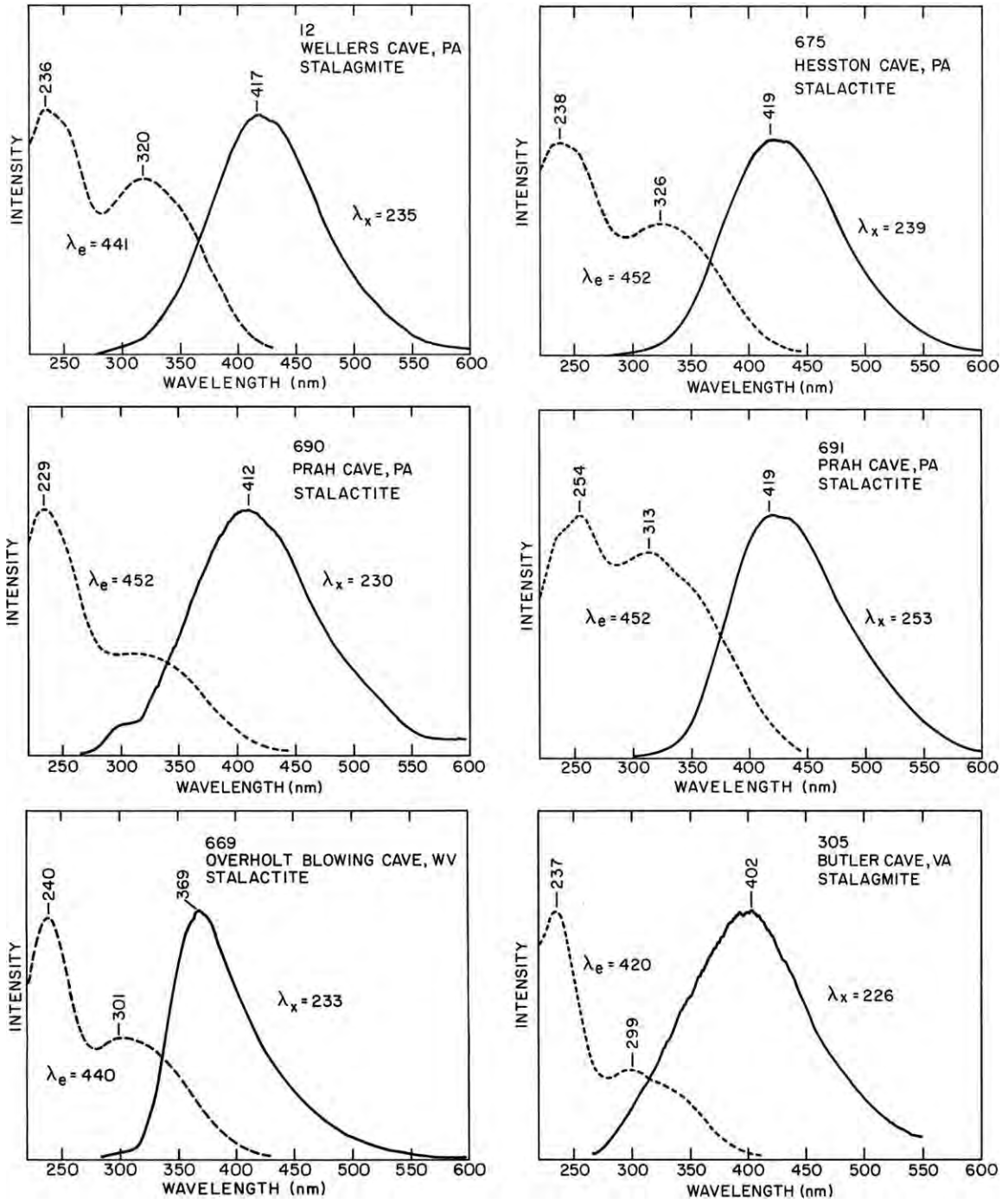

Figure 1. Emission spectra (solid lines) and excitation spectra (dashed lines) for solutions of dissolved speleothems. Excitation wavelength, λ_x, for emission curves and emission wavelength, λ_e used to monitor excitation are labeled on the spectra. Light colored specimens taken from caves in the central Appalachians.

spectra contain at least two bands, one at short wavelengths, 230 to 250 nm, and the other in the mid-ultraviolet, 300 to 340 nm. There are, however, many differences in detail from sample to sample. In some samples, the excitation bands are sharp and well separated; in others they are smeared together. The relative intensities of the two excitation bands vary widely. The emission bands shift in peak wavelength and also vary in the width of the band (full width at half maximum).

The spectra are grouped by climatic setting. Eight spectra of speleothems from the temperate climate of Pennsylvania, Virginia, and West Virginia may be compared with four spectra from tropical speleothems from Jamaica and Venezuela, and these in turn with six spectra of

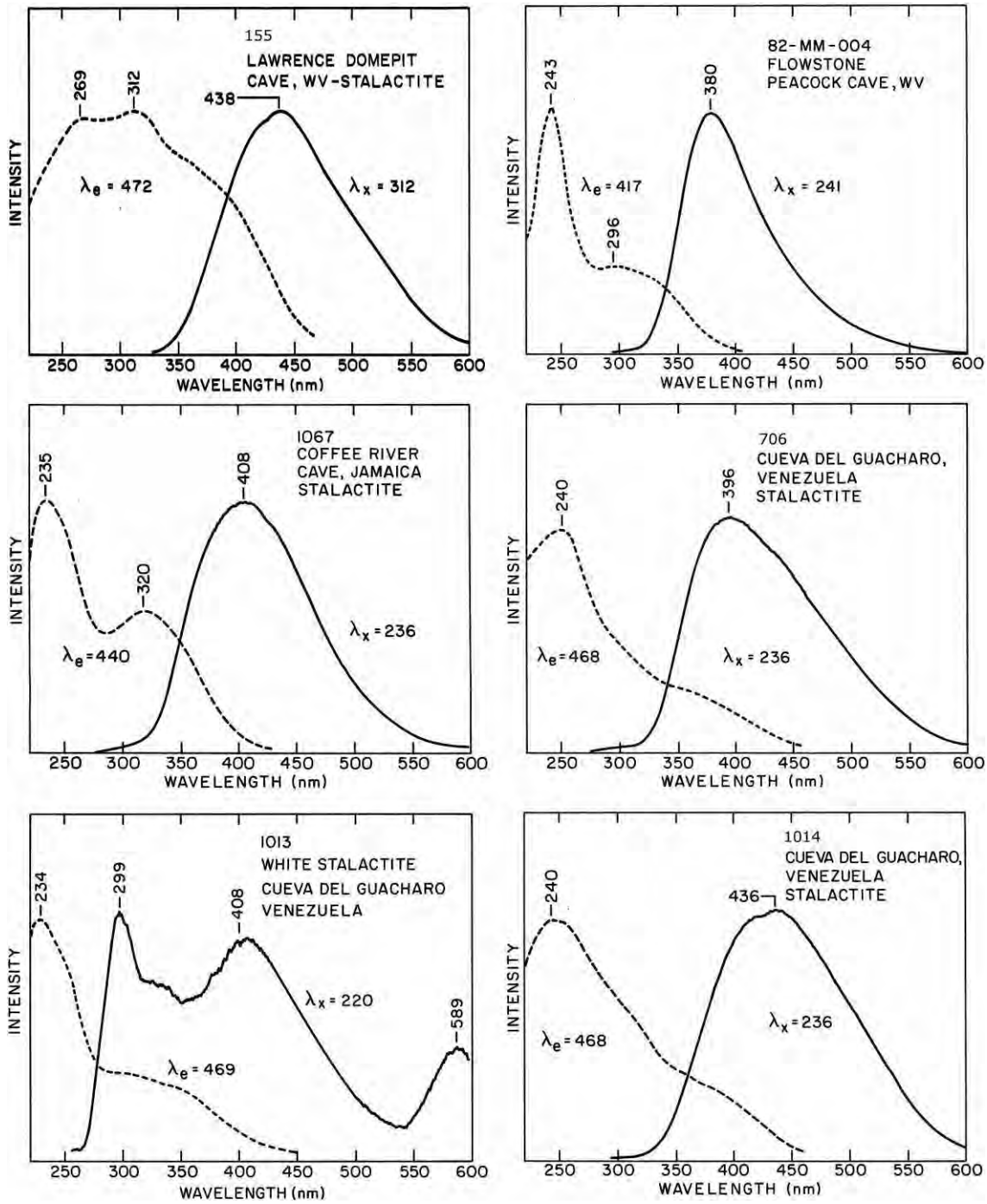

Figure 2. Emission and excitation spectra, as in Figure 1, for solutions of dissolved speleothems. Two dark-colored specimens from the Appalachians and four specimens from tropical caves.

speleothems from the semi-arid climates of Arizona and Utah. There are no systematic differences between these climatic groups.

