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Fauna and conservation in a show cave, Puerto Princesa Underground River Park, Palawan, Philippines

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Abstract

The Philippines are located in a complex biogeographical area, a crossroad for different species of animals from Asia and Australia. The natural isolation of Palawan favors the speciation of fauna (and flora) and the karst area of Mount Saint Paul represents itself an additional "island" for the species connected to the underground environment. Our research, carried out in November 2016 and April-May 2017 in the Puerto Princesa Underground River (the main cave of this karst area) revealed the presence of 17 vertebrate species and 84 invertebrate species, 18 of which at least are new for science: an extraordinary biodiversity hotspot for cave fauna. The heavy tourist presence in the first part of the cave (about 1000 people every day) requires a careful management of the access and the identification of the most suitable bio-indicators to monitor the anthropic impact on the cave fauna. Our study provided fundamental data to develop new tourism management policies: precise access and exit time, behavioral rules inside the cave, monitoring techniques for the most important bio-indicators (7 species of bats and 2 of swiftlets) in order to constantly evaluate the anthropic impact and therefore modulate the tourist presence in the cave.

Résumé

Faune et conservation dans une grotte touristique, Parc de la rivière souterraine de Puerto Princesa, Palawan, Philippines.

Les Philippines sont situées dans une zone biogéographique complexe, un carrefour pour différentes espèces d'animaux d'Asie et d'Australie. L'isolement naturel de Palawan favorise la spéciation de la faune (et de la flore) et la zone karstique du Mont Saint Paul représente elle-même une « île » supplémentaire pour les espèces liées au milieu souterrain. Nos recherches, conduites en novembre 2016 et avril-mai 2017 dans la rivière souterraine de Puerto Princesa (la grotte principale de cette zone karstique) ont révélé la présence de 17 espèces de vertébrés et 84 espèces d'invertébrés, dont au moins 18 sont nouvelles pour la science : une extraordinaire biodiversité pour la faune des grottes. La forte présence touristique dans la première partie de la grotte (environ 1000 personnes par jour) nécessite une gestion attentive de l'accès et l'identification des bioindicateurs les plus adaptés pour suivre l'impact anthropique sur la faune de la grotte. Notre étude a fourni des données fondamentales pour développer de nouvelles politiques de gestion du tourisme : temps d'accès et de sortie précis, règles de comportement à l'intérieur de la grotte, mise en place d'un suivi techniques de monitoring des bioindicateurs les plus importants (7 espèces de chauves-souris et 2 de martinets) afin d'évaluer en permanence l'impact anthropique et réguler la présence touristique dans la grotte.

1. Introduction

Located on the Philippine island of Palawan, Natuturingam Cave, better known as Puerto Princesa Underground River (PPUR), is one of the world's largest subterranean estuaries, with ocean tides that flow over 7 kilometers into the cave (FORTI, 2014). With more than 34 kilometers of passages, the PPUR hosts an extremely complex environment. In 1999, UNESCO recognized PPUR as a World Heritage Site for its biodiverse karst habitat.

The reason for this great biodiversity is primarily due to the location of the Philippines in a complex biogeographical area, a crossroads of various animal species from Asia and Australia. This extraordinary biodiversity has been subjected

to prolonged evolutive processes due to the insular condition of Palawan. Furthermore, the karst area of Mount Saint Paul is an "island" within the island that favors the specific differentiation of fauna and flora and, finally, the cave itself where the environmental conditions are very different from outside, constitutes a formidable laboratory for studying the phenomenon of speciation. The result is the great importance of PPUR for the study of the underground fauna and for the conservation of biodiversity. The PPUR is a poecilotrophic cave characterized by five different ecosystems that create one of the world's most complex underground environments that hosts a huge variety of

living organisms with relationships of competition/predation (AGNELLI & VANNI, 2017 b). The first part of the navigable branch of the cave (about 2 kilometers) has been used as a show cave since the late 1970s (Fig. 1) Visitors are impressed by the length of the great tunnel along which the underground river silently flows, the huge speleothems and the presence of a great number of bats and swiftlets. When a wild cavity is transformed into a show cave, a more or less pronounced loss of its pristine condition usually occurs which, over time, may threaten the living organisms of the cave and the survival of their ecological relationships. In about 40 years, the PPUR has become the most visited show cave of the Philippines and among the most visited of the whole planet (over 300.000 visitors per year since 2015) (Agnelli et al., 2018).

Protecting the cave and its environment has always been a primary concern for local and national authorities. In fact, not a single cement step, steel footbridge, or electric line has been allowed inside the cave. Nevertheless, with the exponential growth of tourism in the last few years, the first evidence of ecological problems, mainly related to the diurnal resting of bats, has appeared during the busiest days of tourist visitation. Therefore, the local government,

2. Materials and methods

Despite the scientific and conservation interest of this complex underground environment, little was known of its fauna. After a first explorative expedition carried out in November 2016, we were able to perform a more focused and more productive biological research mission in spring 2017, aimed at studying the habitats of the cave. The more detailed and widespread biological research was conducted using the appropriate equipment (hand nets of different sizes, pliers of different hardness and length, snake stick, different types of containers, photographic equipment to document fauna and environments, etc.).

We explored a representative sample of all five ecosystems present in the cave, working a total of 42 man-days. To increase the biological sampling, some collecting material was distributed to some other cavers taking part to the mission, who worked also in the most distant branches of the cavity. Every day the collected zoological material was checked for a first systematic classification. The specimens that needed more accurate identification were labeled and preserved in ethylic alcohol (70 or 75%) for a subsequent examination in laboratory.

To better evaluate the human impact on bats and swiftlets, it is fundamental to ascertain their number in the cave, and in which part of the day they use the spaces along the river where the tourists move producing light, noises and any other sort of disturbance. A direct counting of all the specimens present in the cave is impossible due to its enormous size (over 30 km!), and because during the long time necessary for the survey of all the underground environments the animals can move from one shelter to another.

We thus chose to work on the specimens that enter and exit the cave each day, to follow their own circadian rhythms, collecting the number of passages at the entrances of the cave. From the first surveys carried out in 2016, we had

together with the Protected Area Management Board, decided to investigate how to manage the increasing tourism and to limit the environmental impact to the PPUR and its surrounding areas.

At the end of 2015, in the framework of the Philippines-Italy Debt for Development Swap Program, the Support for Sustainable Eco-Tourism in the Puerto Princesa Underground River Area Project was financed. Thanks to the grant, two big expeditions to the PPUR were organized by La Venta association in 2016 and 2017 to define the PPUR's capacity for eco-tourism.

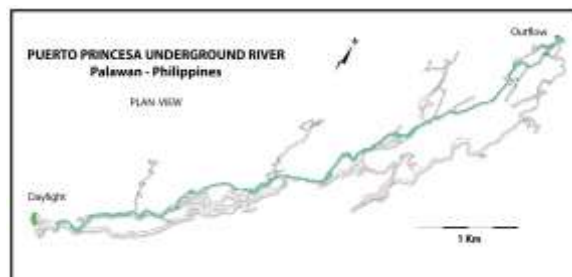


Figure 1: Plan view of the PPUR Cave (green line: the Underground River)

already found that at sunrise the bat flow is distributed over many hours and that the first foraging flights of the swiftlets at the exit of the cave is characterized by continuous back-and-forth that make their tally too difficult and not accurate enough. Therefore, we recorded the evening flow of bats exiting the cave and the evening flow of swiftlets entering (Fig. 2). To achieve this, we used a “FLIR E60BX 1,2” thermographic camera near the two entrances of the cave, the upper karst shaft (named “Daylight”) and the lower river outflow (named “Outflow”). To place ourselves in the most favorable spot for filming at the mouth of the subterranean river, we used an inflatable canoe that allowed a better anchoring to the walls of the cave near the entrance.



Figure 2: recording of the Swiftlets flow in the PPUR Cave with a thermographic camera (Photo by G. Boldrini).

The tally was carried out by playing the video in slow motion. In the approx. two hours of flow we counted, for 10 subsequent times, the specimens flying by in 30 seconds. Thus, the total minutes of survey (5 min) were correlated with the duration both incoming flow of the swiftlets and outgoing flow of the bats. (For more detailed data about the tally techniques, see AGNELLI et al., 2017).

3. Results

The fauna of PPUR is very rich, with a huge variety of species, many of which are resulted new to science. We have classified hundreds of specimens and, in short, we can list 17 species of Vertebrates and at least 84 species of invertebrates! All the 5 Classes of Vertebrates are present, with 3 Families of Fish, 1 of Amphibia, 3 of Reptile, 1 of Bird and 4 of Mammal. Regarding the classified Invertebrates, they belong to 10 Classes, 35 Orders, 52 Families and 43 identified Genera. An extraordinary biodiversity for an underground environment, with a least 18 new species! (8 Arachnida, 3 Diplopoda, 3 Insecta, 4 Malacostraca, 2 Gastropoda).

The faunal list of PPUR is still running and we are waiting for additional reports by specialists who are still examining some specimens (For more detailed data about the faunal list, see AGNELLI & VANNI, 2017 a; AGNELLI et al., 2017).

The evening flow of swiftlets was recorded on May 5 and 9, 2017 and the estimated number entering the “Outflow” was 63,810 specimens. The same tally at the “Daylight” turned out to be 151,988 specimens. The estimate of the total number of swiftlets that daily enter the cave from the two entrances “Outflow” and “Daylight” in this season is thus $63,810+151,988=215,804$ specimens. Additionally, it should be noted that in this season many specimens stay in the cave during the day too, because they are building nests or brooding. Therefore, it can be reasonably estimated that the

population of swiftlets living in PPUR is between 240,000 and 270,000 specimens (Fig. 3).

The count of bats exiting the Outflow was carried out on April 30, 2017, and the estimated number was 123,110 specimens. During the May 9th surveys for the tally of the swiftlets at the “Daylight”, we observed that the number of bats crossing that area is entirely negligible.

It should also be noted that, as a matter of fact, many bats stay in the cave during the night as well. Therefore, it can be reasonably estimated that the population of bats living in PPUR is between 130,000 and 135,000 specimens.



Figure 3: Swiftlets (*Genus Aerodramus*) flying inside the PPUR Cave (Photo by P. Petrignani).

4. Discussions

The study of the fauna of a cave is always very long and laborious. It does not end with the hardworking collection of zoological data but represents the beginning of a long research path that involves many people. The identification of numerous taxa collected in PPUR was performed with the collaboration of at least 30 specialists, thanks to whom it was possible to gain better knowledge on the huge biodiversity of the cave fauna. About half specialists belong to the staff of the Museum of Natural History of the University of Florence or adhere to it as external collaborators. We have involved 16 other scientific institutions, located in 9 nations, representing almost all the continents. The certain and definitive description of some species and the publication of some papers could still require a long time.

We know that the basis of the food chain here, as in many other subterranean environments, is the guano released every day by swiftlets and bats. This organic substance feeds a large number of small animals that, in turn, become food for other larger animals. The same bats and swiftlets are prey for large mygalomorphs, centipedes, and snakes. The presence of swallows and bats is the basis for life in this cave, so their conservation must be a priority.

The increasing human presence represents a source of disturbances for bats and swiftlets. It is therefore necessary to decisively manage the problem. The most critical periods are dawn and sunset when a great flow of bats and swiftlets enter and exit the cave. The animals must not be disturbed during these times. For this reason, the main prescription to minimize human impact is a strict regulation of the opening

and closing hours for the tourist boats. We underline the fact that the main prescriptions, thanks to the Park and its management, are already in place:

- The opening and closing hours for the flow of boats in the cave must be maintained as they currently are, which means beginning (entrance of the first boat) at least two hours after dawn and ending (exit of the last boat) at least two hours before sunset. In this way the inflow and the outflow of bats and swiftlets through the lower entrance remains undisturbed.
- Visitors must always have to stay on the boats, never going ashore, to avoid disturbing bats, swiftlets colonies and terrestrial fauna too.
- A normal tour along the river should only reach the “God’s Highway” gallery up to the fossil remains of the siren (about 1,5 km from the entrance). A further extension of the anthropic presence would limit the spaces that can be used by bats and swiftlets along the river, forcing the animals to spend an excessive amount of energy to avoid human presence and reach their undisturbed shelters.
- The light must only be maneuvered by the guide that drives the boat, while visitors must be forbidden to use other light sources (phones or similar devices), because the light produced by visiting tourists is the main source of disturbance for the animals.
- A careful and respectful behavior must be maintained, without speaking out loudly, because the noise also disturbs the animals.
- The information for visitors must be provided through an audio-guide with headphones, which has been the case for

a few years already. In this way the noise produced by visiting tourists is significantly reduced.

These behaviors will then have to be even more strictly controlled during mating season, which corresponds to the spring period, when the reproductive colonies of bats (mothers with offspring) hang from the ceiling of the cave in the first section after the entrance.

We suggest a simpler fast procedure to count a representative part of the bats of the cave and obtain control data to monitor the impact of tourist frequentation. A direct tally of the bats (Fig. 4) hanging from the vaults and the walls of the cave must be conducted from the "Outflow" up to the fossil remains of the siren in the "God's Highway" gallery. As for the swiftlets, the number of specimens that could be observed hanging in that section of the cave is too scarce.

The tally should be carried out from the boats during the early morning before the tourists enter the cave. This is suggested by the fact that in that moment the animals are less reactive than in the late afternoon. It will be necessary to use lights that are not too dazzling (possibly red lights), and to maintain a quiet behavior to keep the specimens from flying away. It is important that the staff assigned to

these tallies remain the same as far as possible, to limit methodological errors. This monitoring program should be repeated once a month, to record possible seasonal variations and to highlight annual variations in the density of these bat populations. These data sequences could then be related to variations in tourist flow or to the adoption of mitigation measures.



Figure 4: *Hipposideros diadema* (E. Geoffroy, 1813) hanging from the vault of the cave (Photo by P. Petrignani).

5. Conclusion

Thanks to our project, the PPUR National Park is currently one of the best-studied large karst systems in the world. The extraordinary environment of the Underground River Cave must be protected and preserved for its beauty and rich biodiversity. For this reason, it is important to control the

impact of human presence inside it, to the point that even scientific research will have to be adequately regulated. Adopting these recommendations, it will be possible to let visitors fully enjoy the beauty of the cave without excessive impact on the fragile ecosystem it hosts.

Acknowledgments

We gratefully thank the Puerto Princesa Underground River Park for its dedication to the biodiversity conservation and to the appropriate management of this extraordinary world heritage. We also thank the friends Gaetano Boldrini, Leonardo Colavita, Marta Ciaramella, Carla Corongiu, Jo de Waele, Luca Massa, Chiara Paniccia, Alessio Romeo and Marco Vattano for helping us in specimen collection and zoological documentation.

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Cave Microbiome in karsts of Armenia

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Abstract

During 2015 – 2020, faunal changes and microbial communities were studied in caves of Armenia: comparisons were made with research results obtained in 1985. The 3 karst caves of Dahna mountain range (Ararat, Armenia), the underground Urartian Asni Aqueduct and the Stone Door Cave of Aragatsotn, Armenia, were studied. Since 2017, changes in populations of birds, including doves and rooks and by bats have also been studied. Based on observations made in all these karst caves in 2019, the minimal air temperature was 5°C higher than in 1985, and in one of the caves, Bat Hole, the maximal temperature was 18°C higher than in 1985. Carbene dioxide concentration in some parts of the caves was 5 times higher and the water temperature in Asni Aqueduct was 3°C higher. The human and animal pathogens and the level of presence of opportunistic pathogenic microorganisms were found to increase. We performed experiments in which we studied and isolated spore-forming and non-spore forming *Bacillus*: we identified 4 thermophilic (65°C) and 4 psychrophilic (4-22°C) strains. Their microbiological, ecological and genetic characteristics are described in the present article.

Résumé

Le microbiome des grottes des karsts d'Arménie. En 2015-2020, les changements de la faune et des communautés microbiennes ont été étudiées dans des grottes d'Arménie, sur la base de comparaisons avec les résultats obtenus en 1985. Trois grottes du massif de Dahna (Ararat, Arménie), l'aqueduc souterrain d'Urartian Asni et la grotte Stone Door d'Aragatsotn ont été étudiées. Depuis 2017, les changements de l'avifaune (colombes, freux) et des populations de chauves-souris ont aussi été étudiés. Dans toutes ces cavités, les températures minimales relevées en 2019 étaient 5° C plus élevées qu'en 1985, et dans la grotte des chauves-souris, la température maximale était de 18° C plus élevée qu'en 1985. La concentration en CO₂ était, en certains endroits, 5 fois plus élevée et la température de l'eau dans l'aqueduc d'Asni était de 3° C plus élevée. Les pathogènes humains et animaux et le niveau de présence de microorganismes pathogènes opportunistes étaient en croissance. Lors de nos expériences, nous avons étudié et isolé des *Bacillus* sporulés et non sporulés : nous avons identifié 4 souches thermophiles (65°C) et 4 psychrophiles (4-22°C). Leurs caractéristiques microbiologiques, écologiques et génétiques sont décrites dans le présent article.

1. Introduction

Cave ecosystems are very specific and unique for every cave, which is explained by unique microclimate, chemical compounds of media and isolation from different abiotic and biotic, as well as anthropogenic factors of surface. The majority of bacteria, which were identified in caves, are *Pseudomonas* (*P. fluorescens*, *P. aeruginosa*, etc.) and *Bacillus*, *Stenotrophomonas* and others. Some of them are well known as human opportunistic pathogens (JURADO et al., 2010). They show a wide diversity of adaptive mechanisms, including antibiotic resistance enzymes, which genes can be transferred between bacteria of different species. The mentioned bacteria are also able to degrade various chemical substances, including natural as well as synthetic organic compounds. Due to several factors

including climate change, microbial communities of any ecological system also change through time (ITCUS et al. 2018). According to research conducted in recent years in 3 karst caves of Dahna mountain range, Deep Hole, Dove Hole and Bat Hole Ararat, RA (RUGGIERI et al., 2018), in Urartian Asni Aqueduct and in the Stone Door Cave of Aragatsotn, RA, significant fluctuations in temperature and biodiversity were observed (SHAHINYAN, 2019).

In the current study, 8 bacterial strains were isolated from the underground Urartian Asni Aqueduct near Asni Cave Church. Some characteristics of their morphology, physiology, genetics, antibiotic resistance, as well as xenobiotic biodegradation potential were studied.

2. Materials

The isolated bacterial strains were sampled from fossil cave mud from Aqueduct near Asni Cave Church, near Afshar

Village by the specialists of the Speleological Center of Armenia. Then, they were cultivated in large diapason of

temperatures (6°C - 56°C) on liquid and solid nutrient, using selective and mineral media, according to standard protocols. Microbiological identification of morphological and physiological parameters was carried out by optical microscopy and physiological tests (figures 1-3) (YASIR, 2018). All microbiological analyses were carried out according to standard protocols in the Microbe Depository Center (MDC) of "Armbiotechnology" Scientific and Production Center (SPC) of the National Academy of Sciences, Republic of Armenia (NAS RA). Control strains were taken from the National Culture Collection, MDC "Armbiotechnology" SPC NAS RA. Antibiotic and tartaric acid derivatives resistance and biodegradation tests were

carried out according to standard protocols (PEREZ-PANTOJA *et al.* 2010). Xenobiotic biodegradation tests were carried out by substitution of carbon sources in appropriate culture media using benzyl- and cyclohexil derivatives of tartaric acid (BABAYAN *et al.*, 2020) (figures 4-5). Genetic analyses were done by the total and plasmid DNA isolation, purification, electrophoresis, PCR and transformation methods (figure 6) (LUCOTTE and BANEYX, 1993). New tartaric acid antimicrobial derivatives were elaborated at the Laboratory of New Agrarian Pesticide Creation and the Quality Control, National Polytechnic University of Armenia (NPUA) (DASHCHYAN *et al.*, 2014).