The samples were deliberately chosen to represent a range of color (given in Munsell notation in Table 1), and therefore, presumably, the concentration of humic substances. Within the specimen set from Pennsylvania, Virginia, and West Virginia, the specimens from Overholt Blowing Cave and from Butler Cave were very light colored, the Prah Cave specimens were a yellow-orange color, and the Peacock Cave and Lawrence Domepit Cave samples were a deep chocolate brown. In spite of the range in colors, the emission spectra are all roughly similar. There are no distinctive differences between the lightly colored and deeply colored specimens. This is in agreement with the hypothesis that the luminescence arises mainly from low molecular

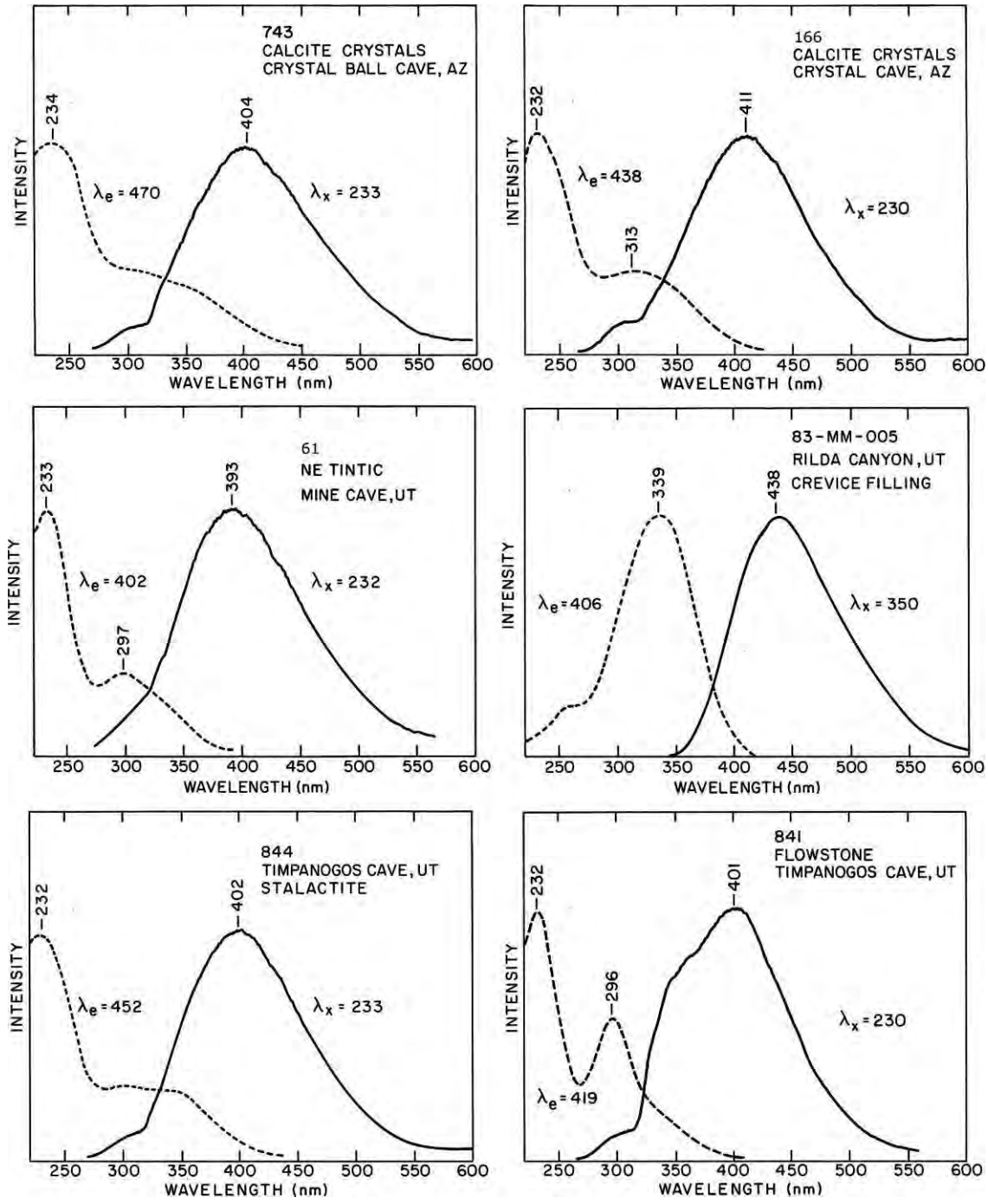

Figure 3. Emission and excitation spectra, as in Figure 1, for solutions of dissolved speleothems. Specimens taken from caves in semi-arid environments.

weight fulvic acids, while the colors arise from mainly high molecular weight humic acids. What is different over the observed range in color is the concentration of humic acids. Self-absorption within the strongly colored specimens may lower the effective intensity of the luminescence.

The Prah Cave samples (specimens 690 and 691 in Fig. 1) are instructive. These two stalactites were taken from the same cave, at a separation of only a few tens of meters, and yet the spectra are distinctly different. The comparison is even stronger with the two specimens from Timpanogos Cave (Fig. 3). Both nearly colorless specimens are from a cave at high elevation in the Wasatch Mountains. It is quite clear that each speleothem has its own individual spectral signature that was determined by local details of soil and flow path. The spectra cannot be used to label a particular climatic or geologic setting.

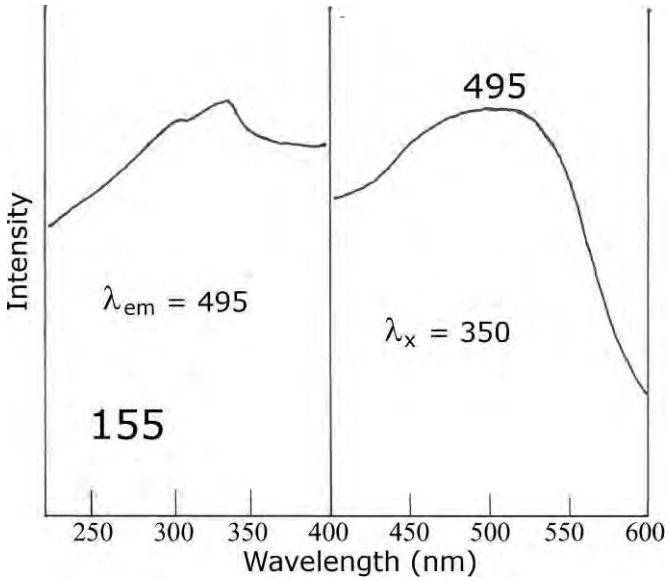

Figure 4. Emission and excitation spectra for solid chips of the chocolate-brown speleothem 155.

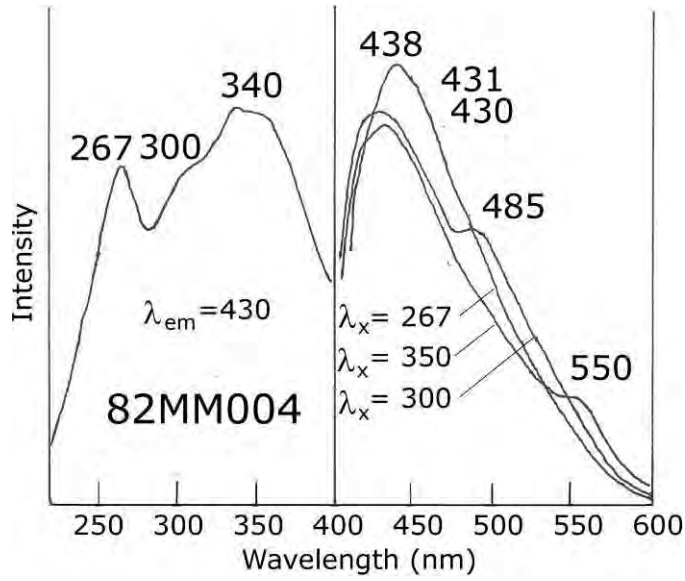

Figure 5. Emission and excitation spectra for solid chips of deep brown, coarsely crystalline speleothem 88MM004. The emission spectrum of specimen 88MM004 displays the emission at three excitation wavelengths.

The three stalactites from Cueva del Guacharo in southeastern Venezuela (Fig. 2) offer the most interesting contrast. Specimen 706 was a yellow-tan, coarsely crystalline stalactite. It produces a single broad luminescence band peaking at the edge of the ultraviolet. Specimen 1013 was a pure white stalactite that had grown as a single crystal, breaking along the rhombohedral-calcite cleavage planes. The emission spectrum is complex, with an emission band at 299 nm, a main band at 408 nm, and a weak emission at 589 nm. Specimen 1014 was a deep orange, almost red color. The broad main luminescence band peaks at 436 nm, but the visible luminescence under long wave UV is a deep yellow.

Fulvic acid is a complex group of molecules based on aromatic phenolic and benzoic acid rings. The luminescence arises from triplet-to-singlet transitions within the rings, and as such, would be expected to exhibit broad, gaussian emission-line shapes. Following the usual custom for luminescent materials, the spectrometer that plotted Figures 1–3 used a wavelength scale. The gaussian shape would be more apparent on a wavenumber scale. However, the range in band widths indicates that these spectra are a superposition of the spectra of a mix of molecules within the samples.

Spectra of Chrstalline Speleothems

The deeply-colored chocolate-brown speleothems were very similar in appearance and were taken from two caves in the same climatic and vegetative environment in eastern West Virginia. Specimen 155, from Lawrence Domepit Cave, has a broad, poorly-defined excitation spectrum and also a broad emission peak that changed little with excitation wavelength. Only one example is shown in Fig. 4. In contrast, specimen 82MM004, from Peacock Cave, has distinct excitation bands and excitation into each of those bands produces a different spectrum (Fig. 5).

The specimens of most interest are those from Cueva del Guacharo. These specimens were obtained from the back section of the cave, which requires a free dive though a 4-meter sump to reach. These speleothems grew at high humidity and (probably) high CO_2 pressure in a section of the cave sealed off from outside air. The calcite is very coarse grained and free of any included dust or other solid matter. Indeed, specimen 1013 was a stalactite composed of a single crystal 10 cm in length. Photographs and a diagram of specimens 1013 and 1014 appear in a review article on speleothem growth (White, 2012).

Excitation into the broad 370-nm excitation band of specimen 706 produces the expected broad luminescence emission at 455 nm, but excitation at 250 nm produced an additional pair of sharp bands at 502 and 520 nm, characteristic of the uranyl, UO_2^{2+} ion (Fig. 6). The visual luminescence of this specimen under short wave (254 nm) UV is the bright green characteristic of uranium salts (Jørgensen and Reisfeld, 1982).

The spectrum of solid chips of the white stalactite, specimen 1013 (Fig. 7), is distinctly different from the spectrum of the dissolved specimen (Fig. 2). There is the expected two-band excitation spectrum and only a single broad emission band at 405 nm. The visual luminescence of this specimen is a weak blue-white. The deep orange-red specimen, 1014, has an almost flat excitation band and a very broad emission band at 518 nm that is almost independent of excitation wavelength (Fig. 8). There is a

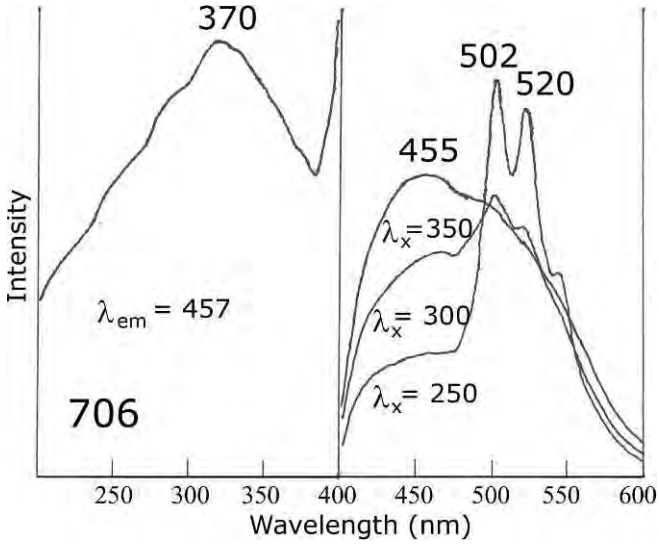

Figure 6. Emission and excitation spectra for specimen 706. The emission spectra exhibit the characteristic fulvic acid band under 350-nm excitation, but the characteristic UO_2^{2+} bands under 250-nm excitation.

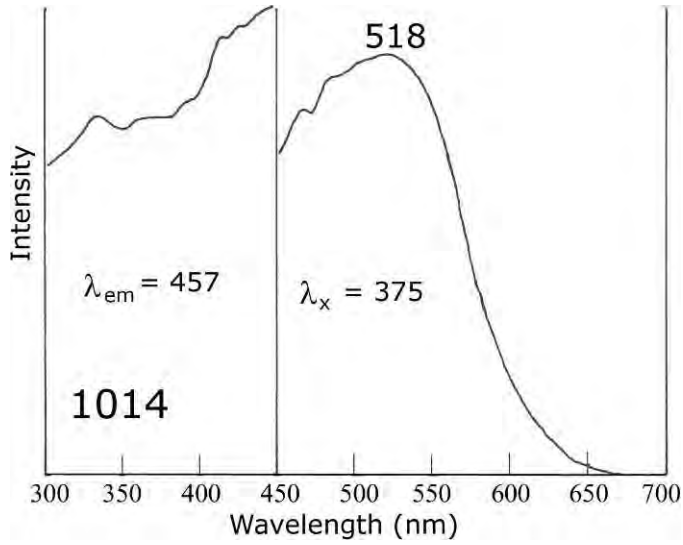

Figure 8. Emission and excitation spectra for specimen 1014, a deep orange-red stalactite. The broad emission band of specimen 1014 shifts slightly with decreasing excitation wavelength: 520 (325), 500 (300), 495 (275), 490 (250), and 485 (225).

shift in peak wavelength from 518 under 375-nm excitation to 485 at 225-nm excitation.

LUMINESCENCE DECAY TIME

Quantitative measurements of the phosphorescence lifetime were made for five specimens (Fig. 9). A single decay process would be described by an exponential function of the form $I = I_0\ e^{-kt}$, where I is measured luminescence intensity at time, t. The rate constant, k, is the reciprocal of the characteristic decay time. The measured decay characteristics vary somewhat from one specimen to another, but all decay curves have the same shape. The decay curves deviate from the single exponential, which would plot as a straight line on the semi-log plot. This implies that there is a distribution of decay rates both within any given specimen and also between specimens and is further evidence for a varying mix of fulvic acid species both within and between specimens.

Fitting the data for each specimen to a single exponential function gives an effective decay constant for that specimen. The decay constant obtained by averaging over all five specimens is $k = 1.51\ s^{-1}$, a decay time of 0.664 s. These values are in the expected range for triplet → singlet transitions and are also in agreement with the visual phosphorescence of the speleothems.

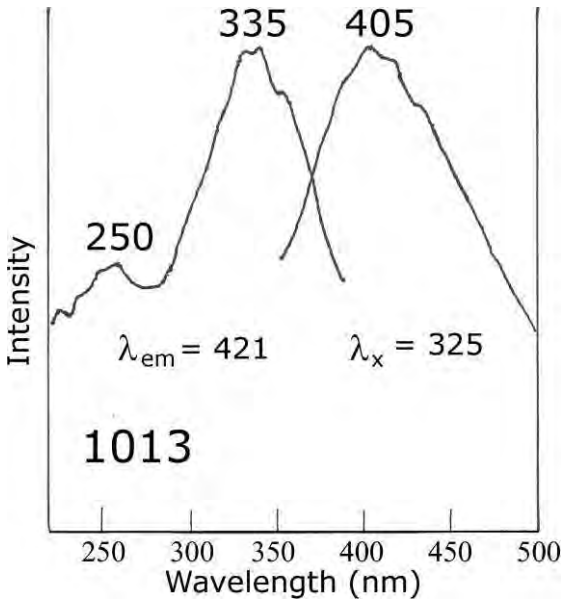

Figure 7. Emission and excitation spectra for specimen 1013, a white single-crystal stalactite.

DISCUSSION AND CONCLUSIONS

Luminescence banding in speleothems, as originally described, provides a useful record of growth rates and seasonal variation in precipitation. However, it is apparent from the data collected by Van Beynan et al. (2001) and in the present investigation that the detailed luminescence spectra are highly specific to not only the individual cave, but also to the specific flow path that feeds the sampled speleothem, to the type and thickness of soil overlying the cave, and to specific details of the vegetation that was growing on the surface at the time the speleothem was deposited. No evidence was found for a regional-scale

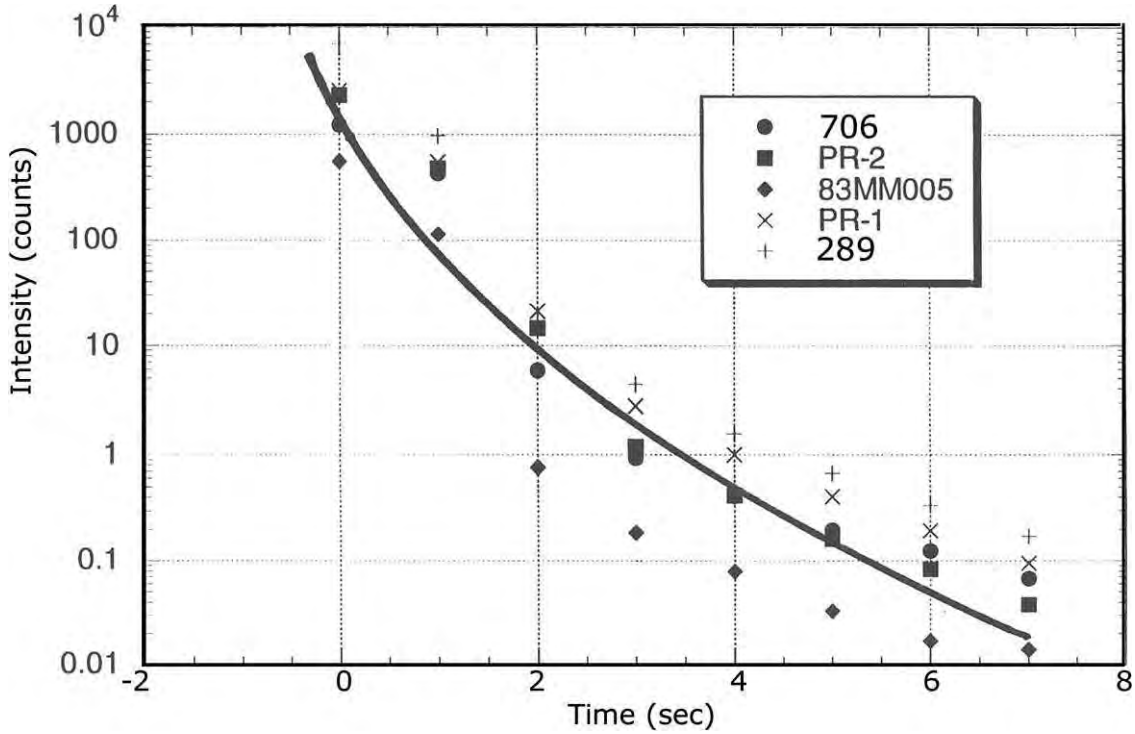

Figure 9. Luminescent decay curves for speleothems 706, PR-2, 83MM005, PR-1, and 289.

pattern that would relate to either the geologic or the climatic setting of the cave.