3. Results

Inside Dove Hole the temperature was 14°C in 2004. In the deep sections of the cave, bat colonies were detected, while at the entrance dove populations were observed. The thickness of guano accumulation was 171 cm. During further measurements made in August 2018, the air temperature inside that particular cave was 24°C. Here multiple accumulations of guano were observed (thickness was 35 cm). The presence of snakes, bats and magpies was also observed. In Asni's Aqueduct, the temperature of water was 9°C in September 1984, while the air temperature inside the cave was 12°C. The water flow was 6.2 L/sec. There were no fungi and animals.

In August 2018 the water flow was 3 L/sec. The water temperature was 10°C, while the air temperature inside the cave was 17°C. On the walls of the cave various species of mold fungi associated with guano accumulations and lichens were present at the cave entrance in the twilight zone. *Bacillus* genus representatives were detected showing various morphology, physiology, lifecycle and antibiotic resistance.

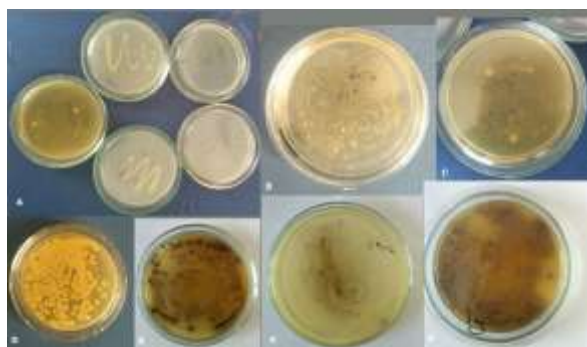


Figure 1: Asni cave bacteria cultivation and purification. A – accumulative culture, with different strains (at 6°C, 11°C, 13°C, 22°C, 37°C, 56°C); B – mix culture of white and yellow bacteria and fungi; C – purified white colonies; D - Purified yellow colonies E, F, G - white colonies with brown-black coloring since 3rd, 4th, 5th, 6th day of cultivation (22-25°C), after isolation at 6°C, 11°C, 13°C.

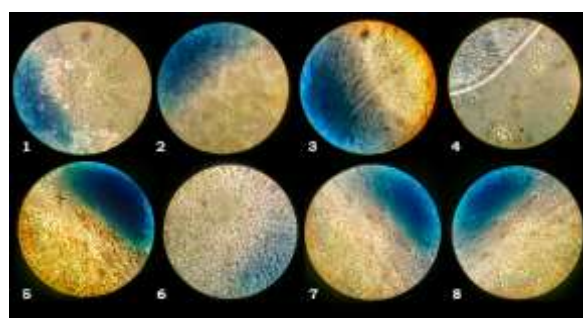


Figure 2: The microscopy of researched cave strains of *Bacillus*, isolated from Asni Cave Church. 1 – white spore forming bacilli, 2 – brown-black colored spore forming colonies, 3 – yellow non-spore forming bacilli, 4 - white spore forming colonies, 5 - ivory colonies, 6 – white shiny oily colonies, 7 – yellow colonies, 8 – white colonies with matt surface and smooth edges.

Using different temperature and culture media supplemented with antibiotics, as well as tartaric acid (TA), 4 cyclic and aromatic complex monoamino salts, multi-drug resistant cave microbes were identified. The studied bacteria were isolated and cultivated at 6°C, 11°C, 13°C, 22°C, 25°C, 37°C, 56°C. As a result, psychrophilic and thermophilic microbes were detected. We found no mesophilic microorganisms (37°C).

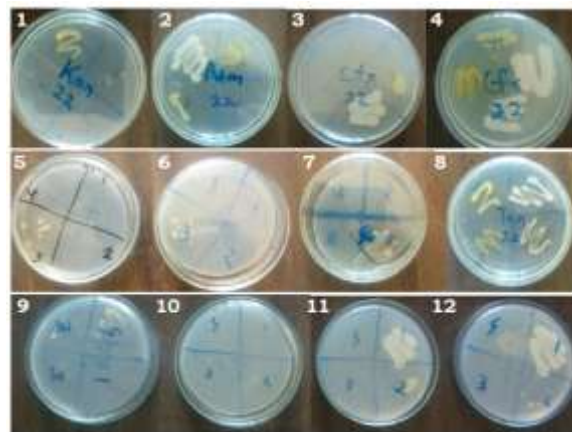


Figure 3: Resistance potential of Asni Cave bacteria. Antibiotic resistance of strains, isolated at 22°C (1, 2, 3, 4)

and 56°C (5, 6, 7); Growth inhibition (samples at 22°C (9, 10); samples at 56°C (11, 12) by the new derivatives of TA.

Using optical microscopy, all detected microbes were rod-shaped. In samples isolated at 6°C, white and yellow colonies were observed, with optimal growth temperatures of 6°C, 11°C, 13°C, 17°C, 22°C and 25°C. White and yellow colonies were detected also in samples isolated at 6°C, and cultivated at 6°C, 11°C and 13°C.

A	1	2	3	4	5	6	7	8	9	10	11	C
I	-	+	+	+	+	+	-	-	-	-	+	+
II	-	+	+	+	+	+	-	+	-	+	+	+
III	-	+	-	+	+	-	-	-	+	+	+	+
IV	-	+	+	+	-	-	-	-	+	+	+	+
B	1	2	3	4	5	6	7	8	9	10	11	C
V	-	-	-	-	-	-	-	+	-	-	-	+
VI	-	-	-	+	+	-	-	+	+	-	-	+
VII	-	-	+	-	+	-	+	+	-	-	+	+
VIII	-	-	-	-	-	-	-	-	-	-	-	+

Figure 4: Antibiotic resistance of Asni Cave Bacillus. A – psychrophilic strains; B – thermophilic strains; 50µg/ml antibiotics: 1 – ampicillin, 2 – amoxicillin, 3 – augmentin, 4 – cefixime, 5 – ceftriaxone, 6 – kanamycin, 7 – Stp/streptomycin, 8 – azithromycin, 9 – ciprofloxacin, 10 – chloramphenicol, 11 – tetracycline; “+” – growth, “-” – inhibition; C – control on nutrient culture media.

In samples isolated at 11°C and cultivated at 22°C, small white round shiny oily colonies, with smooth edges were observed. In samples isolated at 22°C and cultivated at 25°C, white round ivory colonies with matt surface and smooth edges were detected. In the sample, isolated at 17°C and cultivated at 22°C and 25°C, yellow shiny bulging colonies with smooth edges were identified. In samples isolated and cultivated at 25°C, white colonies were detected with the ability to become brown after 3 days of cultivation, with matt folded surface and wavy edges. In samples isolated and cultivated at 65°C, 4 types of spore forming white and yellow colonies of different sizes were detected. The analysis of antibiotic sensitivity showed a wide diapason of resistance, including multi-drug resistant microbes (figure 4). Biochemical tests showed the ability of bacteria to use cellulose as carbon source for samples which were isolated and cultivated at 22°C - 25°C.

4. Discussions

Studies have been conducted to determine whether the temperature increase in 2 studied caves is accompanied by significant changes of fauna, flora and microbial communities. Eight strains of spore forming and non-spore

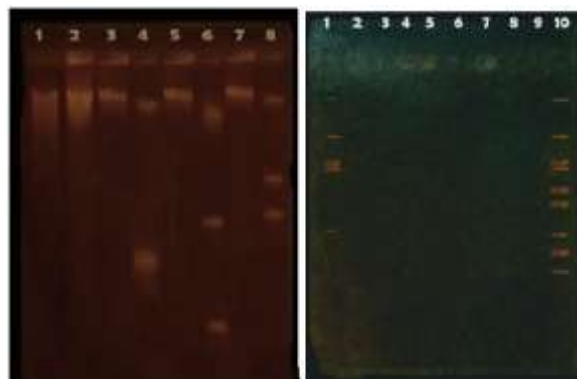


Figure 5: Genetical analyses of Asni Cave Bacillus. left – plasmid profile: 1-4 – psychrophilic microorganisms, 5-8 – thermophilic microbes; right – PCR analysis of resistance genes’ absence: 1, 10 – control samples with 2-5 – psychrophilic strains, 6-9 – thermophilic strains.

Genetic analyses revealed a diversity of plasmid consistence in 8 isolated strains. Transformation results demonstrated the absence of gene horizontal transfer by plasmids from the 8 isolated strains to *Pseudomonas* sp., *Stenotrophomonas* sp. and *Escherichia coli*. PCR analysis demonstrated the absence of aminoglycoside, lactam and amphenicole resistance genes: blaOXA-10, aac(6’’)II, aph(3’’)IV and catB7 (figure 5).

Sample (22 °C)	TA	Na ₂ -TA	BI	CI	BAS	CAS	EDTA	Bz	C
1.	-	+	+	+/-	+	+/-	-	+	+
2.	+	+	+	+/-	+	+/-	-	+	+
3.	+	+	+	+/-	+	-	+	-	+
4.	+	+	+	+/-	+	-	+	-	+
Sample (56 °C)	TA	Na ₂ -TA	BI	CI	BAS	CAS	EDTA	Bz	C
1.	+	+	-	-	+	-	+	-	+
2.	-	-	-	-	-	-	-	-	+
3.	-	+	-	-	+	+	-	-	+
4.	-	-	-	-	-	-	-	-	+

Figure 6: Tartaric acid derivatives (0,5M) effect on Asni Cave bacteria. “+” - growth, “-” – inhibition; C – control on nutrient culture media; TA – tartaric acid, Na₂-TA – Na-tartrate, BI – Benzylimide of TA, CI – Cyclohexylimide of TA, BAS – benzyl amino salt of TA, CAS – cyclohexyl amino salt of TA, EDTA – Sodium ethylenediaminetetraacetate dehydrate, Bz - benzyl chloride.

The effect of the tartaric acid derivatives was different among the 8 studied strains and it was higher for psychrophilic strains. Some of them were able to degrade natural and synthetic derivatives of tartaric acid.

forming psychrophilic and thermophilic *Bacillus* were isolated. Genetic analyses showed the diversity of plasmid profiles in strains, which were resistant and sensitive to tartaric acid and antibiotics. The impossibility of plasmid

replication in *E. coli* and the absence of plasmid replication from surface bacteria in their cells were shown. In isolated strains of microbes, a wide spectrum of resistance to various substances (tartaric acid derivatives, EDTA, benzyl chloride and antibiotics) was detected. PCR analyses have demonstrated the absence of aminoglycoside, lactam and

amphenicol resistance genes in the isolated strains. The detection of thermophilic strains in Asni's Cave could potentially be due to geothermal activity. Their xenobiotic biodegradation properties make these *Bacillus* representatives potentially deteriorative for cave paintings.

5. Conclusion

The present study of 3 caves (Dove Hole, Deep Hole and Bat Hole) suggests temperature may have changed over the last two decades. In microbiome of fossil cave mud, 8 strains of thermophilic and psychrophilic *Bacillus* were detected. The presence of thermophilic strains might potentially be the consequence of ancient geothermal activity and/or modern global temperature increase but these two potential explanations need to be confirmed. The detected wide diapason of biodegradation and resistance, simultaneously with the expected pathogenicity, makes these microbes potentially aggressive for cave visitors and cave paintings. Further detailed research is important for the safety of cave visitors and maintenance of the cultural heritage of the caves, especially due to touristic and religious importance of Asni Cave.

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Gender inequality in the dark: are adaptations to the cave environment sex-specific?

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Abstract

Cave-related morphological adaptations (troglomorphy) are well-studied phenomena in various taxa. However, the effect of cave adaptation on sexual dimorphism – or the sex-dependency of selective forces in caves – have not been extensively examined yet. The *Asellus aquaticus* species complex is a model system for studying evolution in caves and has been examined from many aspects, but most of the morphological studies included males only. Here, we included both sexes (298 females and 358 males) from six cave and nine surface populations to quantify morphological variation. In a previous study, we investigated 16 function-related morphological traits and, by focusing on differences between cave and surface habitats, we found out that the extent of sexual dimorphism was habitat-dependent in eight traits. Here we analyse these eight traits further and focus on differences at the population level. The new results strengthened our previous findings in most cases, but also revealed previously overlooked patterns originating from between-population morphological variation within habitats. We conclude that morphological adaptations to cave life can depend on both habitat and sex, while there are notable population differences within each habitat type. General patterns should therefore be interpreted with caution.

1. Introduction

Detection of conspicuous similarities in the appearance of different subterranean taxa date back to DARWIN (1859). Today, these similar morphological and other organismal changes related to cave life are called troglomorphy (CHRISTIANSEN, 1965). Troglomorphic changes can be regressive or progressive (ROMERO, 2011). These phenotypes result from similar selection forces acting repeatedly and independently in different caves (CULVER & PIPAN, 2015). Troglomorphy can emerge in phylogenetically distant taxa (convergent evolution) or in descendants of a common ancestor (parallel evolution) (ENDLER, 1986). It is widely accepted that the key driver of troglomorphic changes is the natural selection stemming from the unique environmental conditions (permanent darkness, constant climatic conditions, limited amount of food). However, it has been largely overlooked that selection might act differently on individuals within a population, for instance, on males and females resulting in sexual dimorphism (ANDERSSON, 1994), the extent of which

might even differ between cave and surface habitats. A prime model organism for understanding morphological changes after colonisation of subterranean habitats is the primarily surface-dwelling freshwater isopod *Asellus aquaticus* species complex (PROTAS & JEFFERY, 2012). It colonised caves on several independent occasions (VEROVNIK & KONEC, 2019), resulting in parallel divergent evolution. Although many of its cave and surface populations have already been subject to extensive morphological analysis (e.g. PREVORČNIK *et al.*, 2004), sexual dimorphism has only been examined in surface populations (e.g. BERTIN *et al.*, 2002). To our knowledge this is true for all subterranean model organisms. In our previous study (BALÁZS *et al.*, unpubl.) we found habitat dependent sexual dimorphism in the *A. aquaticus* species complex. Here, we examined this dimorphism further, focusing on how cave adaptation might have affected its magnitude and direction in distinct populations.

2. Materials and Methods

To investigate sex-dependent troglomorphy, we collected samples from 15 populations (6 cave and 9 surface) (Fig. 1) of the *A. aquaticus* species complex. SWOROBOWICZ *et al.* (2015) suggest that there might be as many as 13 cryptic species in the complex throughout Europe. Many of these received no taxonomical description yet, while others have various ranks, thus the taxonomy of *A. aquaticus* remains unresolved. In the present study we do not intend to resolve taxonomical questions and therefore we treat all focal populations as part of the *A. aquaticus* species complex. We used cave-surface population pairs for comparison. This means that for the LAB-TIM (IT), the PIV-PLA, ZEL-CER (SI),

the CA-KO (RO) and the MJ-MT (HU) (Fig. 1) population pairs there is no physical barrier between the cave-surface habitats as there is a direct hydrological connection between them. In case of the KRS-LJB (SI) population pair we collected individuals from the geographically closest surface population as there is no known surface population inhabiting the epigean part of the sinking river. Additionally, we included one extra surface population from Romania (TB) and two from Hungary (DL, CS) from geographically close but hydrologically different localities. Only adult animals (≥ 4 mm) were collected. The animals were fixed in RNAlater solution and stored at 4°C. First, whole-body

pictures were taken under standardized setup for body size measurement. Then we dissected antenna I, II, pereopod I, IV, VII and the exopodit of pleopod III (hereafter 'exopodit') from both sides of the body and mounted them in glycerine gelatine on a glass slide. Originally, 16 different function related traits were measured plus the body length as a proxy for body size. All animals with broken appendages (n=110) were excluded, resulting in 656 individuals (298 females, 358 males). In our previous study, we found significant interaction between sex and habitat in eight (antenna II 5th and 6th article length, pereopod I propodus length and width, pereopod IV total length, pereopod IV propodus

arbitrary and polygon length, and exopodit area) out of 16 traits (BALÁZS *et al.*, unpubl.). In the here presented follow-up study, we analysed this subset of traits further with a focus on the effect of population. First, we ran a multivariate linear model (mLM) on the eight traits as response variables with population, sex, population × sex interaction and body length as fixed effects. Since all effects were highly significant in the multivariate approach, we ran univariate linear models (LMs) on the original variables (built similarly to the mLM) one-by-one to investigate the between and within population variation in more detail. For all statistical analysis, SPSS 26 was used.

Population ID	Country	Locality	Habitat	GPS coordinates
MJ	HU	Molnár János Cave	Cave	47°31'4.8"N 19°2'9.877"E
MT	HU	Malom Lake	Surface	47°31'4.8"N 19°2'9.877"E
CS	HU	Csömör Stream	Surface	47°35'35.03"N 19°7'21.78"E
DL	HU	Dunakeszi Lake	Surface	47°36'23.15"N 19°7'24.63"E
CA	RO	Dimitru Ana Well	Cave	43°49'23.59"N 28°34'01.45"E
KO	RO	Kara-Oban Lake	Surface	43°50'46.0"N 28°33'59.1"E
TB	RO	Spring at Baile Turcesti	Surface	43°49'12.15"N 28°29'28.26"E
PIV	SI	Pivka Channel of Planina Cave	Cave	45°49'11.6"N 14°14'44.4"E
PLA	SI	Planina Polje	Surface	45°49'56.2"N 14°15'30.0"E
ZEL	SI	Zelše Cave	Cave	45°47'26.4"N 14°18'12.6"E
CER	SI	Cerknica Polje	Surface	45°46'23.0"N 14°19'31.2"E
KRS	SI	Krka Cave	Cave	45°53'24.0"N 14°46'16.5"E
LJB	SI	Ljubljana Marsh	Surface	45°58'02.9"N 14°32'52.0"E
LAB	IT	Grotta di Trebiciano	Cave	45°41'04.1"N 13°49'42.9"E
TIM	IT	Timavo Spring	Surface	45°47'15.8"N 13°35'28.7"E

Figure 1: Locations of the studied populations of the *Asellus aquaticus* species complex

3. Results

The mLM revealed a significant population × sex interaction (Wilk's $\alpha_{112, 4345} = 0.089$; $p < 0.001$) besides significant main effects (population: Wilk's $\alpha_{112, 34345} = 0.003$, $p < 0.001$; sex: Wilk's $\alpha_{8, 618} = 0.289$, $p < 0.001$; body length: Wilk's $\alpha_{8, 618} = 0.116$, $p < 0.001$).

The LMs revealed that population, sex and population × sex effects were significant (all $p < 0.001$) in all traits, but the sex effect in exopodit ($p = 0.127$). The estimated marginal means and their standard errors are reported in Fig. 2. The results

4. Discussion

In BALÁZS *et al.* (unpubl.) the extent of sexual dimorphism differed between surface and cave animals in all herein examined traits translating to: i) longer propodus of pereopod I and longer 5th and 6th articles of antenna II in caves and males ii) longer pereopod IV and larger propodus of pereopod IV in caves and females, iii) wider propodus of pereopod I in surface and males iv) opposite sexual dimorphism in exopodit area, males having larger exopodit in caves and females having larger exopodit in surface habitats. We use this previous analysis as a framework for the interpretation of the population focused results obtained in this study.

Antenna II: The 5th and 6th articles of antenna II were measured as a proxy for its length, as the flagellum is often broken. The antenna II has a mechanosensory function that aids palpation in both sex and thus its elongation is expected in caves. Additionally, BERTIN & CÉZILLY (2003) reported that in certain surface *A. aquaticus* populations males with longer antenna II have increased pairing success. This explains the male-biased sexual dimorphism. In general,

of the LMs translate to i) longer and wider propodus of pereopod I in males of all populations, ii) longer pereopod IV, and larger propodus arbitrary length in females in all populations except CER, iii) larger pereopod IV polygon length in females in most populations except: CER, DL, KO, TB, iv) longer 5th and 6th article of antenna II in males in all population but MJ and MT, and only for the 6th article in case of TB, v) varying sexual dimorphism of exopodit population-by-population.

both sexes have longer articles in caves and the male-biased sexual dimorphism is more pronounced in cave populations. An exception is the MJ cave population where marginal female-biased sexual dimorphism was observed. There were two surface outliers. In the TB population the 6th article, in the MT population the 5th and 6th articles showed a similar pattern as the MJ population. The similar pattern of MT and MJ populations is surprising as despite them inhabiting the same hydrological system, they are genetically isolated for at least 60.000 years (PÉREZ-MORENO *et al.*, 2017). We also note that contrary to PREVORČNIK *et al.* (2004), who reported that antenna II elongation was not present in LAB population and was modest in CA population, we found that substantial elongation of the 5th and 6th articles is present in both. We conclude that the male-biased sexual dimorphism which is already present in surface populations is strengthened in caves. A possible driver of this evolutionary change might be the low densities of cave populations that require a more effective male mate searching behavior aided by elaborated antenna II.