Luminescence spectroscopy has proved to be a useful method for the characterization of humic substances in soils (Senesi et al., 1991). The application of luminescence spectroscopy to speleothems, especially speleothems that have established ages, would be an approach to the study of changes in soil and vegetative cover over time.

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HIGH PHYLOGENETIC DIVERSITY OF BACTERIA IN THE AREA OF PREHISTORIC PAINTINGS IN MAGURA CAVE, BULGARIA

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Abstract: Magura Cave, situated in the northwest of Bulgaria and containing prehistoric paintings, is a famous tourist site. The present study is the first report on bacterial diversity in guano paintings in a Bulgarian cave using molecular methods. We identified 68 taxa, which is an unusually high number for oligotrophic niches. They are affiliated with eight phyla representing more than half of the bacterial divisions identified in caves. As in many other caves, *Proteobacteria* dominated in this type of ecosystem (about 40%), followed by *Nitrospirae* (22.5%) and *Acidobacteria* (21.5%). Weakly represented were *Actinobacteria* (6.4%), *Chloroflexi* (3.2%), *Planctomycetes* (2.2%), *Firmicutes* (2.2%), and *Gemmatimonadetes* (2.2%). About one third of all DNA sequences recovered in this study were new. Some of them had more than 10% divergence from the closest neighbor, which suggests the existence of new taxa of at least genus level. Bacteria identified in the community expressed various types of metabolism; lithoautotrophic, organotrophic, and methylotrophic.

Introduction

Karst landscapes and caves are formed in soluble rock over millions of years. In the last two centuries, traces of prehistoric life were found in many caves across the continents. The interiors of caves have stable environmental conditions such as temperature, and this has favored their occupation by prehistoric people. Several caves have authenticated paintings connected with religious and everyday habits, some of them representing real masterpieces of ancient art (Schabereiter-Gurtner et al., 2002c; Pike et al., 2012). UNESCO has recognized such caves as a valuable part of the world's cultural heritage. Caves with prehistoric images are found in France, Spain, and Italy. Until recently, the paintings found in Chauvet Cave, France were accepted as the oldest in Europe, but recent studies using radioactive decay rates of uranium revealed that the paintings in El Castillo Cave in Spain date back to at least 40,800 years, making them the oldest known cave art in Europe, five to ten thousand years older than previous examples from France (Pike et al., 2012). The cave microbiota have been well characterized in several Spanish caves, including Tito Bustillo (Schabereiter-Gurtner et al., 2002b), Altamira (Schabereiter-Gurtner et al., 2002a), and La Garma and Llonín (Schabereiter-Gurtner et al., 2004). Rock paintings are found in several Bulgarian caves, with Magura Cave being the largest and having the best-conserved images. These ancient paintings are multi-layered, dating from the Neolithic and Eneolithic Ages to the beginning of the early Bronze Age (Stoytchev, 1994).

Prehistoric paintings in caves have been made predominantly with inorganic pigments including hematite (red), limonite (yellow), manganese oxide, and charcoal (black) (Onac and Forti, 2011). There are many examples of prehistoric paintings with inorganic pigments, including black charcoal images in Chauvet Cave (Sadier et al., 2012) and ochre images in Spanish caves Tito Bustillo, Monte Castillo (Iriarte et al., 2009), Altamira (Schabereiter-Gurtner et al., 2002a), and El Castillo, and the French cave Niaux (Valladas et al., 1992). Two pigments were used in the Cave of Bats, Zuheros, Spain, red ones composed mainly of hematite (iron oxide) mixed with calcite and black ones of undetermined origin (Urzi et al., 2010). To the best of our knowledge, the paintings in only three caves have been created using fossilized bat guano: Magura Cave and Baylovo Cave, Bulgaria (Stoytchev, 2005), and a part of the paintings in Grotta dei Cervi, Italy (Laiz et al., 2000).

Despite its archaeological importance, the knowledge of the microbiology of caves is still incomplete. Although geomicrobiological environmental investigations have been widely published, a significant increase in biospeleological research has occurred within the last two decades (Urzì et al., 2002).

Most caves offer similar conditions for microbial growth, including a relatively constant temperature of 8 to 12 °C, absence of light (preventing photosynthesis), and sparse nutrients. The relatively constant conditions ensure the stability of microbial communities, which is why karst caves can be considered as a long-term home for the same microorganisms. Without photosynthesis, caves are deprived

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of the main energy supporting life on the earth's surface. To overcome those close-to-starvation conditions in caves, selfish competition for food is replaced by cooperative and mutualistic association (Barton and Jurado, 2007). According to Canaveras et al. (2001), caves are usually oligotrophic environments where primary production depends on autotrophic bacteria, and well-established autotrophic communities can support the growth of several chemoorganotrophic microbes (Urzi et al., 2010). Although many authors accept that autochtonic organic carbon is often produced from chemolitotrophic primary productivity in such ecosystems (Sarbu et al., 1996; Schabereiter-Gurtner et al., 2003, 2004; Opsahl and Chanton, 2006; Chen et al., 2009), others point out that wind, flowing and dripping water, or guano can provide organic material (Culver and Pipan, 2009).

According to Lehman (2007), microbial communities associated with different karst zones are distinct from each other, as supported by, for example, a domination of *Firmicutes* in Zuheros Cave, Spain (Urzì et al., 2010) or *Streptomyces* in Grotta dei Cervi, Italy (Laiz et al., 2000). On the other hand, some types of microbes are reported to be widespread in caves. Comparing five caves, Pronk et al. (2009) concluded that *Delta Proteobacteria*, *Acidobacteria*, and *Nitrospira* are specific cave groups. Indeed, the presence of these bacteria in caves has been mentioned by several authors (Schabereiter-Gurtner et al., 2002a, 2004; Pašić et al., 2010). Shabarova and Pernthaler (2009) suggested that three *Beta Proteobacteria* groups (families *Oxalobacteraceae*, *Methylophilaceae*, and *Comamonadaceae*) point toward a highly specialized endemic subsurface microflora. Consideration needs to be given to the possibility of contamination from drip waters or human presence for some pathogenic or enteric bacteria like *Esherichia coli, Enterococcus,* and *Staphylococcus* (Bastian et al., 2009; Kelly et al., 2009).

Among the less well known ecological zones on earth, caves located in Eastern European countries still present an opportunity for searching for taxa that could represent novel biotechnological resources. Although Magura Cave is very popular as a tourist site, its bacterial diversity had not been investigated. This report is the first culture-independent investigation on the bacterial structure of the microbial community from the area of the prehistoric paintings in Magura Cave.

Materials and Methods

Study Site and Sample Collection

Bacterial community samples were collected from the prehistoric paintings in the Gallery, Magura Cave, northwestern Bulgaria (E 22°34′54.84″, N 43°43′41.16″), located close to the village of Rabisha. The total length of Magura Cave exceeds 2.5 km, comprising an area of 28,600 m^2. The cave offers a rich collection of geological formations of all shapes and sizes—stalactites, stalagmites, columns, cave pearls, and flows of moonmilk. Remnants of prehistoric life, traces of settlements, and rock drawings have been found in the cave. One of the caverns, the Gallery, contains prehistoric paintings carved into the walls and decorated with bat guano. The Gallery is located on a branch off of one of the main rooms, the Landslip, and the isolated location has a constant temperature of 12 °C and a humidity of about 94%. It is the highest part of the cave, is 240 m long and up to 24 m high, and has an area of 3750 m^2. The drawings represent religious ceremonies, deities, and hunting scenes and include dancing male and female figures, hunting men, people wearing masks, a large variety of animals, and also suns, stars, tools, and non-figurative, symbolic, and abstract elements. A solar calendar from the late Neolithic found there is the earliest solar calendar discovered in Europe (Fig. 1). Magura Cave was recognized as a natural landmark in May 1960 and placed on the Tentative List for consideration as a World Heritage Site by UNESCO in 1984. The cave was opened for visitors in 1961, but the Gallery was open only for five years (2002–2007).

Figure 1. Some of the paintings in the Gallery, Magura Cave, including solar calendar and dancing figures.

Twelve samples were collected from the areas of different painting groups along the whole length of the Gallery wall (105 m) at locations separated by 5 to 11 m. About one gram was scraped from the area of a painting or the rock within 2 cm of it with sterile scalpels under the supervision of the responsible archeologist from the local museum. The samples were placed in sterile tubes and transported to the laboratory in an insulated bag. All samples were mixed together and vortexted immediately after delivery to the laboratory and considered as a common sample. The procedure of total DNA isolation was initiated immediately using 10 g of the common sample.

The mineral composition of the rock in the area of the paintings was determined to be mainly limestone ($CaCO_3$,

74.02%), with saltpeter (KNO_3, 7.05%), gypsum ($CaSO_4 2H_2O$, 6.25%), hematite (Fe_2O_3, 5.05%), apatite ($Ca_5(PO_4)_3(F,Cl,OH)$, 3.08%), calcium monohydrogen phosphate ($CaHPO_4 \cdot 2H_2O$, 1.45%)] taranakite ($K_3Al_5[(PO_3OH)_3|PO_4]2{\cdot}18H_2O$, 1.28%), and todorokite ($(Mn^{2+}, Ca,Mg)Mn_3^{4+}O_7{\cdot}H_2O$, 1.25%) (Benderev, 2006).

DNA Extraction, PCR Amplification and Construction of Bacterial 16S rRNA Gene Library

Total DNA was extracted from the common sample as described by Selenska-Pobell et al. (2001) with some modifications. A sample (10.0 g fresh weight) was measured aseptically and used for further analysis. The sample material was suspended in 10 ml of 0.12 M sodium phosphate buffer. Lysis of the cells was achieved after adding sodium dodecyl sulfate (final concentration 2%), NaCl (0.5 M), and PEG 6000 (20%). The protocol for extraction of a total-community DNA encompassed three cycles of freezing and thawing (respectively −80 °C and 96 °C), chemical lysis in an extraction buffer, and a proteinase K step. The crude DNA was purified with AXG-100 Nucleobond cartridges (Machery-Nagel, Duren, Germany) following the manufacturer's instructions. The eluate was precipitated by 0.7 volumes of ice-cold isopropanol. The integrity of the DNA was checked by horizontal electrophoresis in 1% agarose (Sigma) gel and visualized with ethidium bromide (0.5 µg/l).

Community ribosomal DNAs were amplified from 1 to 50 ng of bulk DNA in reactions containing (as final concentrations) 1× PCR buffer, 2 mM $CaCl_2$, 4× 200 µM deoxynucleoside triphosphates, 400 nM each forward and reverse primer, 0.5 U Taq polymerase (GenetBio, Korea). Two universal primers for domain *Bacteria* were used: 8F (EUB008, Hicks et al., 1992), and 1492R (EUB1492, Kane et al., 1993). Reaction mixtures were incubated in a BioRad thermal cycler (model T100) for an initial denaturation at 94 °C for 3 min followed by 30 cycles of 94 °C for 0.5 min, 55 °C for 0.5 min, and 72 °C for 1 min, and final extension at 72 °C for 5 min.

For characterization of the bacterial population, a gene library was constructed of the amplified products. The PCR products were cloned in *E. coli* JM 109 using the pJet1.2 cloning kit (Fermentas) according to manufacturer's instructions. Cloned fragments were reamplified using the primers pJet1.2 forward and pJet1.2 reverse located in the vector and surrounding the inserted PCR fragment.

Restriction Fragment Length Polymorphism (RFLP) Analysis

PCR-amplified bacterial inserts were digested using Msp I followed by digestion with Hae III (1 U each) according to the manufacturer's instructions (Fermentas). Restriction profiles were analyzed using 2% agarose gel electrophoresis. The different banding patterns were noted, and clones were grouped according to their RFLP patterns. The sequence of one clone from each group was determined.

16S rRNA Gene Sequencing and Analysis

16S rRNA gene sequences were determined with an Applied Biosystems model 373A DNA sequencer by using the ABI PRISM cycle sequencing kit (Macrogen, Korea), where they were reamplified by using 8F primer. Those of the clones that showed less than 97% similarity to the closest relative after sequencing with the forward primer were additionally sequenced with the reverse primer 1492R to establish if the full gene sequences were new (≥ 3% evolutionary distance of the full gene).

The degree to which RFLP types covered the diversity in the sample was measured with the Shannon index (e.g., Hill et al. (2003): $H = -\text{sum}_i (p_i \ln (p_i))$, where p_i is the relative frequency of the ith in the examined clones and ln is the natural logarithm.

The sequences obtained were checked for potential chimeric artifacts using the CHIMERA_CHECK program from RDPII on the Ribosomal Database web site (http://rdp8.cme.msu.edu/html/analyses.html). The sequences were compared to the closest relatives in the NCBI GenBank database using BLASTn (Altschul et al., 1990) and Ribosomal Database Project resources (Maidak et al., 1994) to determine their close relatives and approximate phylogenetic affiliations. Phylogenetic analyses were conducted using *MEGA* version 4.0 (Tamura et al., 2007) and the Neighbor-joining method (Saitou and Nei, 1987). The 16S rRNA gene sequences of the sequenced clones have the Gen Bank accession numbers HE653817 to HE653888.

Results

Clone Library

To determine the structure of the microbial community in the Gallery in Magura Cave, we analyzed the sequences of 16S rRNA genes obtained by PCR amplification followed by cloning. The isolated 126 correct clones containing 16S rRNA gene were screened by RFLP to identify unique types for sequence determination. rDNA digestion resulted in three to ten bands with the discernible fragment size range of 50 to 1000 bp from each clone (data not shown). A total of 93 different rDNA groups were formed; 77 of them contained only one clone. More than 96% of the clone fragments sequenced with an 8F primer were longer than 900 bp. Sequences for which the similarity to the closest relative was less than 97% for the full gene sequence were defined as new. They represented 31.9% of all clone sequences. Sequences differing only slightly (≤ 2%) were considered as a single relatedness group. Clone analysis revealed the presence of eight bacterial phyla. These were *Proteobacteria, Acidobacteria, Nitrospirae, Actinobacteria, Planctomycetes, Firmicutes, Chloroflexi,* and *Gemmatimonadetes.*

Proteobacteria

Thirty-seven of the clones in the library assigned to thirty restriction groups were related to *Proteobacteria* (Fig. 2), subphyla *Alpha, Beta, Gamma,* and *Delta.*

The taxonomic groups affiliated with *Alpha Proteobacteria* were represented by one clone for each group (nine clones, nine groups). Unlike all other groups, most of the sequences related to *Alpha Proteobacteria* had culturable microorganisms as closest neighbors (orders *Rhizobiales* and *Sphingomonadales*). Identified clones represented metabolic types methylotrophs (*Methylobacterium* and *Hyphomicrobium*) and heterotrophs (*Sphingomonas*). Aerobic metabolism was characteristic for all identified genera.

With respect to *Beta Proteobacteria*, nine clones were spread in six groups and corresponded to two orders, *Burkholderiales* (genera *Methylibium* and *Duganella*) and *Rhodocyclales* (family *Rhodocyclaceae*). More than half of the clones (five out of nine) were affiliated with the genus *Methylibium*, including facultative anaerobes, methylotrophs, or organotrophs with optimal growth at pH 6.5 and temperature 30 °C (Nakatsu et al., 2006). Bacteria from the genus *Duganella* are strict aerobes and also organotrophs. The microorganisms from the family *Rhodocyclaceae* prefer oligotrophic niches and have diverse metabolisms.

Most of the *Proteobacteria* clones referred to the subphylum *Gamma Proteobacteria* (fifteen clones spread in eleven groups). Only two of these sequences grouped together with culturable microorganisms from the genera *Nitrococcus* and *Enterobacter*; all others grouped together with unculturable sequences. Microorganisms from the genus *Nitrococcus* oxidize nitrites to nitrates and play a key role in nitrate cycling. Two of the sequences (HE653823 and HE653851) related to family *Chromatiaceae* are novel. They have 92 to 95% similarity to the closest neighbor. *Chromatiaceae* are sulfur bacteria, oxidizing sulfides.

Four clones distributed in four different groups corresponded to *Delta Proteobacteria* representing anaerobic sulfate-reducing bacteria. All phylogenetic neighbors of the clones from this subphylum were unculturable bacteria. Three of the four sequences in this subphylum are new, with similarity 93 to 96% to the unculturable sulfate-reducing bacteria from the order *Syntrophobacterales* and saprophytic order *Myxococcales*. The role of the *Delta Proteobacteria* is hypothetical due to the low phylogenetic similarity. Sequences related to *Epsilon Proteobacteria* were not identified in Magura Cave.

Nitrospirae

All identified twenty-one sequences from this phylum were referred to the genus *Nitrospira*; nineteen of them had environmental sequences as closest neighbors (Fig. 3). Five clones were only distantly related, with similarity 88 to 95%, suggesting the existence of new taxa in the phylum. Sequence HE653833 showed 89% similarity with the closest unculturable neighbor and 88% with the culturable *Candidatus* Nitrospira defluvii. One of the sequences (HE653821) had already been identified in another cave, Pajsarjeva jama, Slovenia (Pašić et al., 2010). Although a small number of culturable *Nitrospirae* are known, all of them are obligate chemolithotrophs.

Acidobacteria

Twenty of the clones spread in twelve groups were referred to the phylum *Acidobacteria* (Fig. 4). All identified sequences grouped together with environmental sequences, unlike the *Acidobacteria* taxa identified in La Garma Cave, Spain, which were closer to culturable representatives of the phylum (Schabereiter-Gurtner et al., 2004). *Acidobacteria* is a monophyletic phylum comprising eleven deeply branched groups (Zimmerman et al., 2005). Three of them (1, 3, and 7) were identified in Magura's Gallery. Half of the identified *Acidobacteria* sequences showed low similarity to the closest neighbors (92 to 95%), suggesting that the *Acidobacteria* community in Magura Cave differs from those described so far.