POP	SEX	ANT II 5 th article	ANT II 6 th article	PE I prop length	PE I prop width	PE IV length	PE IV prop polygon l.	PE IV prop arbitrary l.	Exopodit of pleopod III
ZEL	F	59.05±0.83	89.18±1.38	59.77±0.78	27.38±0.71	381.78±2.22	84.91±0.89	86.51±0.86	130.29±3.60
ZEL	M	64.65±0.79	101.83±1.31	70.58±0.74	35.44±0.68	357.23±3.05	69.76±0.84	73.62±0.81	130.97±3.42
CER	F	53.63±0.77	80.22±1.27	59.58±0.72	35.09±0.66	330.84±2.97	57.73±0.82	60.80±0.78	146.25±3.32
CER	M	55.23±0.77	83.30±1.27	69.35±0.72	44.37±0.66	334.43±2.98	62.38±0.79	57.78±0.82	156.24±3.34
PIV	F	70.96±0.79	112.94±1.31	66.95±0.74	31.84±0.68	379.15±3.06	88.59±0.81	85.24±0.84	123.19±3.43
PIV	M	77.55±0.82	126.40±1.36	74.96±0.77	34.57±0.71	346.43±3.18	71.67±0.85	67.46±0.88	105.96±3.56
PLA	F	52.83±0.76	78.91±1.27	57.18±0.72	31.52±0.65	330.51±2.96	61.47±0.79	59.06±0.81	133.79±3.31
PLA	M	54.20±0.79	80.75±1.31	72.96±0.74	46.86±0.68	316.00±3.07	59.89±0.82	54.82±0.85	135.55±3.44
KRS	F	59.09±0.83	88.16±1.38	61.94±0.78	31.48±0.71	341.49±3.22	73.83±0.86	70.63±0.89	127.21±3.61
KRS	M	65.68±0.80	102.74±1.33	71.32±0.75	37.87±0.69	328.63±3.10	67.57±0.82	63.26±0.85	133.06±3.47
LJB	F	55.05±0.79	84.76±1.31	58.94±0.74	34.44±0.68	318.46±3.06	59.83±0.81	56.85±0.84	160.35±3.42
LJB	M	57.70±0.76	89.66±1.26	71.06±0.71	45.99±0.65	311.71±2.94	59.04±0.78	55.11±0.81	160.80±3.29
LAB	F	63.46±1.29	94.81±2.13	68.08±1.20	31.88±1.10	373.85±4.97	87.41±1.32	84.39±1.37	130.21±5.57
LAB	M	73.52±0.80	111.04±1.32	82.50±0.74	42.65±0.68	321.55±3.08	68.64±0.82	60.94±0.85	108.14±3.44
TIM	F	58.99±0.79	94.99±1.31	56.84±0.74	29.45±0.68	345.17±3.06	67.45±0.81	65.52±0.84	147.63±3.42
TIM	M	62.27±0.88	101.80±1.36	75.23±0.77	48.90±0.70	305.68±3.18	59.90±0.85	55.32±0.88	131.58±3.56
CA	F	55.52±0.98	88.14±1.63	54.91±0.92	26.98±0.84	352.24±3.80	75.11±1.00	73.38±1.05	206.54±4.26
CA	M	64.95±1.11	104.47±1.83	75.97±1.04	48.28±0.95	318.04±4.28	64.11±1.14	55.27±1.18	342.21±4.79
KO	F	57.54±1.36	86.76±2.24	55.53±1.27	31.76±1.16	326.50±5.24	58.53±1.39	55.56±1.44	157.84±5.87
KO	M	62.01±1.21	95.51±2.01	74.57±1.14	49.72±1.03	305.01±4.69	59.28±1.25	52.72±1.29	157.74±5.25
MJ	F	62.84±1.08	93.37±1.79	63.83±1.01	35.41±0.92	321.82±4.17	71.38±1.11	68.07±1.15	183.03±4.67
MJ	M	62.27±0.85	92.69±1.41	73.26±0.80	43.11±0.73	301.74±3.29	66.80±0.87	61.57±0.91	170.92±3.68
MT	F	56.92±1.01	86.10±1.67	58.13±0.94	35.24±0.86	307.11±3.90	58.93±1.04	55.32±1.08	173.37±4.37
MT	M	56.72±0.86	82.16±1.42	70.18±0.80	48.35±0.74	271.90±3.32	55.70±0.88	48.53±0.92	165.69±3.72
TB	F	56.28±1.25	88.67±2.07	57.58±1.17	36.19±1.07	315.57±4.84	58.25±1.29	54.62±1.33	152.60±5.42
TB	M	57.31±1.13	87.95±1.87	75.73±1.06	55.64±0.97	290.09±4.38	58.39±1.16	51.30±1.21	133.17±4.90
DL	F	59.37±1.11	89.49±1.83	60.80±1.03	38.05±0.95	309.94±4.27	57.89±1.14	53.77±1.18	164.22±4.78
DL	M	60.30±0.93	92.05±1.54	72.66±0.87	51.58±0.80	292.31±3.60	58.08±0.96	51.15±0.99	154.13±4.03
CS	F	57.04±1.16	87.26±1.92	60.21±1.09	37.68±1.00	301.39±4.50	56.64±1.19	52.59±1.24	171.13±5.03
CS	M	58.34±0.92	88.94±1.53	73.30±0.86	54.72±0.79	284.30±3.57	59.70±0.95	51.91±0.98	160.25±3.99

Figure 2: Estimated marginal means and corresponding standard errors deducted from the LMs. They are presented separately for males and females for each population. POP= Population, ANT= Antenna, PE= Pleopod, prop=Propodus, l=length.

Pereopod I propodus: The propodus of pereopod I is related to feeding in both sexes. In males it is also used for grabbing the female in precopulatory mate guarding (BERTIN *et al.*, 2002) and it likely has a role in aggressive behaviour towards male competitors. We found that males had longer and wider propodus of pereopod I than females in all populations. While cave males had larger propodus than surface ones only in some cases, cave females had larger propodus than surface ones in most cases. Said differently, in most cases sexual dimorphism of the propodus was less pronounced in cave than in surface populations, exceptions being propodus length in LAB and both measures in CA. The CA population resembled the pattern of the Romanian surface populations (KO, TB). This similarity probably originates from the close phylogenetic relationship between these populations, as they are descendants of the same ancestral lineage (KONEC *et al.* 2015). Nevertheless, the emerging general pattern is that elongation of the propodus in caves is mostly affecting females, which might be a result of an increased importance of feeding in a low-food environment. At the same time, in males, this trait might be more conserved due to its further functions, a trade-off resulting in lower sexual dimorphism in cave compared to surface populations.

Pereopod IV: Males use this appendage for holding the female in mate guarding, while in females, it has general locomotory function. It is well known that this trait is sexually dimorphic in *A. aquaticus*, females having a longer appendage and males having a more curved propodus. Expectedly, we found that females had longer pereopod IV than males (except CER population). However, it is interesting that the sexual dimorphism is markedly more pronounced in cave populations. To capture the curvature of the propodus we measured its arbitrary (the distance

between the proximal and distal end of the article) and polygon lengths, with higher values depicting a less curved article shape in both cases. In propodus arbitrary length sexual dimorphism is female-biased, except in the CER cave population where no difference was observed between the sexes. The same general pattern was found for propodus polygon length, but with four surface populations (CER, DL, KO, TB) showing differently. Thus, sexual dimorphism of propodus curvature seems to vary population by population at the surface, but it follows a clear trend in caves with males having more curved propodus. The modest sexual dimorphism in surface populations becomes emphasised in caves in both curvature proxies. This might be due to low population density and scarcity of mates in caves: the strongly curved propodus might decrease the chance of the female escaping from the male's hug. We conclude that even though elongation occurred in both sexes, the holding function of this appendage in males restricts its elongation.

Pleopod III exopodit: This appendage is probably related to swimming ability. It could also play a role in respiration and osmoregulation indirectly by circulating the water under the abdomen, as it is suggested for other crustaceans (DAHL, 1977) or directly as a respiratory surface (PREVORČNIK *et al.*, 2009). We found varying and modest sexual dimorphism of the exopodit in all but the CA population, which had an exceptionally large exopodit supporting the findings of TURK-PREVORČNIK *et al.*, (1998). It also showed outstanding male-biased sexual dimorphism. A reasonable explanation for the enlarged exopodit is the extremely low oxygen concentration characteristic for the sulfidic waters of the Movile cave (REISS *et al.*, 1999). Considering this, it seems that certain traits are better explained by local environmental factors than general habitat characterization.

Conclusion

The detected between and within populations differences are probably interplays of various factors such as standing genetic variation of ancestral populations or site-specific ecological conditions (e.g. KONEC et al. 2015). Despite this, we can generally conclude that the patterns found in this study support the previously found appendage elongation of certain traits in caves. We also found that the degree of appendage elongation is sex-specific due to the different functions in males and females. The magnitude of cave related changes is not universal in all populations but mostly

points to the same direction, thus constituting a general trend. Although many details remain unexplained, we suggest that explanations of troglomorphy in sexually dimorphic traits must consider selection forces that might affect the sexes differently. The results of the herein presented population-focused analysis clearly shows the importance of within-habitat replicates as certain populations showed clear deviations from the general habitat-related trends.

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Mammals and birds cave use in the Sonoran desert, Mexico

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Abstract

In Mexico, diversity of species classified as troglolobites and troglolophiles has been reported; but species known as trogloloxenes (occasional users) have not been evaluated to understand their importance in underground environments. During one year (2015-2016), a camera-trap sampling station was installed at entrances of the “Cueva de la Mariana” and “Cueva El Tigre”, located in central of Sonora, Mexico. The cameras were deployed for a total of 662 trap days and capture 237 independent events. We recorded 15 species, 10 medium and large mammal species and five bird species. The most abundant species were the gray fox and the black vulture, while less abundant were the puma and the barn owl. The Cueva El Tigre entrance was used mostly by the gray fox as a shelter for its cubs and feeding site, while the black vulture, provides nesting, breeding, parental care to its offspring until they become adults, and even teaching how to flight. On the other hand, Cueva de la Mariana entrance had similar evidence for the gray fox and black vulture. Our finding show caves are of primary importance for a number of mammals that have long been considered as occasional users. Evidence provides an active movement of the fauna into these arid land caves.

1. Introduction

Extensive biological studies in caves have been made around the world. Mexico, for instance, is considered to have one of the richest cave-dwelling fauna on Earth (HOFFMANN *et al.*, 2004; REDDELL, 2006; PALACIOS-VARGAS *et al.*, 2014). However, information about subterranean fauna in the state of Sonora, Mexico is sparse and scattered (CALVA & CASTILLO-GÁMEZ, 2017; CALVA, 2017). In the field of biospeleology, trogloloxenes are the species that have been least investigated because they are considered as occasional users. Recently it has been documented through direct and indirect observations that certain species use the entrances of the cavities for a variety and important activities related to their life cycle (REDDELL, 2006; KEMPE *et al.*, 2006; PALACIOS-VARGAS *et al.*, 2014).

In addition, trogloloxenes contribute to favoring trophic networks of subterranean ecosystems (CULVER & PIPAN, 2009; ROMERO, 2009).

The trap-camera has become a useful tool to study the ecology and behavior of terrestrial fauna especially in habitats with hard access. Its advantages include the precision in the identification at a specific and frequently individual level, an efficiency of detection of the diurnal and nocturnal fauna use of habitat and the confirmation of species whose tracks and traces do not differ (MONROY-VILCHIS *et al.*, 2009; MONROY-VILCHIS *et al.*, 2011).

The objectives of the present work are to estimate the richness and relative abundance of terrestrial vertebrate species in cave entrances; detect the activity patterns and analyze the behavior of the species identified.

2. Materials and methods

Sampling was carried out from April 11, 2015 to April 12, 2016. Two trap stations were placed with a trap-camera in each of the cave entrances. The trap-camera used (SIMMONS®, Whitetail model) is a digital automatic detection system that operates with a passive infrared sensor. The trap-cameras were programmed to remain active 24 hours a day, with a minimum delay of one minute between shots and a record of 3 photographs per shot. They were checked out once every three months. The photographed species were identified by comparison with the specialized literature.

The total sampling effort is the sum of the trap-days during which each trap-camera remains active. Likewise, the calculation of the relative abundance index (RAI) corresponds to the number of independent photographic records acquired for every 100 trap days. This value is a unit

of standardization for the comparison of data with other studies (MONROY-VILCHIS *et al.*, 2011).

$$RAI = \frac{C}{EM} * 100 \text{ trap-days}$$

C = Independent photographs.

EM = (Number of active days per camera).

In order to estimate abundance more accurately and to avoid counting the same individual several times, only the following cases used by MONROY-VILCHIS *et al.* (2011) will be considered as independent photographic records: 1) consecutive photographs of different individuals, 2) consecutive photographs of individuals of the same species separated by more than 24 hours, this criterion was used when it was not clear whether a series of photographs

correspond to the same individual, so that photographs taken within the same 24-hour period were considered as a single record and 3) non-consecutive photographs of individuals of the same species. In the case of gregarious species, the photographs where more than one individual was observed, the number of independent records was considered equal to the number of individuals observed.

3. Results

The total sampling effort was 662 trap-days, and 2,277 photographs were obtained from 10 species of mammals and 5 species of birds. The total number of photographs, 237 (10 %) were classified as independent records. These species correspond to two orders and six families of mammals and four orders and five families of birds.

In Cueva de la Mariana (CM), the sampling effort was 366 trap-days, recording 462 photographs, with 41 classified as independent events. At the entrance to the CM, 12 species were detected. In Cueva El Tigre (CT), the sampling effort was 296 trap-days, recording 1,815 photographs, 196 of which were classified as independent events. At its entrance 10 species were registered.

The species detected in the CM were: *Ammospermophilus harrisi* (n = 1), *Bassariscus astutus* (n = 1), *Coragyps atratus* (n = 6), *Geococcyx californianus* (n = 6), *Lynx rufus* (n = 19), *Nasua narica* (n = 2), *Neotoma albigula* (n = 4), *Puma concolor* (n = 1), *Spermophilus variegatus* (n = 39), *Spilogale gracilis* (n = 1), *Urocyon cinereoargenteus* (n = 28), and *Zenaida sp.* (n = 1), whereas in CT the following species were recorded: *Bubo virginianus* (n = 10), *Conepatus leuconotus* (n = 6), *Coragyps atratus* (n = 117), *Geococcyx californianus* (n = 1), *Puma concolor* (n = 2), *Spermophilus variegatus* (n = 5), *Spilogale gracilis* (n = 3), *Tyto alba* (n = 2), *Urocyon cinereoargenteus* (n = 48), and *Zenaida sp.* (n = 2). Both caves shared four species of mammals and three of birds. According to the IAR, the most abundant species for the CM were *U. cinereoargenteus*, *L. rufus* and *S. variegatus* and in the case of CT the most abundant species were *C. atratus* and *U. cinereoargenteus*.

4. Discussions

Generally, studies with trap-camera use a large number of sampling stations and cameras in order to have a highest probability of detection of the species of interest (PIÑA *et al.*, 2004; MONROY-VILCHIS *et al.*, 2011). For our study, the objective was different since it consisted in identifying vertebrates that exclusive used the caves entrance during a whole year. PIÑA *et al.* (2004) made a study using 88 sampling stations in three different habitats in the eastern part of the state of Sonora, obtaining 897 total photographs and 437 photographs of only wild species in a sampling effort of 2,348 trap-days during two years. The study registered 18 species of mammals. Comparing our results

To determine the pattern of activity of the species present in the study, only species with at least 11 independent photographic records were used, which has been considered as a minimum number to describe the activity pattern (MONROY-VILCHIS *et al.*, 2009, 2011). Based on some photographic records, the behavior and use of the caves of the species present was determined.

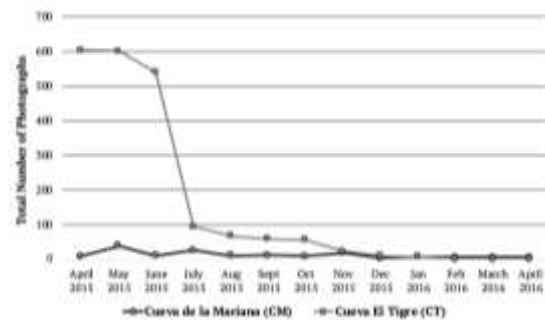


Figure 1: Total number of photographs of all species recorded for both caves, Cueva de la Mariana (CM) and Cueva El Tigre (CT).

The total number of photographs were used to determine the activity patterns of three species in the CM, and two species in CT. For CM, *S. variegatus* was active both day and night, but with a greater presence between 8:01-16:00h. *Lynx rufus* and *U. cinereoargenteus* were mainly active at night, although they also showed activity during the day. In CT, *C. atratus* was recorded only during the day, with a higher activity between 8:01-16:00h, while *U. cinereoargenteus* was active day and night, but unlike the records of the CM, its tendency was to habit the cave during the day between 8:01-16:00h. In turn, considering the total photographs of all the species detected, it was observed that for both, the largest number of records correspond to the months of May, June and July. The total number of photographs, 47 % was registered during these months at the entrance of CM, while for CT it was 30 % (Fig. 1). During the study, in CM, the species *S. variegatus* was the species with the highest number of months recorded (10 months), and in CT, *C. atratus* its presence was for 7 months.

using only two sampling stations in a single habitat type (caves), we had a much lower sampling effort but a greater number of total photographs (2,277) with a record of ten species of mammals of which approximately 50 % were present in the study by PIÑA *et al.* (2004) that took two years to conclude.

Relative Abundance Index

Variations in Relative Abundance Index (RAI) of the species were presented for both caves (Fig. 2). The Cueva de la Mariana (CM) had three species with a high RAI, being *S. variegatus* (10.6), *U. cinereoargenteus* (7.65) and *L. rufus* (5.19). The rest of the species (75%) had an IAR between 0

and 2. The Cueva El Tigre (CT) had the species that were the most abundant throughout the year, *C. atratus* with a RAI of 39.52, followed by *U. cinereoargenteus* (16.22). The rest of the species (80%) had an RAI between 0 and 4. We can infer that *U. cinereoargenteus* shows a preference for inhabiting caves, as in both caves its RAI was between the most abundant species. Because there are no similar studies to compare the presence and abundance in caves of the gray fox, an analogy can be made with the results by PIÑA *et al.* (2004), where the number of records of this species was 88 independent photographs and occupying the three habitats evaluated, observing their capacity to use different habitats present in the region. On other hand, *C. atratus* also appeared in the CM, however its RAI was low (1.63).

Name	Scientific Name	Cave Entrance	Independent Photographs	Relative Abundance Index (RAI)
Rock squirrel	<i>Spermophilus variegatus</i>	CM	39	10.6557377
Gray fox	<i>Urocyon cinereoargenteus</i>	CM	28	7.650273224
Wild cat	<i>Lynx rufus</i>	CM	19	5.191256831
Black vulture	<i>Coragyps atratus</i>	CT	117	39.52702703
Gray Fox	<i>Urocyon cinereoargenteus</i>	CT	48	16.21621622

Figure 2: Relative Abundance Index (RAI) of the most recorded species for Cueva de la Mariana (CM) and Cueva El Tigre (CT).