Actinobacteria

Actinobacteria was weakly presented by six clones spread in five groups (Fig. 4). All five sequences showed highest similarity with environmental sequences; two of them corresponded to order *Actinomycetales* and one to the family *Acidothermaceae*.

Phyla *Planctomycetes, Firmicutes, Chloroflexi,* and *Gemmatimonadetes*

Phyla weakly represented in the community (less than 10%) were *Chloroflexi* (3 clones, 3 groups), *Planctomycetes* (2 clones, 2 groups), *Firmicutes* (2 clones, 2 groups), and Gemmatimonadetes (2 clones, 1 group) (Table 1). One of the *Chloroflexi* sequences (HE653852) showed 92% similarity to the closest neighbor, and one of the *Planctomycetes* sequences (HE653854) showed 95% similarity.

Discussion

The culture-independent approach to microbiological investigations of caves with prehistoric paintings has revealed an unusually complicated structure of bacterial communities (Gurtner et al., 2000; Schabereiter-Gurtner et al., 2002a, b; 2003; 2004) despite the commonly observed low degree of diversity for oligotrophic niches. Our phylogenetic analysis of the bacterial community in the painted Gallery in Magura Cave revealed the highest number of identified phylotypes (68) among all comparable caves. Despite the fact that the number of the analyzed clones (126) was higher than that for caves Llonín (27), La Garma (58), Altamira (21), and Tito Bustillo (41), a comparatively low diversity measure was estimated (Shannon index $H = 4.43$). This measure, which expressed the certainty of appearance of a random selected sequence, reflects the significant variety in the taxa in Magura Cave.

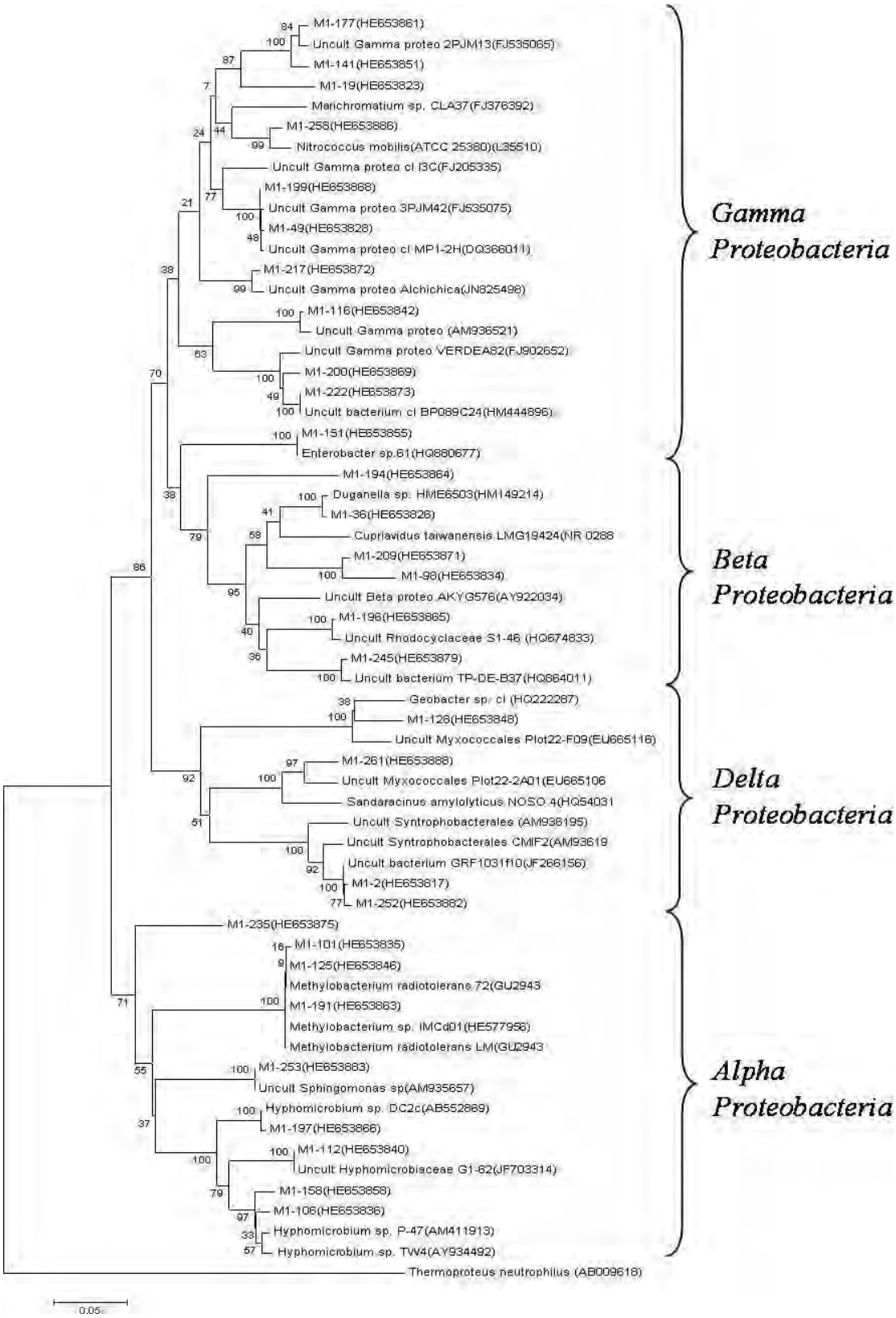

Figure 2. Neighbor-joining tree, calculated using evolutionary distances, based on the *Proteobacteria* 16S rRNA gene sequence types from the Magura Cave sample. Scale bar 5% substitutions in nucleotide sequence. Bootstrap values are shown at branching points (percentage of 1000 resamplings). Sequence accession numbers are given in parenthesis. *Thermoproteus neutrophilus* was used as the out-group.

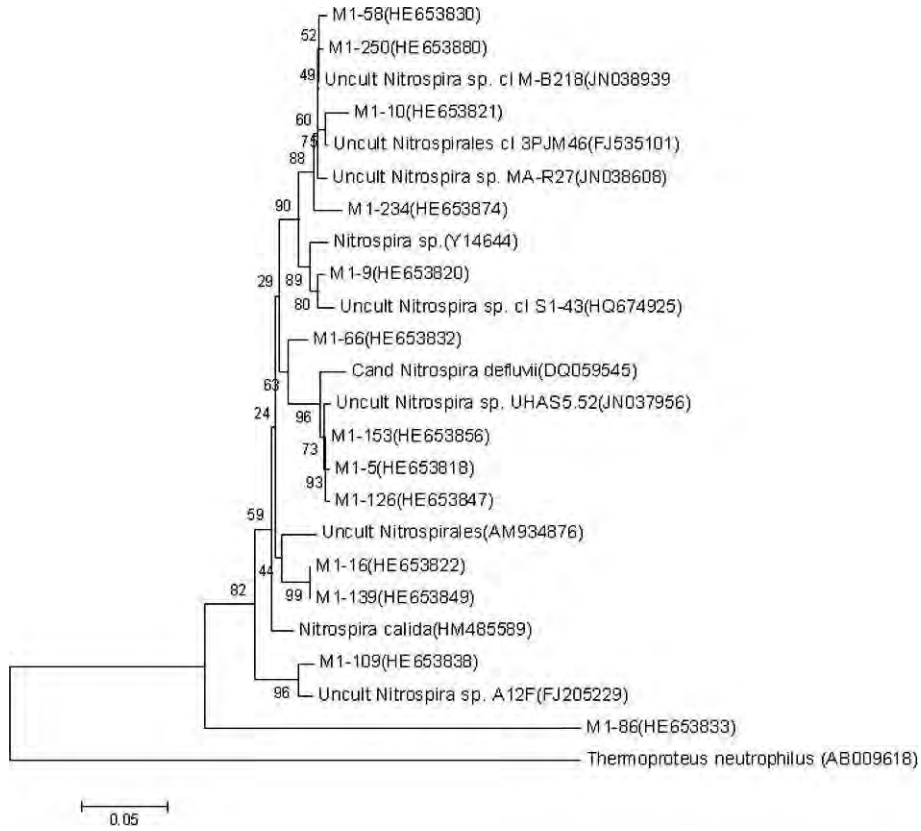

Figure 3. Neighbor-joining tree, calculated using evolutionary distances, based on *Nitrospirae* 16S rRNA sequence types from Magura Cave. Scale bar 5% substitutions in nucleotide sequence. Bootstrap values are shown at branching points (percentage of 1000 resamplings). Sequence accession numbers are given in parenthesis. *Thermoproteus neutrophilus* was used as the outgroup.

Most of the sequences identified in Magura Cave are phylogenetically close to environmental sequences (57 out of the presented 68 phylotypes, 84%). A predominance of unculturable phylogenetic neighbors was also observed for Altamira, Spain (62%) (Schabereiter-Gurtner et al., 2002a), while unculturable and culturable neighbors were equally presented in the caves Llonín and La Garma, Spain (Schabereiter-Gurtner et al., 2004). Culturable neighbors of the sequences from the Magura Cave samples predominated only in the subphylum *Alpha Proteobacteria* and were fully absent in *Delta Proteobacteria*, *Acidobacteria, Actinobacteria, Planctomycetes*, *Chloroflexi*, *Firmicutes,* and *Gemmatimonadetes.* Branching of the sequences almost exclusively between unculturable bacteria confirms that a great variety of microorganisms remain undiscovered and uncharacterized in various natural microbial habitats (Ranjard et al., 2000). Our results confirm the wide spectrum of still unculturable bacteria and the importance of the culture-independent approach to understanding the whole bacterial diversity in caves.