Activity patterns

To determine the pattern of activity of the species present in the study, only species with at least 11 independent photographic records were used. The gray fox (*U. cinereoargenteus*) recorded an activity during the 24 hrs, with a higher record at night and at twilight. However, its activity should also be mentioned during the day. The same pattern was found in both entrance of CM and CT. The wild cat (*L. rufus*) had activity similar to *U. cinereoargenteus*, with a tendency to enter the CM at night and in the twilight. The squirrel (*S. variegatus*) in CM and the black vulture (*C. atratus*) in CT were recorded exclusively on the day (6:01 to 18:00 h). In the case of birds, four of the five reported species for both caves had daytime records with the exception of *T. alba* in CT that was present between 01:00 and 06:00 only. It can be considered that their activities are influenced by temperature, and the availability of food inside the caves (MONROY-VILCHIS *et al.*, 2011).

Behavior

To analyze behavior, two species had the highest abundance and activity patterns. The first one is the gray fox (*U. cinereoargenteus*), where in CT had several records from the beginning of May to July 2015. In May, two individuals were observed to move in and out of the cave, both in the day as in the night, but at night it was common to observe the species with prey to feed inside the cave. During June, specifically in June 2015, a female was recorded with a cub, using the cave entrance as shelter and since then, their presence was constantly recorded (Fig. 3); in the middle of the month the presence of the same female was observed but now with two cubs. During their visit to the cave, the individuals remained a few meters from the entrance, and their main activity was rest. Their recording ended until July

7, 2015 when they did not appear again during the period of the trap-camera. The black vulture (*Coragyps atratus*) shared the use of caves with *U. cinereoargenteus* in CT. At the end of May, a constant visit of the cave (CT) by a female black vulture was observed and at May 27, 2015 the presence of two offsprings of the vulture were registered. In the month of June, their presence was 24 of the 30 days of that month. The parental care was provided only by the female where the feeding, the flight teaching and the thermoregulation by unfolding their wings were observed. The change of feathers to adult by the offspring was obtained on June 2015 but its presence continued until the beginning of July where they left the cave (Fig. 3). An interesting behavior was that in the month of June, both *C. atratus* and *U. cinereoargenteus* shared the cave without problems of territory. Both use CT as a refuge for the protection of their young. *Coragyps atratus* reappeared in CT several months later (September, October, November, January) but only one individual, possibly using CT as a shelter from the adverse conditions of the external environment. Unfortunately, the last 60 days of the trap-camera of CT entrance did not complete due to failure in the memory used, but by direct observation on February 20, 2016 two eggs of *C. atratus* appeared, located at ground level.



Figure 3. Cubs (*U. cinereoargenteus*) and offsprings (*C. atratus*) of two species with highest abundance and activity patterns inside Cueva El Tigre.

The eggs were observed in the deep part of the section corresponding to the entrance at the same place where their presence was previously registered in survey (2014) of Cueva El Tigre. The hatching of the eggs of the current year is not known with certainty. However, on April 12, 2016, two new offsprings were observed. Several authors mention that the time of reproduction and nesting of this species varies according to the latitude in which it is found. In the case of Sonora, it is reported during the months of January and February, incubating 1 to 3 eggs, which corresponds to what we observed.

The recurrence during three consecutive years of *C. atratus* in the El Tigre Cave and its preference to use this cave as a nesting and breeding site for their offspring, may be due to the bats that inhabit that cave. This cave is visited by several species of bats, mainly by *Tadarida brasiliensis*, which has

been reported with populations of thousands to millions within that cave (COCKRUM & BRADSHAW, 1963; CALVA, 2017; CALVA & CASTILLO-GÁMEZ, 2017), producing tons of guano. The decomposition of guano produces high concentrations of ammonium so it limits the invasion of other predators to deeper parts. This was observed during the exploration to the cave, where the offsprings from 2015

were located in the deepest parts of the entrance, where MCFARLANE *et al.* (1995) has reported a high concentration of ammonium of 1270 ppm, a concentration that still continues. Apparently, the black vulture is not affected by this high concentration, and may even be a strategy to avoid predators during the early stages of their lives.

5. Conclusion

Several species were related to the use of both cavities. Cueva El Tigre has more species of birds, while Cueva de la Mariana has greater number of mammals, this may be due to the difference between both entrances, the concentration of ammonium and its connection with the surrounding environment, despite sharing the same external climate.

Due to the richness of species, the frequency of records and use detected by the most abundant species, we will continue to analyze the troglomen that visit the caves of Sonora, Mexico. Evidence provides an active movement of the fauna to these arid land caves, which should provide different food sources for other species that live in the deeper parts of the caves.

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Données complémentaires sur la biodiversité cavernicole du Pays basque

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Résumé

Depuis la fin du XIX^e siècle, les grottes et gouffres du Pays basque ont été fréquentés par les plus éminents biospéléologues. Leurs études ont révélé une faune endémique particulièrement riche et variée. Depuis 2018, nous avons souhaité mener des recherches complémentaires dans certaines cavités des massifs des Arbailles et d'Urkulu, de la colline de Gaztelu, d'anciennes mines de cuivre et de fer, et de la côte basque. Certaines études sont totalement inédites (grottes du phare de Biarritz et mines de Banca). D'autres ont été conduites dans des cavités déjà étudiées pour lesquelles nous avons pu apporter des informations complémentaires et nouvelles. Les investigations entreprises ont permis une connaissance accrue de la biodiversité cavernicole locale : plus de 100 taxons ont été recensés avec de nombreux Invertébrés troglobies. Ces recherches ont également permis de révéler la présence soit d'espèces mentionnées pour la première fois sur le territoire métropolitain (Diplopodes), soit d'espèces nouvelles pour la science (Araignées, Collemboles).

Abstract

Additional information on the cave biodiversity of the Basque Country. Since the end of the 19th century, the caves and chasms of the Basque Country have been frequented by the most eminent biospeleologists. Their studies revealed endemic fauna particularly rich and varied. Since 2018, we have been conducting further research in some cavities of the massifs of Arbailles and Urkulu, of the hill of Gaztelu, of ancient copper and iron mines, and of the Basque coast. Some studies are completely new (caves of the Biarritz lighthouse and mines of Banca). Others were carried out in caves already studied, on which we have been able to bring additional and new informations. The investigations undertaken have resulted in increased knowledge of the local cave biodiversity: more than 100 taxa were identified with numerous troglitic Invertebrates. This research also revealed the presence of either species mentioned for the first time in the metropolitan area (Diplopoda) or species new to science (Spiders, Collembola).

Resumen

Información adicional sobre la biodiversidad cavernícola del País Vasco. Desde finales del siglo XIX, las cuevas y simas del País Vasco han sido frecuentados por los bioespeleólogos más destacados. Sus estudios revelaron una fauna endémica particularmente rica y variada. Desde 2018, hemos querido realizar investigaciones adicionales en algunas cuevas de los macizos de Arbailles y de Urkulu, de la colina de Gaztelu, de antiguas minas de cobre e hierro, y de la costa vasca. Algunos estudios son completamente inéditos (cuevas del faro de Biarritz y minas de Banca). Otros fueron realizados en cuevas ya estudiadas sobre las cuales hemos podido proporcionar información adicional y nueva. Las investigaciones realizadas han permitido un mayor conocimiento de la biodiversidad cavernícola local: se determinaron más de 100 taxones con muchos Invertebrados troglobios. Estos estudios también contribuyeron a revelar la presencia de especies mencionadas por primera vez en el territorio metropolitano (Diplopoda) o de especies nuevas para la ciencia (Arañas, Collembola).

1. Introduction

L'étude de la faune cavernicole du Pays basque a débuté entre le milieu et la fin du XIX^e siècle : Simon signale la présence d'un nouvel Opilion troglobie dans la grotte de Sare en 1879 (LUCANTE, 1880). Les plus éminents spécialistes vont fréquenter de nombreuses cavités du Pays basque et publieront les résultats de leurs études dans *Biospeologica*, dont une synthèse générale sera réalisée par René Jeannel (JEANNEL, 1926). On remarquera que ces études ont surtout été menées dans le massif des Arbailles et dans les grottes de Sare. Ainsi, peu d'études ont été menées dans le massif d'Urkulu. Éric Dupré a publié un

inventaire de la faune souterraine des Pyrénées basques (DUPRÉ, 1991).

À partir de 2018, nous avons entrepris une étude complémentaire de la biodiversité cavernicole de cette région en nous intéressant à quelques cavités présentant des environnements très variés et complémentaires : altitude et bord de mer, cavités déjà étudiées ou inédites, cavités karstiques et anciennes mines.

D'autre part, nous avons souhaité étudier l'ensemble des Invertébrés cavernicoles et ne pas nous limiter à un ou des

groupes spécifiques, en nous intéressant plus particulièrement à la faune de petite taille, souvent ignorée, telle que les Collemboles ou micro-Gastéropodes.

2. Matériel et méthode

Les prélèvements ont été effectués selon deux procédés : à vue à l'aide de pinces souples ou avec un aspirateur à bouche, et par piégeage à l'aide de croûtes de fromage. Tous les spécimens ont été fixés en alcool à 70° ou à 90°.

Les tris ont été effectués à la loupe binoculaire et les spécimens ont été envoyés aux différents spécialistes (voir remerciements) aux fins de détermination.

Cavité	Abrév.	Commune	N	W	Z
Haspia	Has	Assurucq	43.129576	-0.9526060	820 m
Oyanbeltza	Oya	Saint-Michel	43.059333	-1.239066	1 170 m
Mine des 3 Rois	3RS	Banca	43.129853	-1.373698	254 m
Mine de Méhatzé	Meh	Banca	43.085418	-1.344228	1 170 m
Mines de Larla	Lar	Saint-Martin-d'Arrossa	43.243639	-1.329687	440 m
Grotte de Lakarra	Lak	Lacarre	43.1846728	-1.1624087	210 m
Colline de Gaztelu*	Gaz	Saint-Martin-d'Arberoue	43.352719	-1.206172	139 m
Grotte de la Vierge	Bon	Bonloc	43.368961	-1.266889	49 m
Grotte à Lulu	Lul	Saint-Pierre-d'Irube	43.463712	-1.444093	10 m
Grotte Lezea	Sar	Sare	43.268122	-1.571574	201 m
Grotte d'Urio Gaïna	Sar	Sare	43.266697	-1.581802	290 m
Grotte du Phare 1	Bia	Biarritz	43.493656	-1.555026	8 m
Grotte du Phare 2	Bia	Biarritz	43.494296	-1.553893	19 m

* : 4 cavités étagées : Aldabia, Oxocelhaya, Isturitz et Figuier.

Figure 1 : Tableau récapitulatif des cavités étudiées avec leurs coordonnées GPS.

3. Résultats

La figure 3 récapitule l'ensemble des taxons récoltés, observés ou mentionnés antérieurement dans les cavités des sites étudiés.

4. Discussions

La richesse de la biodiversité cavernicole au Pays basque était déjà connue et reconnue (BERTRAND, 2003 et 2020). Notre étude a permis de compléter ces connaissances dans les 12 sites que nous avons étudiés.

Ainsi, nous avons repéré : 106 taxons, dont une vingtaine peuvent être qualifiés de troglobies.

Cette étude a également permis de : confirmer la présence du Diplopode *Mesoiulus cavernarum* sur le territoire métropolitain (GEOFFROY, 2020) ; mentionner une Araignée

Nesticidae probablement nouvelle, récoltée dans la mine des 3 Rois à Banca ; mentionner la présence de 7 espèces probablement nouvelles de Collemboles rien que pour les grottes de Sare ; d'autre part, des compléments d'étude sont en cours pour *Coecobrya* dans la grotte du Phare N°2 à Biarritz ; révéler la présence de l'Isopode *Trichoniscoides saeroeensis*, alors qu'il n'avait plus été retrouvé sur nos côtes depuis les années 1950.

Remerciements

Nos remerciements vont aux spécialistes qui ont assuré l'identification des espèces : Alain Bertrand et Sophie Gansoinat (Gastéropodes), Fabio Stoch (Copépodes et Amphipodes), Florian Malard (Isopodes aquatiques), Olivier Gargominy (Gastéropodes), Samuel Danflous et Sylvain Dejean (Opilions et Aranéides), Emmanuel Séchet (Isopodes terrestres), Jean-Jacques Geoffroy (Myriapodes), Thomas Wesener (Diplopodes), Louis Deharveng (Collemboles) et Arnaud Faille (Coléoptères). Ces travaux scientifiques s'inscrivent dans le cadre de l'inventaire national du patrimoine naturel (inpn.mnhn.fr). Ils ont bénéficié en 2019 d'un soutien de l'UMS PatriNat (AFB, CNRS, MNHN).

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Figure 2 : Collembole *Tomocerus* sp. Grotte Urio Gaïna Sare (64). Cliché : Louis Deharveng.

Aquatique	Genre	Espèce	auteur(s), date		Has	Oya	3RS	Meh	Lar	Lak	Gaz	Bon	Lul
Urodela	<i>Calotriton</i>	<i>asper</i>	(Al. Dugès, 1852)	1, 2	Sp		X						
Gastropoda	<i>Bythinella</i>	sp.		1, 2	Sb								
	<i>Bythinia</i> ou <i>Mercuria</i>	sp.		2	Sp					X			
	<i>Potamopyrgus</i>	<i>antipodarum</i>	(Gray, 1843)	2	Sp							X	
	<i>Radix</i>	sp.		2	Sp							X	
Copepoda Cyclopoida		sp.		2	Sp								
Copepoda Calanoida		sp.		2	Sp								
Isopoda	<i>Proasellus</i>	<i>chauvini</i>	Henry & Magniez, 1978	1, 2	Sb	X							
	<i>Proasellus</i>	<i>colffaiti</i>	Henry & Magniez, 1972	1	Sb								X
Amphipoda	<i>Echinogammarus</i>	sp.		2	Sp								
	<i>Niphargus</i>	<i>puteanus</i>	(Koch, 1836)	1	Sb								
	<i>Niphargus</i>	<i>robustus</i>	Chevreaux, 1901	1, 2	Sb								
	<i>Pseudoniphargus</i>	<i>africanus</i>	Chevreaux, 1901	1	Sb								

Figure 3, faune aquatique : Récapitulatif des Invertébrés mentionnés ou récoltés dans les différents sites étudiés. Sb : Stygobie ; Sp : Stygophile ; Tb : Troglobie ; Tp : Troglophile ; 1 : mentionné dans la littérature ; 2 : récolte ou observation en 2018, 2019 et 2020 ; en rouge, espèce probablement nouvelle pour la science.

Nitrogen Dynamics in a Tropical Karst Cave Stream

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Abstract

The ongoing global change in the amount of nitrogen cycling in the biosphere forecast serious impacts to the structure and functioning of aquatic ecosystems. Nitrogen transport, transformations and transferences along karst cave streams will have a significant influence in the health of karst surface streams and basin ecological processes. An assessment of the nitrogen energy-yielding processes (nitrification–denitrification) and uptake efficiency was conducted at the El Convento cave stream, a tropical lotic system which receives large inputs of nitrogen-rich bat guano. The findings showed that nitrogen cycling was sustained at a near equilibrium condition where nitrogen export (82 % of inputs) is favored over retention (18 % of inputs). Results indicated that an optimum and efficient nitrification process (ranging from 8.9 to 68.3 mg-N/kg/d) is favored by the cave environment when compared to similar surface streams. The nitrogen uptake length, velocity and areal uptake rate, which averaged 165 m, 4.9 mm/min and 15.3 mg -N m²/min respectively, is by far much higher compared to first and second-order surface streams. As virtually all nitrogen transformations and transferences are biotically mediated the results provide a comprehensive insight into the ecological relevance of benthic microbial communities in tropical cave streams..

1. Introduction

The ongoing global change in the amount of nitrogen cycling in the biosphere forecast serious impacts to the structure and functioning of aquatic ecosystems (Matson *et al.*, 1999). In tropical karst terrains, many cave streams receive large inputs of nitrogen-rich organic material from bat's metabolic wastes (Sakoui *et al.*, 2020) while linkages between surface and subterranean drainage systems are very strong. The impact of excessive nitrogen loads on cave streams ecosystem and receiving surface and nitrogen-limited coastal waters will primarily depend on the dynamics of nitrogen transport, transformations and transfers that occur within the speleotitic (*i.e.*, cave stream) environment. The lack of nitrogen-cycling studies in tropical cave streams makes these studies extremely valuable from a global nitrogen cycle point of view and for a better understanding of the ecological processes occurring in tropical karst basins. As virtually all nitrogen transformations and transferences are biotically mediated such appraisal provides a comprehensive insight into the ecological relevance of benthic microbial communities in tropical cave streams. In order to improve the knowledge of nitrogen-cycling dynamics in a speleotitic system an assessment of the magnitude of the energy-yielding nitrogen transformations (nitrification-denitrification) and nitrogen uptake-retentive capacity was conducted at the El Convento cave stream (Fig. 1), a representative tropical speleotitic system located at the southwestern coast of Puerto Rico. As the main feature

of the most prominent karst terrain in the region the cave stands out as a major bat roosting site where its perennial stream receives a constant input of nitrogen-rich organic material in the form of bat guano. The following paper summarizes fundamental aspects of the doctoral dissertation Nitrogen Dynamics in a Tropical Cave Stream presented to the Graduate Program of Biology of the University of Puerto Rico, Rio Piedras.

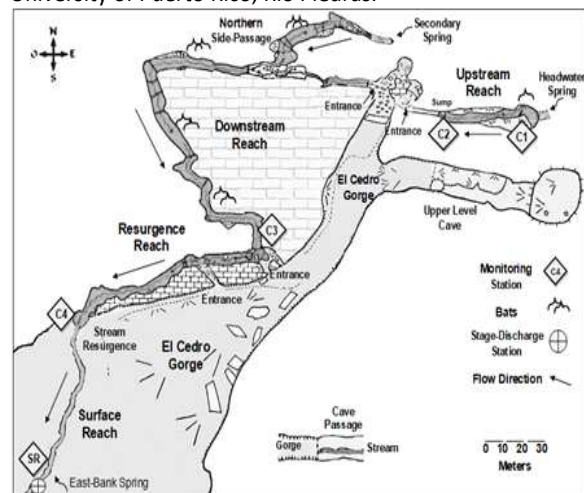


Figure 1: Plan-view map of the El Convento speleotitic system, Guayánilla-Peñuelas, Puerto Rico..

2. Methods and Procedures

The monitoring events were conducted under base-flow conditions; that is, when the cave stream was recharged only by groundwater. For assessing the magnitude of the energy-yielding nitrogen transformations (nitrification-

denitrification) achieved by the benthic microbial community bottom, sediment cores were collected at the cave upstream (C1 and C2) and downstream (C3) reaches, and at the surface reach (SR) of the speleotitic system. The

nitrogen uptake-retentive capacity was determined at the cave upstream and surface reaches.

Nitrification and Denitrification

Nitrification is the biological oxidation of ammonium (NH₄⁺) and occurs under aerobic conditions. The process has two distinct steps, each mediated by a specific group of chemoautotrophic bacteria that use nitrogen compounds as energy source for the synthesis of organic matter by the associated reduction of carbon dioxide (CO₂). The nitrosomonas group, oxidizes NH₄⁺ to nitrite (NO₂⁻) while the nitrobacter group oxidizes NO₂⁻ to nitrate (NO₃⁻) (Duff and Triska 2000). Denitrification is the reduction of oxidized nitrogen anions with concomitant oxidation of organic matter, almost occurring exclusively under anaerobic conditions. Denitrifiers include a diverse group of facultative anaerobic heterotrophic genera that use NO₃⁻ as oxidizing agent during respiration. The process represents a permanent removal of NO₃⁻ from the system in the form of dinitrogen (N₂) or nitrous oxide (N₂O) gas.

Potential nitrification rate (PN_Tr) was determined by measuring rates of nitrate accumulation in the overlying water at selected time intervals (Henriksen et al., 1993). The sediment slurry flasks (amended to 5 mg/L of ammonium) were incubated aerobically in the dark using the shaken soil-slurry method (Schmidt and Belser, 1994). The potential denitrification rate (PD_Nr) was determined by the Acetylene Block technique, where sample preparation and incubation procedures followed those described by Groffman et al. (1999). The assays were conducted at the Water Resources Research Center of the University of New Hampshire. Nitrate and ammonium were measured using a colorimetric automated discrete analyzer (Smart Chem™) following standard methods. Measurement of the evolved N₂O was performed on a Hewlett Packard 5890™ gas

3. Results

Nitrification and Denitrification

With a highest average rate of 68 mg-N per kg of sediment per day, results show that nitrification increases downstream the cave reaches while decreasing at the surface reach (Fig. 2). Such trend correlated well with the ammonium fluctuation measured along the speleolitic system. The observed differences between the cave and surface reaches (ANOVA, F = 6.6, df = 1, p = 0.015) and between the upstream and downstream reaches (ANOVA, F= 28.7, df = 1, p < 0.001) were found to be statistically significant. Compared to surface tropical and temperate pristine streams, results are indicative that nitrification is highly favored in a tropical cave stream (Fig. 3).