Currently domain *Bacteria* comprises twenty-nine validly described taxonomic divisions (Ciccarelli et al., 2006), and according to Engel (2010) almost half of them have been identified in caves. The retrieved sequences from Magura Cave corresponded to eight bacterial phyla (Fig. 5), more than a half of the divisions reported for caves. Investigations of the samples from five caves located in different places have revealed that the number of phyla represented varied from six in Altamira Cave (Schabereiter-Gurtner et al., 2002a) and Tito Bustillo (Schabereiter-Gurtner et al., 2002b) to seven in Llonín and eight in La Garma (Schabereiter-Gurtner et al., 2004) and Magura Cave. A general lack of phyla unique to a given cave was observed in the phyla presented in the compared caves. Distinct phylogenetic clusters are repeatedly detected in the caves, like *Proteobacteria*, *Nitrospirae*, and *Actinobacteria. Proteobacteria* was found to dominate in all compared caves (Table 2). Almost 40% of the sequences retrieved in Magura samples were identified with this phylum. Phyla *Nitrospirae* and *Acidobacteria* also were presented as dominant groups (more than 10%). Five other phyla (*Actinobacteria, Planctomycetes, Firmicutes, Chloroflexi* and *Gemmatimonadetes*) were weakly represented.

The highest similarity in the presented bacterial groups and their degrees of domination was observed between Magura Cave and La Garma, Spain (Schabereiter-Gurtner

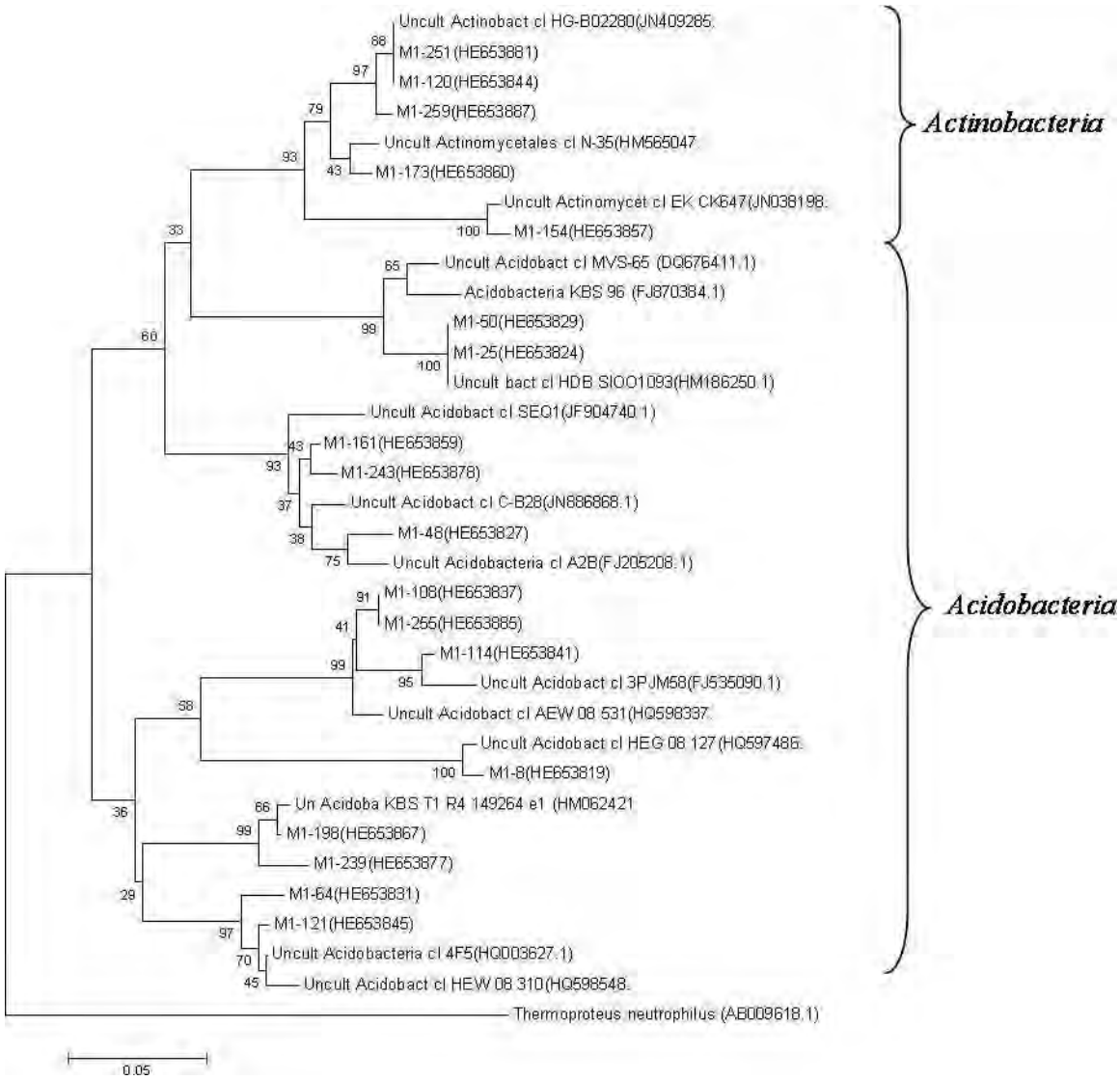

Figure 4. Neighbor-joining tree, calculated using evolutionary distances, based on *Acidobacteria* and *Actinobacteria* 16S rRNA sequence types from Magura Cave. Scale bar 5% substitutions in nucleotide sequence. Bootstrap values are shown at branching points (percentage of 1000 resamplings). Sequence accession numbers are given in parenthesis. *Thermoproteus neutrophilus* was used as the out-group.

et al., 2004). However, *Actinobacteria* was among the dominant groups and *Nitrospirae* was weakly represented in La Garma.

Nitrospirae is not a phylum established in all caves. It was found to be a dominant group only in Magura Cave. It was reported as weakly represented in Llonín and La Garma (Schabereiter-Gurtner et al., 2004) and was absent in Altamira and Tito Bustillo. Although a small number of culturable *Nitrospirae* are known, all they are obligate chemolithotrophs, receiving energy for their growth from nitrite oxidation.

The degree of *Acidobacteria* domination is similar in Magura, Altamira (Schabereiter-Gurtner et al., 2002a), Tito Bustillo (Schabereiter-Gurtner et al., 2002b), and La Garma (Schabereiter-Gurtner et al., 2004), but this phylum was absent in Llonín. Despite the small number of isolated culturable representatives of the phylum *Acidobacteria*, related sequences have been identified from various environments, such as soil, fresh water sediment, and sewage sludge (Tringe et al., 2005; Janssen, 2006). Initially, low pH was accepted as an obligate condition for their growth (Kuske et al., 1997), but 16S rRNA genes related to *Acidobacteria* have been isolated from neutral soils (Dunbar et al., 1999). Quaiser et al. (2003) suggested including non-acidophilic microorganisms as a separate group in the phylum. As this phylum was recently

Table 1. Phylogenetic affinity of 16S rRNA gene sequences of bacteria identified in the Magura Cave to sequences from the phyla *Planctomycetes*, *Firmicutes*, *Chloroflexi*, and *Gemmatimonadetes*. Sequences with similarity to the closest neighbor less than 97% are noted in bold.

Clone and sequence	Closest identified phylogenetic relatives	Phylum	Fragment length (bp)	Clone number
M1-146 (HE653854)	Uncultured bacterium clone FOOS7B_88 - 95%	*Planctomycetes*	1476	1
M1-117 (HE653843)	Uncultured Planctomycetes bacterium - 97%		972	1
M1-237 (HE653876)	Uncultured Gemmatimonadetes bacterium clone g55 - 99%	*Gemmatimonadetes*	1065	2
M1-26 (HE653825)	Uncultured Gram-positive bacterium clone FTL22 - 97%	*Firmicutes*	1420	1
M1-204 (HE653870)	Uncultured bacterium clone SWB36 - 98%		1412	1
M1-144 (HE653852)	Uncultured Chloroflexi bacterium clone II10F - 92%	*Chloroflexi*	1393	1
M1-145 (HE653853)	Uncultured Gram-positive bacterium clone FTL22 - 97%		1458	1
M1-181 (HE653862)	Uncultured bacterium clone HDB_SIPP610 - 99%		1401	1

discovered, their ecology and metabolism are not yet sufficiently clear.