Although along the cave waterway denitrification increases downstream, the highest average rate (0.528 mg-N/kg/d) was documented at the surface reach from fine particle bottom sediments (Fig. 4). Even when the observed trend is in accordance with the increase in nitrate along the speleolitic system the observed differences between the cave and surface reaches (ANOVA, F= 3.65, df= 1, p= 0.065) and within cave reaches (ANOVA, F= 1.22, df= 1, p= 0.283)

chromatograph equipped with an electron capture detector.

Nitrogen Uptake and Retention

The uptake length is the distance traveled by a nutrient in the abiotic form before it is removed by biotic immobilization. The uptake metric provides an estimate of nitrogen retention efficiency at a reach scale, where nitrogen-retentive streams are expected to have a shorter uptake length. The Short-Term Nitrogen Addition technique (Tank et al., 2006) was selected for conducting the uptake length (m) measurements and to calculate the associated parameters of uptake velocity (efficiency at which nitrate is removed from the water column via biotic demand) and areal uptake (amount of nitrate immobilized by a streambed area) rates. As the prevailing and main mobile form of nitrogen in aquatic environments, nitrate was chosen as the target solute to be released. For the solute-tracer solution nitrate background concentration at the cave (2.8 mg/L) and surface (4.0 mg/L) reaches was enriched by 0.5 mg/L while for the conservative tracer chloride the background concentration (36 mg/L) was enriched by 10 mg/L. Nitrate uptake length was calculated based on downstream changes in concentration along the experimental length (50 m). The in-stream annual nitrogen mass balance was computed by adjusting the average nitrogen concentration of the headwater and side passage springs inputs and the surface reach output to their associated mean daily discharges. Nitrogen inputs included the magnitude of direct deposits of bat guano to the stream (2.86 grams of N/m²/day) adjusted to the receiving water-surface area of the upstream and downstream reaches (about 930 m²). The balance assumes that bat population and distribution within the cave remained constant during the year.

were not statistically significant. Although the values fall within ranges reported for surface streams (typically up to about 6.5 mg-N/kg/d), when compared to them denitrification process in the speleolitic system occur at relatively low rates.

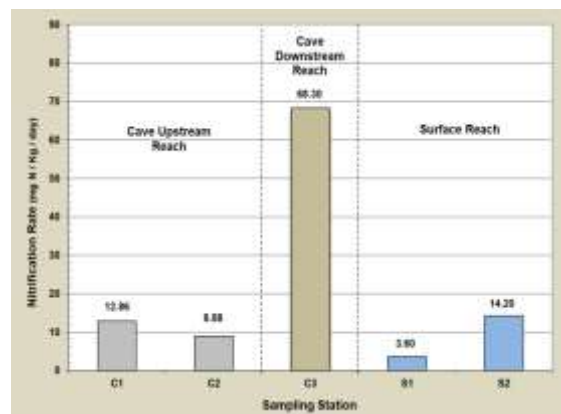


Figure 2: Potential nitrification rates from selected sites at the El Convento cave stream, Puerto Rico.

Location		Nitrification Rate		Remarks
		µg-N/m ² /min	mg-N/kg/d	
El Convento Speleologic System Puerto Rico	Cave Upstream	76.8	10.9	1 st Order stream: Microcosm study
	Cave Downstream	484	68.3	2 nd Order stream: Microcosm study
	Surface Reach	63.0	8.9	2 nd Order stream: Microcosm study
Bisley Creek, Luquillo Experimental Forest, Puerto Rico		20.1	0.25	2 nd Order stream: Field Study (Merriam and others, 2002)
Upper Bell Creek, North Carolina, USA		0.8	na	Field Study (Tank et al., 2002)
Mack Creek, Oregon, USA		11.5	na	3 rd Order stream: Field Study (Ashkins et al., 2004)
Walker Branch, Tennessee, USA		4.1	na	Field Study (Muholland et al., 2000)
High White Creek, North Carolina, USA		na	0.95	Microcosm study (Stary 2004)

Figure 3: Comparison of nitrification rates from the El Convento speleologic system with tropical and temperate headwater surface streams rates.

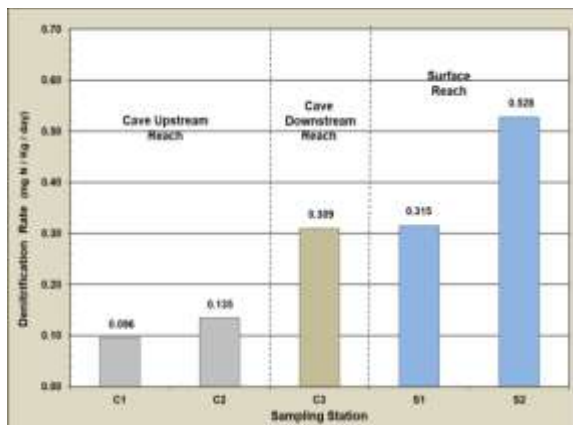


Figure 4: Potential denitrification rates from selected sites at the El Convento cave stream, Puerto Rico.

Nitrogen Uptake and Retention

The nitrate uptake length for the cave upstream and surface reaches averaged 165 and 174 m, respectively. Uptake velocity averaged 4.9 mm/min for the cave reach and 2.4 mm/min for the surface reach, while the areal uptake rate averaged 15.3 and 9.62 mg -N m²/min, respectively. The uptake length was slightly longer at the surface reach suggesting the occurrence of a higher biological demand along the cave reach. However, the mean values of the uptake parameters were not significantly different among the cave upstream and surface reaches (ANOVA, H = 0.60, df = 1, p = 0.67). A comparison of the measured parameters

4. Discussions

Nitrification and Denitrification

The correspondence of the highest nitrification average (68 mg-N/kg/d) with the location (C3) where ammonium highest average concentration (0.48 mg/L) was recorded sustain the availability of ammonium as the leading in-

stream force controlling nitrification rates. The fact that the cave and surface reaches share the same hydrogeological conditions, major nitrogen source, similar water-quality and heterotrophic metabolism points to the absence of sunlight (the fundamental force that differentiate the cave and surface ecosystems) as a major factor responsible for the

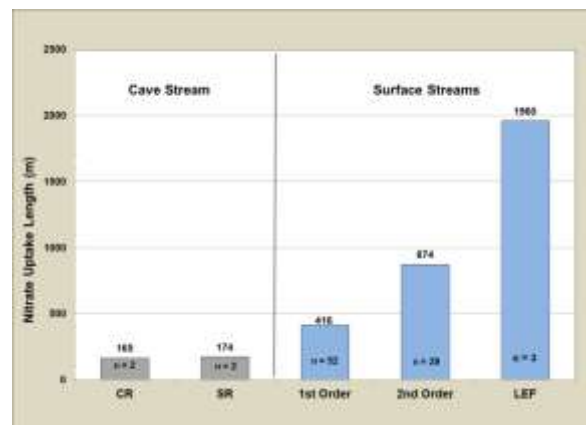


Figure 5: Nitrogen uptake length for the El Convento upstream and surface reaches, grouped 1st and 2nd order surface streams and a tropical 2nd order stream (LEF).

The computed magnitude of the nitrogen input and output loads and corresponding annual mass balance are provided in Figure 6. The assessment show that about 70 percent of the nitrogen is brought in by the groundwater recharge, while 30 percent is provided by the nitrogen-rich bat guano. From the input load, about 80 percent is exported to the surface environment, while 20 percent is retained at the speleologic system.

Nitrogen Inputs				Nitrogen Output (B)	Nitrogen Retained (R = A - B)
Headwater Spring	Side Passage Spring	Bat Guano	Total Input (A)		
1,254	384	920	2,557	2,076	481

Figure 6: Base-flow annual nitrogen mass balance (Kilograms per year) at the El Convento cave stream.

stream force controlling nitrification rates. The fact that the cave and surface reaches share the same hydrogeological conditions, major nitrogen source, similar water-quality and heterotrophic metabolism points to the absence of sunlight (the fundamental force that differentiate the cave and surface ecosystems) as a major factor responsible for the

occurrence of higher nitrification rates at the tropical cave stream, considering that such condition is responsible for the absence of photoautotrophic algae that may compete for the utilization of ammonium. In contrast, denitrification process exhibited a relatively low potential rate believed to be associated with oxic conditions due to the predominant coarse texture of the bottom sediment, a major limiting factor for the presence of higher densities of denitrifiers. Thus the fundamental force (absence of sunlight) that differentiates the cave and surface ecosystems seems not to be a factor influencing denitrification rates. The significant differences found in the potential nitrification and denitrification rates at all the sub-samples sets suggest that at a small spatial scale, the population of nitrifiers and denitrifiers followed a patchy distribution pattern. Such pattern is associated with distinct microhabitats related to the type of substrate, availability of nutrients in the water and within the substrate itself.

5. Conclusion

This study provides a comprehensive account of nitrogen cycle dynamics in a tropical cave stream. As nitrogen is transported downstream it is recycled between the abiotic and biotic compartments while organic and inorganic forms are subject to transformations, microbial uptake and retention processes. Although the majority of the nitrogen is provided by groundwater recharge, concentrations of nitrogen forms along the cave stream are consistent with the size of bat populations and magnitude of direct inputs of

Nitrogen Uptake and Retention

As for speleolotic systems where benthic populations are expected to be at near equilibrium conditions, nitrogen export was found to be favored over retention, where the magnitude of nitrogen assimilatory uptake is limited by the availability of organic carbon. Like cave streams most headwater surface streams are heterotrophic systems that rely almost entirely upon allochthonous sources of energy. However, contrary to the nutrient-limited first and second-order surface streams, the large and constant input of bat guano that enters the cave stream result in a carbon-rich environment. Thus, the relatively higher nutrient availability within the speleolotic system and the expected presence of a greater standing-stock of benthic heterotrophs stand-out as the main promoters of the observed higher efficiency and magnitude of nitrate uptake, particularly evidenced in the areal uptake rate.

guano. Despite the environmental differences among cave and surface ecosystems, nitrogen cycling in a cave stream proceeds in a similar pattern as in headwater surface streams. However, some of the associated processes are enhanced within the speleolotic system by cave related factors, such as a greater environmental stability, a large and constant input of nitrogen-rich organic material and a larger benthic microbial population.

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Stygobites of Puerto Rico: Natural History and Relationship with Caribbean Species

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Abstract

In the Caribbean island of Puerto Rico, the documented stygobites are crustaceans belonging to the orders Mysidacea (1 sp.), Thermosbaenacea (2 sp.) Amphipoda (3 sp.) and Decapoda (1 sp.). Of the seven species, five are known from cave habitats: the mysidacean *Stygiomysis holthuisi*, the amphipods *Alloweckelia gurneei*, *Metaniphargus bousfieldi* plus an unidentified *Metaniphargus* and the decapod *Typhlatya monae*. The two thermobenean species belong to the genus *Tethysbaena* and are known only from phreatic habitats. Hypotheses on the origin of the local forms and dispersion mechanisms are addressed and supported. The relationship with their Caribbean relatives is examined in terms of distribution and dispersal of the ancestral form, speciation processes, and morphological variation as well as habitat particularities. The up-to-date distribution data of local troglobitic amphipods support the hypothesis that specimens of the genus *Metaniphargus*, isolated in coastal areas due to regression of the sea during the Pleistocene, are the parental species of the troglobitic forms, as well as of our endemic fresh-water genus *Alloweckelia*. Long term observations of the cave shrimp *Typhlatya monae* that validate fundamental assertions concerning longevity, reproduction and activity patterns of troglobites are disclosed. Postulates about the origin, evolution and distribution of the *T. monae* are discussed.

1. Introduction

Cave fauna can be characterized in terms of the degree of adaptation and restriction to the underground environment. According to these criteria we classify as troglobites those organisms that have undergone genetic changes that confine them to the underground environment. Their most outstanding features are the loss of vision and pigmentation. Of the 14 cave invertebrates known in Puerto Rico, seven are stygobiontic crustaceans, belonging to the orders Mysidacea (1 sp.), Thermosbaenacea (2 sp.), Amphipoda (3 sp.) and Decapoda (1 sp.). They exhibit distinctive troglobitic characters and were so far only collected from wells or springs (phreatobites). In order to contribute to a better knowledge of these evolutionary relicts as well as to contribute to their

protection and conservation, relevant information concerning the natural history of the stygobites of Puerto Rico was compiled.

The relationship with their Caribbean relatives is examined in terms of distribution and dispersal of the ancestral form. The species are grouped by Order and observations on their natural history, speciation processes, morphological variation and habitat particularities are provided. Special attention is given to the amphipod species and to the stygobiontic shrimp *Typhlatya monae*, from which long-term observations validate fundamental assertions concerning longevity, reproduction and activity patterns of troglobites.

2. Stygobites of Puerto Rico

Order Mysidacea

Stygiomysis holthuisi (GORDON, 1958)

Characterized by a vermiform body with a carapace covering much of the thorax. The species is found in brackish, alkaline and low oxygen anchialine cave pools and within the conduit system of the limestone aquifer (CONDE-COSTAS & GONZALEZ, 1996). Also known in St. Martin, Anguilla and Grand Bahama (PECK, 1981; WAGNER, 1992). Similar species: *Stygiomysis mayor* (Jamaica), *Stygiomysis clarkei* (Caiicos Island and Providenciales Islands) and *Stygiomysis aemete* (Dominican Republic).

Order Thermosbaenacea

Tethysbaena colubrae WAGNER, 1994

Tethysbaena coqui WAGNER, 1994

Characterized by an elongated body with no discernible distinction between the thorax and abdomen, with *T. colubrae* larger in size (WAGNER, 1994). The species are endemic phreatobites that show morphological modifications suitable for their subterranean life. Populations are limited to brackish groundwater aquifers of the southwestern coast of Puerto Rico and Culebra Island (WAGNER, 1994). Similar species: *Tethysbaena gaweini* (Dominican Republic) and *Tethysbaena scitula* (Virgen Gorda).

Order Amphipoda

The group is characterized by a dorsally compressed body and ventrally folded abdomen without a carapace (Fig. 1).

Metaniphargus bousfieldi Stock, 1977

The species is endemic to Puerto Rico, its distribution being limited to the Guánica State Forest caves (southwest coast) (Peck, 1981). It is found in brackish, alkaline and low oxygen anchialine cave pools and within the conduit system of the limestone aquifer. The species has been observed in small briefly flooded soil depressions (CONDE-COSTAS & GONZALEZ, 1996). Similar species: *Metaniphargus longipes* (Aruba) and *M. beattyi* (Santa Cruz) (PECK, 1981; VÉLEZ, 1988).

Metaniphargus sp.

Although not fully described, the species is believed to be an endemic form. Specimens are found in a cave located inland in the northern karst belt, within the Rio Camuy basin. The aquatic habitat is a freshwater, well oxygenated pool above the phreatic zone. Of the 20 known Caribbean species (ORTIZ et. al., 2007), no similar species are found.

Alloweckelia gurneei Holsinger & Peck, 1968

The only representative of this endemic genus is found at the Rio Camuy Cave System (northern karst belt). Populations are known from well oxygenated and shallow freshwater cave pools, well above the phreatic zone. The species exhibit a great capacity to migrate within wet conduits of the vadose zone and colonize pools as much as 30 m above the cave stream. No similar species are found.

Order Decapoda

Typhlatya monae Chace, 1954

Characterized by an elongated body with extremely long antennae and a pigmented point on the eye stem (Fig. 2). The species is found at the Guánica State Forest (southwest coast) in brackish, alkaline and low oxygen anchialine cave pools and within the conduit system of the limestone aquifer at Mona Island. In the Caribbean region, it is also known in Dominican Republic, Barbuda, Curacao and San

Andres islands (BOTELLO et al., 2013). Similar species: *Typhlatya garciai* (Cuba) and *Typhlatya mitchelli* (Mexico) (based on morphologic characters).



Figure 1: Stygobiontic amphipods of Puerto Rico and distribution.



Figure 2: Specimens of the troglobitic shrimp *Typhlatya monae* from el Refugio Cave, Guánica State Forest.

3. Origin and Development

The acquisition of troglobitic features by the Caribbean stygofauna is a distinct evolutionary process. The modifications involve not only the disappearance or regression of traits no longer useful underground but also the acquisition of progressive or advantageous characteristics that will enhance their chance of survival. The most prominent regressive features of troglobitic species are the reduction or loss of pigmentation and vision.

Genetic mutations are random events that tend to be destructive in nature. The mutation responsible for visual organ degeneration is considered fairly common, while no mutation is known to replenish the loss of visual organs (MOORE & SULLIVAN, 1997). The incorporation of such

regressive attributes in troglobitic populations can be reconciled by the "Direct Selection" hypothesis which states that the disappearance or degeneration of disused organs favors the affected organism as it entails an energy economy (SKET, 1985). Therefore, the assimilation of regressive traits by stygobiontic populations is in fact supported by natural selection process as the elimination of unused elements would be an advantage to the organism.

Caribbean stygobites originate from marine epigeal ancestors that managed to invade and colonize coastal groundwater habitats. After the establishment of the ancestral form the acquisition of troglobitic features required genetic isolation between the surface and

subterranean populations. In other words, mating between members of both populations must cease. In the Caribbean region, periods of sea regression between the Pliocene (Tertiary Period) and Pleistocene (Quaternary Period) about 5 and 2 million years ago respectively, were responsible for isolating the ancestral marine species that would give rise to the current stygobites species (HOLSINGER & PECK, 1968; PECK, 1974; WAGNER 1994). The disappearance of the epigeal form suggests its inability to compete with other organisms or extermination by saltwater during the Pleistocene floods (CHACE & HOBBS, 1969).

Consummated the isolation, the natural selection promoted the inception of progressive and regressive mutations. The absence of light and food shortage of the subterranean realm is the most prominent selective force. Among the progressive adaptations for a life in total darkness stand out the elongation of appendages, optimization of sensory structures and a thinned cuticle (MOORE & SULLIVAN, 1997). Attributes that respond to a food shortage include a slower metabolism, smaller body size, higher efficiency in

4. Distribution and Dispersal

It is postulated that the distribution of the mysidacean *Stygiomysis holthuisi* in the Caribbean is the result of pelagic dispersal of the ancestral marine form, probably during the Pleistocene era (PECK, 1981). In evolutionary terms, due to the short time since the ancestral form was isolated in the groundwater habitats, the morphological variations between the Caribbean populations are not yet significant enough to declare distinct species (WAGNER, 1992).

The genus *Tethysbaena* is represented in the Caribbean by 13 species (WAGNER, 1994). Their distribution is the result of vicarious isolation (geographical separation) of the marine ancestor, as today's stygobitic species (adapted to fresh or brackish water) do not have the ability to disperse across the sea (WAGNER, 1994). The Caribbean species diverged between the Pliocene (Tertiary Period) and the Holocene (Quaternary Period), over a period between 5 million years and about 10,000 years.

The marine ancestor of the *Metaniphargus* amphipods was isolated in coastal habitats as a result of the regression of the sea during the Pleistocene (HOLSINGER & PECK, 1968). Of the 20 known Caribbean species of the genus the majority are still restricted to anchialine brackish waters, while 3 species are adapted to freshwater habitats (ORTIZ, et. al., 2007). It is postulated that *Metaniphargus* is the precursor of the endemic freshwater genus *Alloweckelia*, which is not a relict species (survivor of an ancient phylogenetic line) isolated in freshwater habitats during the Pleistocene (HOLSINGER & PECK, 1968). The discovery in 1985 of a freshwater *Metaniphargus* species in a cave located in the Rio Camuy basin, close to the place where *Alloweckelia* is found, supports the hypothesis. The fact is that specimens of *Metaniphargus* adapted to freshwater and moved inland along the conduit system of the limestone aquifer and promoted speciation processes. The dispersion of

digestion and nutrient assimilation. Compared to their surface relatives, the life cycle of the troglotic species shows other progressive adaptations such as a prolonged period between reproductive events, longer gestation time, smaller litters, larger eggs, late maturity and longevity.