A high degree of representation of the phylum *Actinobacteria* was reported for the Paleolithic Cave of Bats, Zuheros, Spain (Urzi et al., 2010). As typical heterotrophs, *Actinobacteria* actively participate in the carbon cycle by degradation of organic wastes. Although Groth and Saiz-Jimenez (1999) described *Actinomycetes* as a dominant group in caves due to low temperature and high redox potential, this group was poorly presented in the Magura Cave. Despite the fact that *Streptomyces* representatives are commonly accepted as obligate habitants

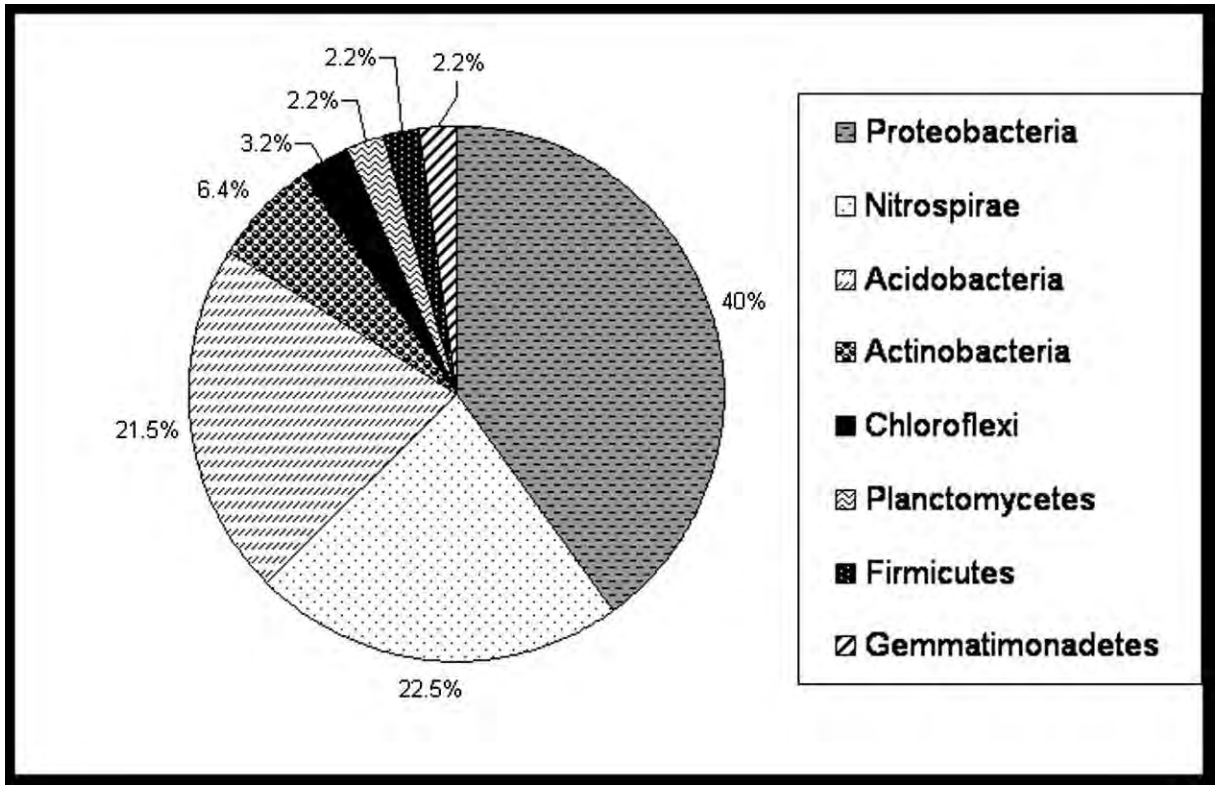

Figure 5. Comparative representation of the bacterial phyla in the sample from the area of the prehistoric paintings in Magura Cave.

Table 2. Represented percentages of bacterial phyla in several European caves with prehistoric paintings.

Represented groups, %	Magura Cave, Bulgaria[a]	La Garma, Spain[b]	Llonín, Spain[b]	Altamira, Spain[c]	Tito Bustillo, Spain[d]
Proteobacteria	39.8	32.7	59.2	52.3	48.8
Nitrospirae	22.5	3.4	3.7	0	0
Acidobacteria	21.5	24.1	0	23.8	29.2
Actinobacteria	6.4	19.0	22.2	4.8	9.8
Planctomycetes	2.2	0	0	4.8	2.4
Firmicutes	2.2	13.8	3.7	0	0
Chloroflexi	3.2	1.7	0	4.8	7.3
Gemmatimonadetes	2.2	0	0	0	0
Cytophaga-Flexibacter-Bacteroides	0	3.4	11.1	9.5	2.4

[a] This study.
[b] Schabereiter-Gurtner et al., 2004.
[c] Schabereiter-Gurtner et al., 2002a.
[d] Schabereiter-Gurtner et al., 2002b.

of caves, especially near the entrance (Urzì et al., 2002), their absence in Magura Cave's Gallery could be explained by its deep position.

Magura Cave is the only one among the compared caves in which representatives of the phylum *Gemmatimonadetes* were identified. Isolated clones related with the phylum *Gemmatimonadetes* grouped with unculturable clones. Despite the fact that only one culturable representative, *Gemmatimonas aurantiaca* gen. nov., sp. nov has been isolated (Zhang et al., 2003), identification of sequences from this phylum from different niches suggests that it is widely distributed in nature.

Some authors suggest that the anthropogenic factor has little or no influence on bacterial communities (Laiz et al., 2000; Schabereiter-Gurtner et al., 2004). However, other authors accept identification of some bacteria, such as *Bacillus*, *E. coli*, and *Staphylococcus aureus*, as indicators for human presence (Lavoie and Northup, 2006). Ikner et al. (2007) suggested that *Proteobacteria* is slightly represented in highly visited caves (more than 200,000 visitors per year) instead of *Firmicutes*. However, a low frequency for the phylum *Firmicutes* was observed not only for Magura and Llonín; it was totally absent in Altamira and Tito Bustillo, two caves intensively visited in the past. Culturable representatives of this phylum were isolated from an area without paintings in Altamira (Canaveras et al., 2001). A low number or absence of *Firmicutes* sequences in cave communities suggest that this phylum does not play a key role for the functioning of cave communities. A sequence related with *Enterobacter* (HE653855) was isolated from Magura Cave. Identification of pathogens could be attributed not only to visitors but also to bats and insects inhabiting the caves (Jurado et al., 2010). People could also change structure of the community by their actions, for example the major changes of microbial structure in the cave Lascaux, France, where *Acidobacteria*, *Actinobacteria,* and *Nitrospira* were replaced by *Beta* and *Gamma Proteobacteria* after use of fungicides to prevent fungi invasion (Dupont et al., 2007).

Together with the high number of phylogenetic groups represented in Magura Cave, the current investigation revealed a presence of sequences suggestive of unknown bacterial taxa. Novelty of sequences is usually discussed in three levels of distance: 3%, 5% and 15%, corresponding to thresholds for novelty on the levels of species, genus, and phylum respectively (Hugenholtz et al., 1998; Pašić et al., 2010). The degree of novelty of the sequences from Magura Cave deposited in GenBank was up to 12% phylogenetic distance (sequence HE653833). We didn't establish phylogenetic types having $< 85\%$ identity to the known sequences, suggesting that all sequences could be affiliated with known phyla. More than 30.9% (21 clones) showed $< 97\%$ similarity with the sequences deposited in GenBank; similarity was less than 95% for thirteen of them, suggesting existence of novel taxa at the genus level in Magura Cave. Novel phylotypes were identified in six out of the eight phyla presented in the cave. New sequences represented half of the identified sequences in two of the phyla, *Nitrospira* and *Acidobacteria*. Two of *Nitrospira* sequences showed more than 10% divergence. Some of the new sequences were represented only once in the clone collection; but others like M1-48 (*Acidobacteria*) and M1-139 (*Nitrospirae*) occurred in four and three clones, respectively, proving a comparatively high abundance of these new lineages in the cave.

Lack of enough nutrients in caves determines metabolic flexibility of bacterial communities. The bacterial community in Magura Cave comprises bacteria with different metabolisms, both autotrophic and heterotrophic. The Gallery is dark, and we expected lithotrophic microorganisms,

rather than phototrophic ones, to be the primary source of organic productivity. Bacteria participating in oxidation of sulfur, sulfide, manganese, iron, nitrite, and ammonium ions have been identified in several caves (Northup et al., 2000; Vlasceanu et al., 2000; Engel et al., 2001; Holmes et al., 2001) and represent an important component of the bacterial cave community. The bacteria from phylum *Nitrospirae* and the genus *Nitrococcus* in the *Gamma Proteobacteria* identified in Magura Cave oxidize nitrites to nitrates. Sulfur bacteria are considered a main group participating in karst solubility and formation of secondary cave structures. Sulfur bacteria from the family *Chromatiaceae* were detected in Magura Cave. Other identified participants in the sulfur cycle were sulfate-reducing bacteria from the order *Syntrophobacterales*. The well-established chemolithotrophic part of the community provides nutrients to support chemoorganotrophic growth. Organotrophs affiliated with *Alpha* and *Beta Proteobacteria*, *Planctomycetes*, and *Firmicutes* were identified in Magura Cave. Interpretation of this information, however, should be very careful, as sometimes related organisms can express different physiologies; moreover, the same group can express different phenotypic properties in different niches (for example *Actinomycetales* and *Actinobacteria*).

Although Barton and Northup (2007) suggest that very few microbial species in caves are capable of assimilating limited but chemically complex nutrients, our phylogenetic analysis of the bacterial diversity in Magura Cave revealed that its habitants are phylogenetically diverse, with lineages referred to a number of different bacterial phyla and to different metabolic types.

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