Observations by the author of a small population of *Metaniphargus bousfieldi* and *Typhlatya monae* held in captivity since 1995 evidenced some of the progressive adaptations. After 25 years in captivity all the 15 specimens of *T. monae* are still alive as well as many of the amphipods. No reproductive event has been observed while their activity pattern does not follow circadian rhythms. Worthy of mention is that the species must have physiological adaptations that allow them to survive under extreme conditions. Their anchialine cave habitat sometimes became practically anoxic (0.6 ppm O₂) and concentrations of carbon dioxide (62 ppm CO₂) and hydrogen sulfide (H₂S) reached levels considered lethal to many organisms (CONDE-COSTAS & GONZALEZ, 1996).

amphipods is enhanced by their ability to migrate through interstitial spaces and to climb wet walls (MOORE & SULLIVAN, 1997). Currently a molecular assessment is taking place by the author to establish the phylogenetic relation of the local stygobitic amphipods.



Figure 3: Distribution of *Metaniphargus* in the Caribbean region.

Concerning the distribution of the cave shrimp *Typhlatya monae* in the Caribbean region, it is postulated that it originated from the pelagic dispersal of the ancestral marine form, probably during the Pleistocene era (PECK, 1981). The slight differentiation of the current populations suggests that they are relicts of the same marine ancestor which adapted to an existence in salty or fresh waters, subsequently invading the coastal aquifers. As stated before, due to the short time since the ancestral form invaded the present localities, morphological variations between these populations are not yet significant enough to declare them different species. A phylogenetic analysis of *T. monae* populations from the Dominican Republic and the

Guanica Forest in Puerto Rico showed very low genetic divergences (BOTELLO, et. al., 2013).

Because of its phreatic life, *T. monae* dispersion is limited by the extension and connectivity of the conduit system and the presence of fresh groundwater. The species was not found at the less-salty environments located farther from the coastline (CONDE-COSTAS & GONZALEZ, 1996). On the other hand, the captive specimens survived under seawater mineral conditions (TDS of 30,000 mg/L). The evidence suggests that *Typhlatya monae* still have not developed osmoregulatory mechanisms to allow them to colonize fresh groundwater habitats.



Figure 4: Distribution of *Typhlatya monae* in the Caribbean region.

5. Conclusion

The study of the stygofauna of the Caribbean region provides significant and useful information for the evaluation of hypotheses concerning processes of evolution, speciation, adaptation and dispersal mechanisms. Moreover, the natural history of stygobites is unique and can be related to the climate changes and geological events

that shape the region. Their life record provides a better understanding on how species can adapt to environmental changes and survive under extreme conditions. Such evolutionary relicts deserve to be managed as rare and vulnerable species.

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Subterranean invertebrates of Quercy (France)

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Abstract

Quercy is a large karstic plateau of low altitude in south-western France. Its subterranean fauna has long been considered as moderately rich but remained relatively understudied. Sampling campaigns since 1985, done in the frame of small regional projects, enabled us to collect a huge amount of data. All subterranean micro-habitats were sampled, including some frequently overlooked ones, like hanging roots, soil in caves and deep waters, using a large range of collecting techniques. Outside soil sampling was systematically associated to cave sampling, for a better evaluation of the ecological status of subterranean microarthropods. Several species new to science have been discovered. We summarize here and discuss in a broader context the main results of our studies.

Résumé

Invertébrés souterrains du Quercy (France). Le Quercy est un grand plateau karstique de basse altitude du sud-ouest de la France. Sa faune souterraine a longtemps été considérée comme modérément riche, mais est restée relativement peu étudiée. Plusieurs campagnes d'échantillonnage depuis 1985, réalisées dans le cadre de petits projets régionaux, ont permis de collecter une énorme quantité de données. Tous les micro-habitats souterrains ont été échantillonnés, y compris certains souvent négligés, comme les racines pendantes, le sol dans les grottes, les siphons et les eaux profondes, en utilisant une large gamme de techniques de collectes. L'échantillonnage du sol extérieur a été systématiquement associé à l'échantillonnage en grotte, pour une meilleure évaluation du statut écologique des microarthropodes souterrains. Plusieurs espèces nouvelles pour la science ont été découvertes. Nous résumons ici et discutons dans un contexte plus large les principaux résultats de nos études.

1. Introduction

Quercy is a karstic plateau of moderate altitude (70 to 450 m), divided into compartments by the river valleys of Dordogne, Célé, Lot and Aveyron (Fig. 1) and stretching on 5000 km² from 44.000° N to 45.100° N on the western border of Central massif in France. It is mainly composed of Jurassic limestones with marly levels, especially in the Lias and Kimmeridgian, and Cretaceous alterations locally. Towards the southwest, it disappears under lacustrine marls and limestones of the Oligo-Miocene. Waters of the impluvium are collected in several hydrological systems and emerge as big springs at the base level in the valleys, like the Fontaine des Chartreux at Cahors (1 to 50 m³/s). Kilometers of underwater passages make of these systems a highly praised destination worldwide for cave diving. Hundreds of dolines and caves are scattered on the plateau. Several subterranean rivers have been explored on more than 10 km, like the gouffre de Padirac (> 50 km of passages) or the système de l'Ouyse (70 km in different large caves). Many "phosphatières", exploited in the past, constitute one

of the richest continental paleontological deposit of Eocene and Oligocene in the world and have been classified as Geological National Nature Reserve in 2015. The Quercy landscape is also marked everywhere by ancient human occupation, including many megaliths and prehistoric caves like the cave of Pech-Merle. All these features led to the creation of the Parc Naturel Régional des Causses du Quercy (PNRCQ) in 1999 and its nomination as a Global Geopark UNESCO in 2016.

Research on cave fauna of Quercy began with the description by Lucas (1866) of the Opiliones "*Scotoleman querilhaci*". A detailed faunistic inventory of the gouffre de Padirac was published 36 years later by Viré (1902). Detailed faunistic results of biospeological sampling campaigns in southern Quercy were published by Bou in 1966. The last important contributions are those of Deharveng (1986) on the regional fauna of cave Collembola, of Lebreton (1986) who summed up our knowledge on cave fauna of central Quercy, and of Bou (2004) about subterranean aquatic

fauna of S Quercy. But most of the huge biological material collected since the 1980s remains unpublished. The present

paper is an overview of the most interesting results obtained so far in the study of this material.

2. Objectives, material and methods

The sampling design was set up with four objectives. First, covering as evenly as possible the four compartments of the Quercy karst (Fig. 1). Second, sampling all subterranean habitats in each of these compartments. Third, associating inside to outside cave samplings for a better evaluation of species ecology. Fourth, solving taxonomic problems detected in several of the most abundant cave taxa.

For aquatic fauna, we sampled puddles, streams, and to a lesser extent phreatic and hyporheic habitats, using nets, Karaman-Chappuis method, pumping, filtering and hand

picking. Sumps and deep water bodies were sampled by divers. For terrestrial fauna, we sampled oligotrophic habitats (walls and ground, puddles, scattered debris), wet oligotrophic habitats, hanging roots, soil in caves, guano and flood debris inside caves, cave entrances habitats and screes of the "Milieu Souterrain Superficiel" outside caves. We used baited and unbaited traps, direct collection by brush and aspirator, soil washing and massive faunal extraction of the soil substrate and guano on Berlese funnels. Specimens were stored in ethanol 95 %, sorted, and identified or sent to specialists for identification.

3. Results and discussion

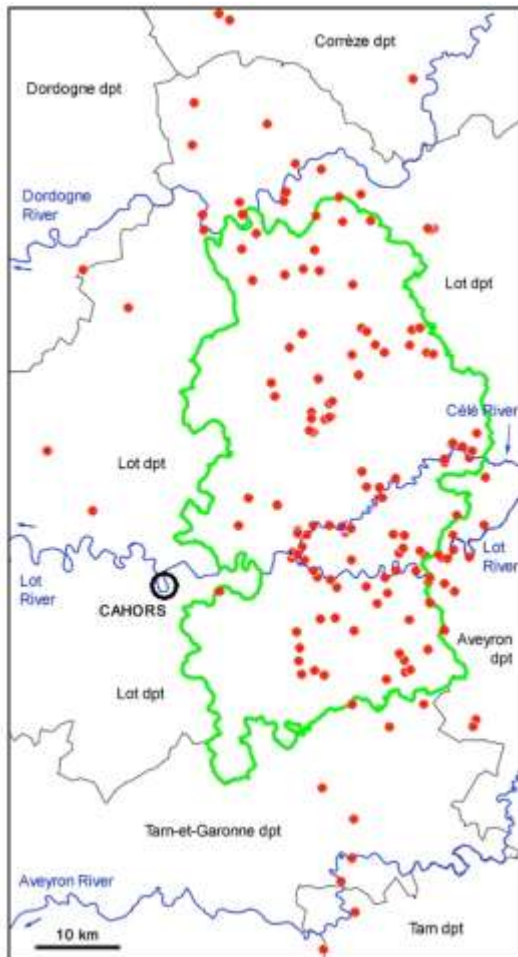


Figure 1: Sites sampled in Quercy. Red dots, sampled sites; green line, PNRCQ; blue lines, main rivers; dpt, department.

We got a total of 2166 records from 560 samples done in 180 subterranean sites (Fig. 1), not including 115 samples of deep soil outside caves. Flood debris, cave entrance habitats

and MSS are not included in the faunistic analysis. A large part of the material is still under identification, but the dataset at hand already gives a good insight into the Quercy subterranean biodiversity.

Subterranean terrestrial communities (Fig. 2)

Invertebrate communities associated to different habitats have been recognized from our material and are briefly characterized below. Their most remarkable species are listed in Figure 2.

1/ Oligotrophic habitats (OH), widespread in Quercy caves. Characteristic taxa are isopods (*Oritoniscus*), and springtails (*Deuteraphorura* spp, *Folsomia candida*, *Pseudosinella dodecophthalma* and *Pygmarrhopalites pygmaeus*), with a rich accompanying fauna including several species of interest.

2/ Wet oligotrophic habitats (WO), known from a few caves in Quercy. Characteristic taxa are springtails (*Schaefferia subcoeca*, *Isotomiella barivierai*, *Oncopodura pelissiei*) and the mite *Rhagidia paralleloseta*. The proportion of endemics is high (see l'Oule in Fig. 2), but accompanying fauna is never important.

3/ Hanging roots (HR), frequent in shallow caves and in "phosphatières" (phosphate mines). Characteristic taxa are mites and springtails (*Megalothorax* sp., *Pseudosinella* sp., *Pygmarrhopalites pygmaeus*).

4/ Soil in cave (SC). Present everywhere, this habitat has been almost completely neglected so far in biospeological studies. It was extensively sampled in "phosphatières" and small fossil caves. In Quercy, it hosts minute blind life-forms and genera reminding those of deep soil layers outside caves, mostly mites and springtails (*Pygmarrhopalites pygmaeus* and several remarkable species listed in Fig. 2).

5/ Guano (GU). Guano accumulations usually host a diversity of taxa, often living in very dense populations. Characteristic taxa are mites and Diptera (unidentified), springtails (*Ceratophysella bengtssoni*, *Mesogastrura ojcoviensis*), millipedes (*Trachysphaera lobata*).

Subterranean aquatic communities

Aquatic fauna has been extensively studied in southern Quercy (BOU, 2004). Data are much sparser in other parts of Quercy. Most microinvertebrates of our samples are not yet identified (Gastropoda, Annelida, microcrustacea), but significant new data on the distribution of several remarkable stygobionts have been obtained and are commented below.

Dendrocoelum regnardi, a large blind flatworm known from a few sites in S Quercy, was collected further north in several caves (Font d'Erbies, Lestang, Marut, Saint-Sauveur), but not retrieved in its type locality, the Saint-Géry cave.

Bythinella are abundant in many of the sampled caves. CO1 sequences of specimens from Font d'Erbie and Bouffio diverged significantly from *Bythinella padiraci* of Padirac, type locality of the species.

Sphaeromides raymondi. Described from southeastern Massif Central caves, this large isopod, was subsequently discovered in the Ressel sump in Quercy (NOTENBOOM et

al. 2006). It has been more recently collected from two other hydrogeological systems at Aujols and Crégols.

Caecosphaeroma burgundum rupisfucaldi, another isopod of large size, reaches its southeastern limit in Quercy, where it was collected from two sites of the Dordogne valley. We got it from a third site, the karstic resurgence of Lanzac in the Lot department.

Stenasellus virei virei. Described from the gouffre de Padirac, it was later found widespread in SW France and N Spain. Bou (2004) reports it from a few phreatic and hyporheic sites in S Quercy. Saint-Sol Igue where we retrieved it is the second karstic site for this species in the region.

Niphargus. This amphipod genus is frequent in Quercy caves. Distance tree of barcoded specimens shows five well separated clusters, as divergent as species. Frequent inconsistencies were observed between morphology and barcoding among our material, confirming a high morphological variability already stressed by Bou (1966). Further analyses are in course to fix this problem in order to get accurate specific identifications.

4. Conclusions

The presence of 7 narrow endemics species in a single small system of S Quercy (BOU, 2004) points to a high richness of the regional aquatic fauna. In contrast, subterranean terrestrial habitats were considered as poor by Bou (1966). Our study does not confirm this last observation. With 5 species new to science, 4 species of relictual or disjunct distribution, 21 endemic and 24 only known so far from subterranean habitats (Fig. 2), the subterranean fauna of Quercy appears clearly much richer than predicted, comparing even with some of the richest karsts of France. Whether it is the result of an intense sampling effort or the reflection of a true high species richness cannot be established at the moment.

The most diversified group in terrestrial subterranean habitats of Quercy, Collembola, were the object of extensive investigations, but several other groups like copepods remain understudied in most part of the region. They are in focus for the coming months. Unevenness in sampling effort is a second concern, with the aquatic fauna of central and northern Quercy clearly under documented. Sampling campaigns will be reactivated in 2021 to fill these gaps. At least, problems of species delimitation and taxonomic confusion affect to various degrees several genera like *Bythinella*, *Oritoniscus*, *Niphargus* or *Deuteraphorura*, and obviously call for a more extensive molecular exploration of cave populations, which would benefit to future biodiversity inventories well beyond Quercy.

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Taxa	N	Ns	Ecol	Habitat	Dist	Locations
Acari						
<i>Rhagidia paralleloseta</i> Zacharda, Grafitti & Piva, 2011	10	3	TB	WO	end	L'Oule/TL, ?Aujols, ?Gelée
Araneae						
<i>Porrhomma ransenhaueri</i>	9	6	TB	OH	wid	Aujols, Geniez, Pégourié ...
<i>Troglohyphantes solitarius</i>	6	1	TB	OH	end	(Presque/TL), Montbleu at Thémines
Chilopoda						
<i>Lithobius</i> sp.	8	8	TP?	HR, GU	?	L'Oule, Pégourié, Toulouse ...
Coleoptera						
<i>Duvalius cadurcus</i>	6	4	TB	OH	end	St Géry/TL, Jamblusse, Loygues
<i>Duvalius lespesi</i> Fairmaire, 1863	bib	bib	TB	OH	end	(Trois Cloches/TL) ...
<i>Trechus delhermi</i> Saulcy, 1880	1	1	TP	soil?	end?	(Robinet/TL), Pouline
Collembola						
<i>Deuteraphorura</i> spp.	1096	80	TP	OH, GU +	?	Aujols, Robinet, Pégourié ...
<i>Folsomia candida</i> Willem, 1902	370	53	TP	OH +	wid	Bret, Goudou, Pouline ...
<i>Heteromurus nitidus</i> (Templeton, 1935)	796	60	TP	OH, (HR) +	wid	Pégourié, Racine, Robinet ...
<i>Isotomiella barivierai</i> Deharveng, 1988	207	14	TB	WO	end	L'Oule/TL, Aujols, Besaces? ...
<i>Isotomiella</i> n.sp.	301	27	TP	SC	end	Cloup d'Aural, Lébratières at Concots, Mémerlin
<i>Lepidocyrtus</i> n.sp.	49	3	TB	GU	end	St Géry
<i>Micronychiurus</i> n.sp. 1	6	2	TB	WO	end	L'Oule
<i>Micronychiurus</i> n.sp. 2	98	7	TP	SC	end	Pouline, Trou Madame
<i>Mucrosomia garretti</i> (Bagnall, 1939)	168	2	TB*	OH	disj	Vitarelles at Gramat
<i>Neonaphorura hexaspina</i> Arbea & Mateos, 1991	28	5	TP	SC	wid	Devèze at Caniac, Palmès, Cour-Igüe
<i>Oncopodura pelissiei</i> Deharveng, 1988	50	13	TB	WO	end	L'Oule/TL, Aujols, Bret ...
<i>Proisotomodes exilis</i> (Gisin, 1960)	54	4	TB?	SC	wid	Mémerlin, Œil de la Dou, St Géry ...
<i>Pseudosinella balazuci</i> Gisin & Gama, 1969	182	43	TB	OH	end	St Géry/TL, L'Oule, Bret ...
<i>Pseudosinella dodecapthalma</i> Gisin & Gama, 1969	970	106	TB?	OH, GU +	end	Pégourié/TL, Aujols, St Géry ...
<i>Schaefferia emucronata</i> Absolon, 1900	13	9	TB*	OH	disj	Diane, Geniez, Palmès
<i>Schaefferia subcoeca</i> Deharveng & Thibaud, 1980	352	13	TB*	WO	disj	L'Oule, Pouline, Loygues at Puylagarde ...
Diplopoda						
<i>Brachychaeteuma caduriense</i> Mauriès, 1967	bib	bib	TB	OH	end	(Roc de Corn/TL), ?Merdalou, ?Réveillon ...
<i>Geoglomeris subterranea</i> Verhoeff, 1908	33	18	TB	OH	wid	Bret, Cloup d'Aural, Robinet ...
<i>Metaiulus pratensis boui</i> Mauriès, 1965	bib	bib	TB	OH	end	(Berthasses/TL)
<i>Metaiulus pratensis henroti</i> Demange, 1958	11	7	TB	OH	end	Robinet/TL, Brasconies, Vayssière ...
Diplura						
<i>Litocampa humilis</i> (Condé, 1948)	234	31	TP	OH +	wid	Pech Merle/TL, Confayrou, Diane ...
Isopoda						
<i>Oritoniscus magnei</i> Legrand, 1964	807	109	TB	OH +	end	Pégourié/TL, ?Aujols, ?Robinet ...
<i>Oritoniscus vandeli vandeli</i> Legrand, 1942	19	7	TP	OH +	end	Villeneuve-sur-Lot/TL, ?Aujols, ?Font d'Erbie ...
<i>Oritoniscus virei occidentalis</i> Vandel, 1947	145	12	TB	OH	end	St Géry/TL, Gourgue, Vipère ...
<i>Trichoniscoides albidus speluncarum</i>	5	3	TB	OH	end	Briandt at Chasteaux, Couze at Noailles
Opliones						
<i>Holoscotolemon querilhaci</i> (Lucas, 1866)	72	31	TP	OH	wid	Aujols, Marut, Robinet ...
Pseudoscorpiones						
<i>Neobisium cavernarum</i> Koch, 1873	1	1	TB	OH?	disj	Robinet
<i>Roncobisium</i> n.sp.	1	1	TB	OH	end	Diane

Figure 2. Remarkable terrestrial cave invertebrates of Quercy.

N (number of specimens collected), and Ns (number of samples): bib, from bibliography – Ecol (ecological category): TB, troglitic; TB*, troglitic in Quercy; TP, troglitic — Habitat (preferential habitat): see text for abbreviations); +, very frequent — Dist (distribution): end, endemic of Quercy and surroundings; wid, wider distribution — Locations: cave toponyms after Bou (1966) and Taisne (2006), otherwise associated with communes; TL, type locality; between brackets, species not retrieved in TL; otherwise, species collected in TL; ... , other sites not listed; ?, uncertainty on species identification in the site, particularly problematic for *Oritoniscus* spp.

Les araignées cavernicoles des "Catacombes de Paris" et des carrières souterraines franciliennes : mise à jour de l'inventaire

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Résumé

En Europe, les cavités souterraines ont un fort potentiel écologique, avec 486 espèces d'araignées cavernicoles inventoriées. La région Ile-de-France n'a que très peu de cavités naturelles, mais présente un grand nombre d'habitats souterrains artificiels, avec des milliers de carrières souterraines (e.g. des carrières de calcaire, craie, gypse). Les cavités anthropiques sont traversées par des micro-karsts, où les araignées cavernicoles peuvent être observées. Depuis 2016, nous avons mené un inventaire des invertébrés que l'on trouve sous Paris. L'objectif de cet article est de présenter un bilan des araignées troglodiles et troglobies des catacombes de Paris et carrières de banlieue. Au total, 16 taxons ont été identifiés, dont deux espèces troglobies du genre *Leptoneta* (*L. olivacea*, *L. abeillei*), endémiques des grottes du sud de la France. *Leptoneta olivacea* a été collectée pour la première fois en région parisienne par J. Sigwalt en 1947, dans les catacombes de Chaillot. Une des hypothèses d'introduction de l'espèce était l'apport de matériels décoratifs provenant des grottes, pour l'exposition universelle de 1900. Dans notre inventaire, nous avons trouvé *L. olivacea* dans plusieurs autres carrières isolées. La découverte de l'espèce *L. abeillei* dans les souterrains de Sèvres (Hauts-de-Seine) est une nouvelle occurrence pour l'Ile-de-France. Ces résultats montrent que le genre *Leptoneta* pourrait être plus largement distribué que ce que l'on pensait.

Abstract

Cave-dwelling Spiders from 'The Catacombs of Paris' and suburb quarries: Update of the fauna inventory. In Europe, underground spaces demonstrate a strong ecological potential with 486 cave-dwelling spiders species. Ile-de-France region has few natural caves, but comprises a large number of underground habitats, with thousands of artificial cavities (e.g. limestone, chalk, gypsum quarries). Anthropogenic cavities cross micro-karsts, where cave-dwellers can be found. Since 2016, a survey of invertebrates under Paris has been carried out. The main objective of this work is to present an overview of the troglodile and troglobiont spiders of the 'Catacombs of Paris' and suburb quarries. Altogether 16 taxa were identified, including two troglobiont species of the genus *Leptoneta* (*L. olivacea*, *L. abeillei*) endemic from caves of southern France. *Leptoneta olivacea* was first collected in Paris area by J. Sigwalt in 1947 in the catacombes de Chaillot. One hypothesis of its introduction was the material from caves brought for the 1900 Universal Exposition. In our inventory we found *L. olivacea* in four other separate quarries of Paris. The discovery of the species *L. abeillei* in Sèvres underground (Hauts-de-Seine), is a new occurrence for Ile-de-France. These results highlight that the genus *Leptoneta* could be more widely distributed than previously thought.

1. Introduction

Les premières données connues concernant les arachnides présents dans les carrières souterraines franciliennes ont été collectées en 1896 par Armand Viré et étudiées par E. Simon (VIRÉ, 1896). Au cours du 20^{ème} siècle, plusieurs publications évoquent des araignées identifiées dans les catacombes de Paris (BALAZUC et al, 1951, DRESCO, 1983, 1987) mais certaines carrières souterraines demeurent inexplorées par ces auteurs, et d'autres qu'ils ont

prospectées ne sont plus accessibles. L'objectif de notre campagne de prospection est de compléter les connaissances de la biodiversité des aranéides souterraines de cette région. Établir des inventaires à des périodes espacées de plusieurs décennies permet de faire un point sur l'établissement de nouvelles espèces introduites, leur dispersion dans le milieu ou éventuellement la disparition d'autres espèces.

2. Matériels et Méthodes

Les caractères anatomiques utilisés pour identifier une espèce d'araignée sont le plus souvent la morphologie des organes génitaux : la forme de l'épigyne des femelles et des bulbes copulateurs des mâles qui ont une structure complexe et très spécifique. Les collectes ont été pratiquées

sur le terrain, à vue, fixées dans de l'éthanol à 75% et étudiées au laboratoire du Muséum d'Histoire Naturelle de Paris par Christophe Hervé. Les échantillons sont ensuite conservés en collection, étiquetés avec leur lieu de provenance et date de récolte.

3. Résultats : Lieux d'observation

Toutes les observations sont réalisées en carrières souterraines. La nature des roches exploitées est précisée sur la figure 1.

Famille Amaurobiidae

Amaurobius ferox (Walckenaer, 1830)

Paris : Carrière du **Grand réseau sud** (GRS), (29/10/2016)

Hauts-de-Seine : Carrière de Bagneux (01/01/2016)

Val-de-Marne : Carrière du GRS, Arcueil (01/01/2016), carrière des Géants, Arcueil (08/09/2018)

Famille Nesticidae

Kryptonesticus eremita (Simon, 1880)

Paris : Carrière du GRS, (31/12/2015, 29/10/2016, 01/06/2017, 01/06/2018), carrière de Place d'Italie, (01/05/2017, 07/09/2018)

Hauts-de-Seine : Carrière de Brimborion, Meudon (01/09/2017), carrière de Bagneux (24/03/2018), Caves du roi, Sèvres (09/09/2018)

Val-de-Marne : Carrière des géants, Arcueil (01/08/2018), carrière de Gravelle, Charenton-le-Pont (15/04/2018).

Val-d'Oise : Carrières de L'Isle-Adam (07/04/2018)

Nesticus cellulanus (Clerck, 1757)

Paris : Carrière du GRS, (01/01/2016)

Seine-et-Marne : Carrière des Gondonnieres, Larchant (19/08/2018)

Hauts-de-Seine : Carrière de Brimborion, Meudon (01/09/2017)

Val-d'Oise : Carrière de L'Isle-Adam (07/04/2018)

Famille Linyphiidae

Leptyphantes leprosus (Ohlert, 1865)

Val-d'Oise : Carrière de L'Isle-Adam (07/04/2018)

Lessertia dentichelis (Simon, 1884)

Paris : Carrière du GRS sous le parc Montsouris, (23/09/2018)

Palliduphantes pallidus (O. P. Cambridge, 1871)

Hauts-de-Seine : Carrière d'Arnaudet, Meudon (12/12/2018), carrière Brimborion, Meudon (01/09/2017)

Palliduphantes sp. :

Paris : Carrière du GRS, (29/08/2018)

Yvelines : La pissote, St-Germain-en-Laye (08/12/2019)

Famille Leptonetidae

Leptoneta abeillei (Simon, 1882)

Hauts-de-Seine : carrière de la cité, Sèvres (06/12/2018, 31/07/2020)

Distribution endémique connue :

Gard : Tharoux : grotte du Cimetière, Saint-Privat-de-Champclos : grotte du Serre-de-Barri, Méjannes-le-Clap : grotte de la Salamandre, baume des Italiens. Saint-Jean de Maruéjols : Grotte inférieure d'Avejan, Montclus : grottes du Prével et de la Bruge ou le Soulier (JEANNEL 1926).

Ardèche : Vallon-Pont-d'Arc : grotte du château d'Ebbou, grotte Nouvelle, grotte de Saint-Martin, Saint Alban : grotte de Beaumefort, Grospierres : grotte de Voidon, Banne :

grotte de Banne, grotte du Saut-du-bœuf, grotte de l'assiette, grotte du cuivre (bois de Païolive), Labeaume : grotte du soldat, Rosières : grotte de Remène. (FAGE 1913, JEANNEL 1926).

Leptoneta olivacea (Simon, 1882)

Paris : Carrière de la Brasserie, (19/04/2018), carrière du GRS : 14^{ème} (01/05/2017, 21/06/2018, 16/06/2018, 17/10/2020, 01/09/2017), 15^{ème} (08/08/2020), Carrière de la place d'Italie : 13^{ème} (01/03/2017, 07/09/2018, 12/11/2020), Carrière de Chaillot : 16^{ème} (10/09/2017, 03/12/2020)

Hauts-de-Seine : Carrière de la cité, Sèvres (06/12/2018, 31/07/2020)

Distributions endémiques connues :

Var : Hyères : grotte des fées, Broussan : grotte du Saint-Trou, Orvès : Saint-trou (FAGE 1913 & 1931, SIMON 1914, DENIS 1933, DRESCO 1987)

Bouches-du-Rhône : Allauch : Baume Sourne, Grotte des Escaouprès (LUCZAK et VEDOVINI 1964).

Et premières observations en Ile-de-France :

Carrières de Chaillot à Paris (BALAZUC 1951, 1962, DRESCO 1983).

Famille Tetragnathidae

Meta menardi (Latreille, 1804)

Paris : Carrière de la place d'Italie, (07/09/2018)

Yvelines : Carrière des lions, Louveciennes (30/05/2020), Carrière Dédé, Bougival (06/11/2020)

Meta bourneti (Simon, 1922)

Yvelines : Carrière de Pinton, Le Pecq (16/05/2020)

Metellina merianae (Scopoli, 1763)

Seine-et-Marne : Carrière des Gondonnieres, Larchant (19/08/2018)

Val d'Oise : Carrière de L'Isle-Adam (07/04/2018)

Famille Pholcidae

Pholcus opilionoides (Schrank, 1781)

Yvelines : Cave du roi, Sèvres (09/09/2018)

Pholcus phalangoides (Fuesslin, 1775)

Paris : carrière du GRS, (01/10/2016)

Val-de-Marne : carrière du GRS, Arcueil (01/01/2016)

Psilochorus simoni (Berland, 1911)

Paris : Carrière de la place d'Italie (07/09/2018), carrière du GRS (29/10/2016), carrière du Jardin des plantes (01/01/2015, 01/11/2016).

Hauts-de-Seine : Cave du roi, Sèvres (09/09/2018)

Famille Theridiidae

Steatoda grossa (C. L. Koch, 1838)

Paris : carrière du Val-de-Grâce, (11/11/2016), carrière de la place d'Italie (07/09/2018)

Famille Zoropsidae

Zoropsis spinimana (Dufour, 1820)

Val-de-Marne : Carrière du GRS, Arcueil (janvier 2016)

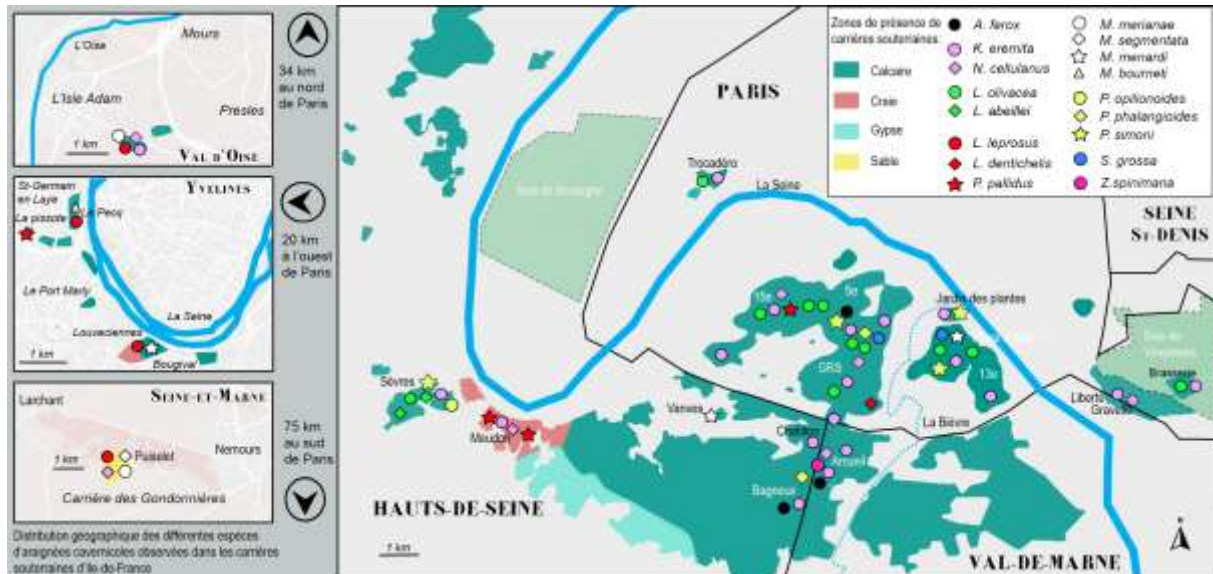


Figure 1 : Carte du contexte de l'étude présentant les observations

4. Discussion

Parmi les taxons observés, il y a une majorité d'espèces courantes des milieux anthropisés, à très large distribution : **A. ferox**, **P. opilionoides**, **L. leprosus**, et à distribution moins large : **Z. spinimana**. Parmi les espèces cosmopolites, certains taxons exotiques ayant trouvé des conditions plus favorables dans le milieu souterrain sont observés : **P. simoni**, **S. grossa**, et **P. phalangioides**.

P. simoni semble avoir élargi son aire de répartition depuis les campagnes d'observation de Balazuc (BALAZUC et al. 1951).

L'observation en carrières souterraines d'espèces très répandues dans les grottes en France confirme les similitudes entre ces biotopes (exemple : **K. eremita**, **M. menardi**, **M. bourneti**, **M. merianae**, **N. cellulanus**, **P. pallidus**, **L. dentichelis**). La plupart des araignées avaient déjà été inventoriées par Balazuc en 1951, bien que les carrières visitées et stations soient différentes. Un nouveau taxon pour la région a été observé (**L. abeillei**) et deux nouveaux taxons pour les carrières souterraines de la région (**P. opilionoides** et **Z. spinimana**). **Z. spinimana** était déjà connu en surface à Paris. **K. eremita**, n'a pas été noté par Balazuc mais a été observé dans les carrières de Paris en 2002 et 2003 (données de l'Inventaire national des araignées de France métropolitaine, Claire Jacquet). Cette espèce troglophile méridionale a certainement été introduite également ces dernières décennies. Certaines des espèces troglophiles recensées par Balazuc n'ont pas été retrouvées (e.g. : **P. egeria**, **P. rosenhaueri**, **C. cicur**, **T. silvestris**) (BALAZUC et al. 1951). Notons que les espèces présentes proches des accès extérieurs boisés ne sont pas les mêmes que vers les espaces urbanisés. Par exemple : **Meta sp.**, **M. merianae**, **L. leprosus**, **L. dentichelis** sont absentes quand il n'y a pas de bois à proximité, et sont remplacées par **A. ferox** et **P. phalangioides**. Dans la mesure où la surface est une des sources de colonisation des milieux souterrains, l'évolution de son paysage impacte les faunes cavernicoles. Par conséquent, l'augmentation de la pression

anthropique en surface peut modifier les distributions d'espèces en souterrain.

Certaines espèces ne se trouvent jamais ensemble dans les carrières prospectées (e.g. : **P. simoni** n'est jamais avec **L. olivacea**). Ces observations induisent de nouveaux objectifs : définir les niches écologiques de chaque espèce et les co-distributions de celles-ci.

Des occurrences inédites pour le Val-de-Marne et de nouvelles stations pour Paris de **L. olivacea** ont été observées.

L. olivacea a été observée pour la première fois en Ile-de-France dans la carrière de Chaillot en 1947 par J. Sigwalt. L'équipe de Balazuc est retournée la récolter en 1951 pour s'assurer qu'elle ne résultait pas d'une erreur d'étiquetage. Ils l'ont récoltée dans la galerie sous l'avenue de Malakoff uniquement. Balazuc évoquait que cette espèce ait pu être introduite accidentellement dans les carrières de Chaillot par l'aménagement de l'exposition universelle de 1900 (BALAZUC et al., 1951, DRESCO, 1983). En effet des matériaux (stalactites, stalagmites, wagonnets de mines, boisages, détritiques...) ont été rapportés en 1900 des grottes du sud-est de la France pour constituer la décoration de l'exposition. Ces matériaux auraient pu protéger les cavernicoles, de la lumière et de la dessiccation, en attendant d'être dans de nouveaux souterrains. Dans nos prospections nous avons observé des populations bien installées de **L. olivacea**, encore cette année 2020, dans différentes stations de la carrière de Chaillot (à Z', la Hague Bar, vers les restes de moulages en plâtre, et vers la 'mine de charbon' de l'exposition universelle, en plus de l'avenue de Malakoff). De plus, nous avons observé l'espèce dans des carrières indépendantes de celle de Chaillot (e.g. La carrière de la brasserie, Carrière de Place d'Italie, carrières du GRS de Paris, carrières de Sèvres). Ces différentes populations sont séparées par des barrières infranchissables par le sous-sol : des cours d'eau comme la Seine (discontinuité des couches géologiques) ou la Bièvre (collecteur souterrain

bétonné). Ces observations invalident l'hypothèse qu'elles aient colonisé ces lieux par voie souterraine exclusivement. Balazuc précise que *L. olivacea* n'a été vu dans aucune autre des localités que les carrières de Chaillot pendant plusieurs années de recherche. Cette constatation suggère que leur dispersion pourrait être postérieure à 1951. A-t-elle vraiment été réintroduite dans chacune de ces carrières par des hommes y progressant (ouvriers, clandestins...) ou était-elle déjà ici avant, mais plus discrète ? Si les ouvriers se font moins nombreux sous terre depuis le 20^{ème} siècle, en revanche la population clandestine y est multipliée. Cette fréquentation humaine apporte de la matière organique attirant la vie (déchets de repas, boisage et outils de travaux)

5. Conclusion

Ce travail préliminaire permet de faire le point sur les espèces présentes dans le milieu souterrain de la région. 16 taxons ont été observés, dont 2 nouvelles pour les carrières souterraines franciliennes (*P. opilionoides* et *Z. spinimana*) et une nouvelle pour la région (*L. abeillei*).

Plusieurs hypothèses sont proposées pour expliquer l'introduction et la dispersion des espèces de *Leptoneta*, mais aucune n'est à ce jour confirmée.

La mise au point d'un travail de barcoding afin d'estimer les divergences génétiques potentielles entre populations permettra peut-être d'apporter de nouveaux éléments de réponse. Un des futurs enjeux sera d'établir une chronologie des peuplements à partir des groupes d'haplotypes et d'évaluer le temps écoulé depuis leur introduction.

La notion de troglobie inféodé exclusivement au monde souterrain et la classification des organismes cavernicoles est questionnée. En effet, l'exemple des *L. olivacea*

et implique le déplacement éventuel des matériaux d'une carrière à une autre. Dans notre étude, elles sont toujours observées aux abords de matériaux boisés en décomposition, où se trouvent d'autres espèces qui représentent leur proie.

La mise en place d'un élevage de *L. olivacea* réalisé en surface à la lumière, nous a permis de constater la survie des araignées quatre semaines avant d'être relâchées, en supportant les températures estivales (entre 12,3°C et 39,1°C). Nos observations suggèrent que les *L. olivacea* supportent, au moins temporairement, des conditions écologiques de surface.

démontre que ces araignées doivent être capables de se déplacer à l'extérieur pour coloniser d'autres souterrains. Bien que cette espèce n'est pas été observé en surface, d'autres micro-habitats (litières, éboulis, sous-bois) pourraient potentiellement lui convenir. La catégorie de cette espèce serait à re-caractériser en troglophile ou endogée, comme d'autres espèces de ce genre. D'autre part il faut garder à l'esprit que la répartition des espèces dans des milieux peu étudiés était encore lacunaire dans les années 1950, mais l'est toujours en 2020. Nous allons encore essayer d'élargir l'inventaire aux carrières encore non prospectées, notamment pour essayer de retrouver les espèces troglophiles trouvées par Balazuc. Nous allons également établir un plan d'échantillonnage et des mesures physiques pour décrire plus précisément le biotope de chaque espèce.

Remerciements

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Bioprospecting and genome mining of culturable bacteria from a quartzite cave

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Abstract

Oligotrophic, aphotic, subterranean environments represent promising habitats for microbiology studies including the discovery of novel microbial metabolic pathways and bioactive molecules. In this work, eighty bacterial strains were isolated from siliceous speleothems collected from the pristine and recently explored orthoquartzite Imawari Yeuta cave in the Venezuelan tepuis. These isolates were further analyzed for the capacity to solubilize silicates and to produce antimicrobial molecules. Twenty-five isolates were able to generate silica solubilization halos on solid minimal growth medium plates supplied with magnesium trisilicate, whereas four bacterial isolates inhibited the growth of several human pathogens in soft-agar assays. Whole genome sequencing of these four bacterial isolates was performed to get insight into their taxonomy definition (using a phylogenomics approach) and their genomic traits associated with oligotrophic growth and antimicrobials production. These isolates belonged to the genera *Paraburkholderia* and *Sphingomonas*, the latter strain representing a novel species. Phenotype Microarray analyses were also performed to assess their metabolic activities in the presence of different carbon sources and stressors (*i.e.*, metals, detergents, antibiotics). These results open new insights into the investigation of quartzite pristine caves as a source of novel metabolic activities and microorganisms able to produce bioactive molecules.

1. Introduction

The terrestrial subsurface microbiome has gained a considerable amount of interest in recent years as it represents a rich potential resource for biomining novel genes and enzymes with biotechnological interest (GHOSH *et al.* 2017). On the contrary to the microbial ecology in cave ecosystems that has attracted extensive research interest, information on the metabolic potentials of cave microorganisms is still limited. Specific cultivation approaches are needed for the isolation of microorganisms with interesting metabolic activities so that they can be further investigated in pure culture experiments to assess a more detailed understanding of bacterial metabolism and physiology. On the other hand, microorganisms' and microbiota's metabolic potentials can be predicted using

information derived from microbial (meta)genomics through bioinformatic tools that allow genome-wide detection and annotation of secondary metabolite biosynthetic gene clusters (BGCs) (BLIN *et al.* 2019).

This work provides a preliminary overview on the isolation and characterization of eighty bacterial strains from siliceous speleothems and biofilms collected from the recently explored orthoquartzite Imawari Yeuta cave in the Venezuelan tepuis (SAURO *et al.* 2018; GHEZZI *et al.* 2021). Genome-wide bioinformatic analyses and Phenotype Microarray assay were performed for the most interesting isolates to get insight into their taxonomy and specific metabolic activities (*i.e.*, carbon source utilization, stress tolerance, antimicrobials production).

2. Materials and Method

2.1 Isolation and molecular identification

Isolation of culturable microorganisms was performed on 18 samples collected from different mats and biofilms in Imawari Yeuta cave (Fig. 1). For strain isolation, ISP2 and diluted R₂A media were used as well as heat and/or

antibiotic treatments to eliminate fast-growing bacteria. Plates were incubated for two weeks at 30°C. The isolated strains were then screened for the capacity to produce amylase, cellulase, lipase, proteinase and urease and to solubilize silica by using starch and CMC agar, skim milk agar, tween 80 agar or by adding magnesium trisilicate to ISP2 and

SIM agar plates. Antimicrobial compound production was evaluated through top agar overlay assay. The taxonomy of the isolates was defined by amplifying and sequencing the 16S and ITS rRNA gene. Colony PCR was performed using bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTACGACTT-3') and fungal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). After purification, the resulting PCR products were Sanger sequenced at BMR Genomics (Padova). Sequence alignments were performed with ClustalW. The neighbor-joining method with 1000 bootstrap in the MEGA X was used for phylogenetic tree construction.

2.2 Phenotype microarray

The metabolic profiles of four bacterial isolates were investigated by using GEN III MicroPlates™ (Biolog, Inc., Hayward, CA), including 71 carbon sources and 23 antimicrobial chemicals. Biomass of *Paraburkholderia* and

Sphingomonas strains were inoculated in a solution composed of IF-0 and DyeA (Biolog, Inc.) at a cell density of T=85% and T=95%, respectively. Inoculated GENIII plates were incubated for 72 h at 37 °C in an Omnilog automated incubator/reader. Data were analysed with Omnilog software and analyzed with the R package OPM.

2.3 Whole genome sequencing, phylogenomic analysis and functional annotation

Approximately 7 million of paired end (PE) reads (2 × 150bp) per strain were generated through Illumina sequencer NextSeq. After sequencing adapter removal and quality trimming, high quality PE reads were assembled using SPAdes v3.14.4. The RASTtk pipeline and eggno3 were used for the prediction of open reading frames and functional annotation, respectively. AntiSMASH v5.0 was used to identify biosynthetic gene clusters (BLIN *et al.* 2019). Phylogenomic analysis was performed through the Type (Strain) Genome Server (TYGS).

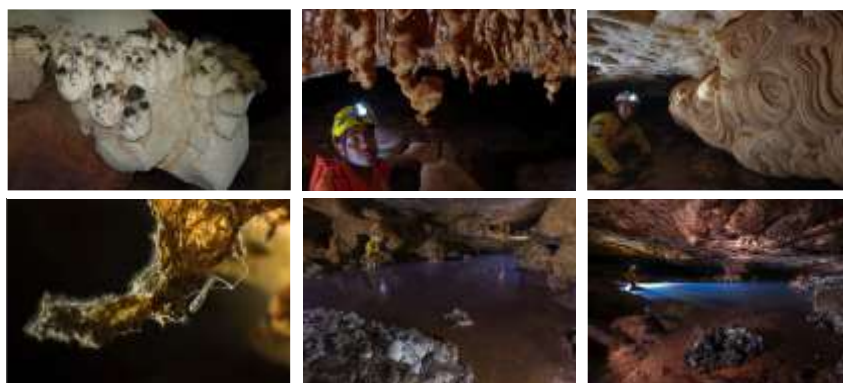


Figure 1: Examples of speleothems, biofilm and microbial mats collected from Imawari Yeuta cave in 2014 and 2016 (Photo by V. Crobu, R. de Luca and A. Romeo – La Venta Esplorazioni Geografiche).

3. Results and Discussion

3.1 Metabolic screening of eighty microbial strains from Imawari Yeuta cave

Eighty microbial strains were isolated from the 18 Imawari Yeuta cave samples and were investigated for specific metabolic activities. In particular, they were screened for enzymes amylase, cellulase, protease, lipase and urease activities, and for the capacity to produce antimicrobials and to solubilize silica (Fig. 2, Fig. 3). As a result, 39 % (31/79) of the isolates were positive to the cellulase screening test, and 42 % of isolates showed lipase and urease activities. Notably, urease activity is well known to be associated with biomineralization processes (REEKSTING *et al.* 2018; OMOREGIE *et al.*, 2020). Twenty-two strains produced extracellular proteases while 16.5 % (13/79) of the isolates showed amylase activity. Additionally, 32 % of the library isolates (25/79) were able to perform silica solubilization on SIM agar plates. Four bacterial isolates were found to be antimicrobial producers as they were able to inhibit the growth (visible as a zone of growth clearance around the tested cave isolate in the soft-agar assay) of model

pathogens (*i.e.*, *Escherichia coli* NCTC12923, *Staphylococcus aureus* ATCC29213, *Pseudomonas aeruginosa* PAO1 and *Klebsiella pneumoniae* ATCC 700603) (Fig. 2). Some of these activities were previously related to possible metabolic strategies to support the growth under acidic pH (*e.g.*, urease activity) and under nutrient-limited conditions (*e.g.*, protease activity) (BARTON *et al.* 2014).



Figure 2: Representative plates showing microbial strains able to solubilize silica (left), to produce extracellular proteinase (middle), and to produce antimicrobials (right).

Among the silica solubilizing microorganisms, we identified strains belonging to bacterial genera *Pseudomonas*, *Serratia*, *Burkholderia*, and *Paraburkholderia*, and to fungal

genera *Penicillium* and *Talaromyces*. Strains active against model pathogens belonged to the genera *Paraburkholderia*, *Sphingomonas* and *Penicillium*.

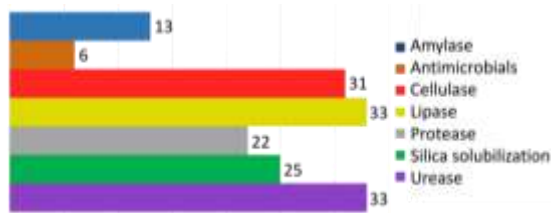


Figure 3: Number of microbial strains able to solubilize silica, to produce antimicrobial molecules and/or enzymatic activities.

3.2 Whole genome sequencing and phylogenomic analyses

High quality Illumina sequencing reads were assembled to obtain the draft genomes of *Paraburkholderia* (*P.*) *bryophila* strains CA002, CA010 and CA067 and *Sphingomonas* (*S.*) *sp.* strain QA11 (Fig. 4).

	CA002	CA010	CA067	QA11
Size (Mbp)	8.0	8.3	8.3	5.6
GC content (%)	63.0	62.9	62.9	64.8
Contigs	88	85	49	42
Protein coding genes	7580	7891	7819	5255

Figure 4: Table indicating assembly and annotation statistics in *Paraburkholderia* *sp.* strains CA002, CA010, CA067, and *Sphingomonas* *sp.* strain QA11

According to the digital DNA-DNA hybridization (dDDH) values, CA002, CA010 and CA067 strains belong to the species *P. bryophila*, sharing dDDH similarity values with *P. bryophila* LMG 26344 of 77.1 – 79.8 %, which is higher than the intra-species cut-off (70 %). On the other hand, none of the *Sphingomonas* ‘type strain’ showed a significant phylogenetic relatedness with *Sphingomonas* *sp.* strain QA11, suggesting that this strain belongs to a new *Sphingomonas* species.

3.3 Metabolic potentials of *P. bryophila* and *Sphingomonas* strains

The metabolic potentials of *P. bryophila* and *Sphingomonas* strains were dissected by identifying genetic traits involved in the adaptation to oligotrophic environments and synthesis of antimicrobials. Consistent with their isolation source, genes linked to oligotrophic lifestyle were identified in *P. bryophila* strains CA002, CA010 and CA067. All *P. bryophila* strains carried a broad array of high-affinity ATP-binding cassette (ABC)-type transporters involved in the scavenging of amino acids and peptides (di- and oligo-), uptake of mono- and oligosaccharides, and assimilation of inorganic and organic phosphate forms such as phosphonate and 2-aminoethylphosphonate (data not shown). *Sphingomonas* *sp.* strain QA11A genome possessed

high-affinity ABC-type phosphate transporter genes (*pst*) and phosphotransferase system (PTS) for the uptake of simple sugars such as glucose and fructose (data not shown). Additionally, bacterial competition strategies (*i.e.*, scramble competition and contest competition) might represent crucial aspects of microbial life under nutrient-limited conditions (HIBBING *et al.*, 2010). Scramble competitions describe the capability of bacteria to use limited nutrients without antagonistic interactions against competitors. Contest competition involves direct and antagonistic interactions between bacteria. In this context, *Sphingomonas* and *Paraburkholderia* strains genomes were mined for the presence of biosynthetic gene clusters involved in the biosynthesis of secondary metabolites with antimicrobial activity.

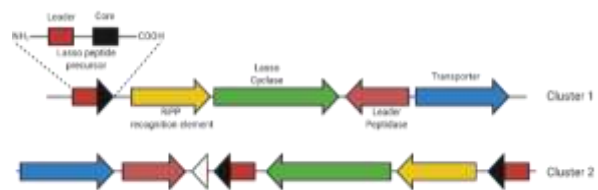


Figure 5: Lasso peptides biosynthetic gene clusters in *Sphingomonas* *sp.* strain QA11. Hypothetical protein coding genes in white.

Our analysis showed that *Sphingomonas* *sp.* strain QA11 carries two gene clusters involved in the synthesis of lasso peptides (LP) (Fig. 5), a class of ribosomally-synthesized and post-translationally-modified peptides, which typically possess antibacterial activity against a wide range of bacterial pathogens and exhibit high stability under extreme conditions of temperature and pH (CHENG & HUA, 2020). While the exact mechanism of action of most LPs remains unknown, the most important enzyme inhibitory activity seems to be the inhibition of the RNA polymerase in Gram-bacteria (CHENG & HUA, 2020). The presence of LP biosynthetic clusters and the limited number of high-affinity ABC transporters suggest that QA11 might have evolved a contest competition strategy to survive in a nutrient-limited environment such as Imawari Yeuta cave.

3.4 Phenotype Microarray analysis of the cave isolates producing antimicrobials

Phenotype microarrays analysis assessed the capacity of *Sphingomonas* and *P. bryophila* strains to utilize different carbon sources and to resist different antibiotics. In respect to carbon sources, CA002, CA010 and CA067 strains outperformed QA11 (Fig. 6). In particular, the *P. bryophila* strains showed higher metabolic activity towards sugars and amino acids, while *Sphingomonas* *sp.* strain QA11 showed greater tolerance against antibiotics (Fig. 6). These capacities are partially in line with the metabolic predictions based on genomic data, *i.e.*, the higher amount of ABC transporter involved in the uptake of amino acids and sugars in *P. bryophila* strains that might confer growth advantage in the presence of a limited amount of organic carbon (NOELL & GIOVANNONI, 2019).

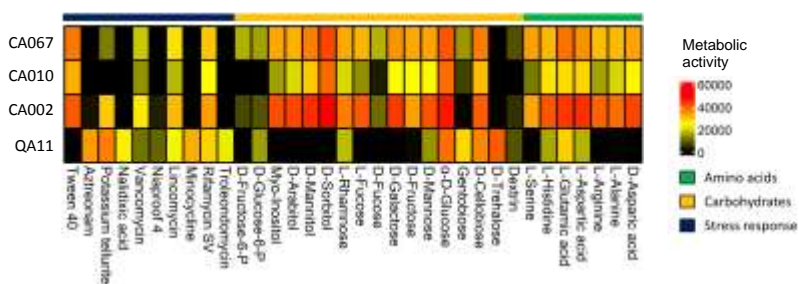


Figure 6: Metabolic activity profiles of the *P. bryophila* and *Spingomonas* sp. strains (indicated on the left) in the presence of amino acids, carbohydrates and stressors.

4. Conclusion

This is the first work describing microbial isolates from the pristine and unique (*i.e.*, isolated and nutrient-limited) Imawari Yeuta orthoquartzite cave. Around eighty isolates were obtained which possessed different enzyme activities (*i.e.*, amylase, cellulase, protease, lipase and urease) that might favor the growth and microbial cooperation in Imawari Yeuta cave. We also detected four bacterial strains able to produce antimicrobials. Due to the broad range of inhibitory activity against Gram+ and Gram- human pathogens, the chemical nature of these antimicrobial

molecules is presently under study for possible biotechnological applications. By whole genome sequencing and phylogenomics, we identified these strains as three *Paraburkholderia bryophila* strains and one *Spingomonas* strain belonging to a novel species. Genome-wide mining and Phenotype Microarray analysis showed that *P. bryophila* and *Spingomonas* strains possess genetic features that might be associated with the adaptation to nutrient-limited conditions (*e.g.*, numerous ABC transporters) and to the production of antimicrobial molecules.

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Distribution, ecology, and diversity of the genus *Gallasellus* (Isopoda, Asellidae)

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Abstract

Until the nineties, *Gallasellus* (Isopoda, Asellidae) was known from a single species, *G. heilyi*, which was known only from the type-locality, the subterranean River of Bataillé (Gournay-Loizé, Deux-Sèvres, France). In 1992, *Gallasellus* was collected in a well in Oléron Island (Saint-Denis-d'Oléron, Charente-Maritime) at a distance of 100 km from the type-locality, indicating that the genus was more widely distributed. Here, we synthesize research conducted on *Gallasellus* since the 2010s and provide new findings on the systematics, distribution and ecology of that genus. *Gallasellus* is phylogenetically closely related to the North American genus *Caecidotea* and may contain at least 6 putative species. Its range spans over a maximum linear extent of nearly 200 km in limestone aquifers of the Poitou-Charentes Region. *Gallasellus* is a primary consumer feeding on organic detritus and microbial biofilm; it is preyed upon by *Niphargus ladmiraleti* (Amphipoda). The finding that *Gallasellus* is a potentially species-rich genus containing a mix of wide- and narrow-range species requires adapting conservation strategies.

Résumé

Distribution, ecologie et diversité de genre *Gallasellus* (Isopoda, Asellidae). Jusque dans les années 90, le genre *Gallasellus* (Isopoda, Asellidae) contenait une seule espèce, *G. heilyi*, connue d'une seule localité, la rivière souterraine de Bataillé (Gournay-Loizé, Deux-Sèvres, France). En 1992, *Gallasellus* fut collecté dans un puits de l'île d'Oléron (Saint-Denis-d'Oléron, Charente-Maritime) à une distance de 100 km de la localité type, indiquant que le genre était plus largement distribué. Dans les années 2010, un large programme d'échantillonnage a été mené à l'échelle de la région Poitou-Charentes et nous résumons ici les nouvelles connaissances acquises sur la systématique, la distribution, et l'écologie du genre.

1. Introduction

The term *Gallasellus* means literally Asellid of Gaul (i.e., “of France”). However, from the description of its type species, as *Asellus heilyi*, by LEGRAND (1956), to the description of the genus by HENRY & MAGNIEZ (1977), the genus *Gallasellus* was found to share many morphological similarities with the North American genus *Caecidotea*. Particularly, the endopod tip of male reproductive pleopod II has three processes (cannula, mesial and caudal processes) that are typically observed in the genus *Caecidotea* (WILLIAMS, 1970).

Until the nineties, *Gallasellus* was known from a single species, *G. heilyi*, which was known only from the type-locality, the subterranean River of Bataillé (Gournay-Loizé, Deux-Sèvres) (Fig. 1). In 1992, *Gallasellus* was collected in a well in Oléron Island (Saint-Denis-d'Oléron, Charente-Maritime) at a distance of 100 km from the type-locality, indicating that the genus was more widely distributed (MAGNIEZ & HENRY, 2001). Since then, some more specimens were collected in 2008-2009, and the first phylogenetic tree of Aselloidea showed a sister relationship between *Gallasellus* and *Caecidotea* (MORVAN *et al.*, 2013).

Date	Event	References
1955	Description of <i>Asellus heilyi</i> as a single-site species	Legrand (1956)
1970	Re-description as <i>Gallasellus heilyi</i> , morphological affinity with North American asellids, ecology and biology	Henry & Magniez (1970, 1977)
1992	Discovery of a new distant locality, distribution range extended to 100 km	Magniez & Henry (2001)
2010	Molecular systematics of Aselloidea	Morvan <i>et al.</i> (2013)
2013	Extensive sampling: distribution, ecology and biology	Lefebvre <i>et al.</i> (2016), Ercoli <i>et al.</i> (2019)
2013	Molecular diversity within <i>Gallasellus</i>	Morvan <i>et al.</i> (2013), Eme <i>et al.</i> (2018), this study

Figure 1: History of the genus *Gallasellus*.

From 2013 to 2015, an extensive collaborative sampling survey was carried out in the Poitou-Charentes Region. Here, we present the main findings of that survey and

summarize knowledge on the biology, distribution, ecology and diversity of the genus *Gallasellus*.

2. Materials and methods

Biology and ecology

Data on sex ratio and body size sexual dimorphism were obtained from morphometric measurements of specimens collected during extensive sampling by LEFEBVRE *et al.* (2016). Knowledge on the biology and behavior of *Gallasellus* stems mainly from observations made by HENRY & MAGNIEZ (1977) during rearing of specimens from the type-locality. Knowledge on its trophic ecology is from stable isotope analyses conducted by ERCOLI *et al.* (2019). These authors used mixing models to estimate the proportions of available food resources exploited by *G. heilyi*.

To characterize habitat preferences of *Gallasellus*, we calculated its frequency of occurrence (number of positive sites / number of sampled sites) in consolidated rocks (mostly limestone, n=118 sampled sites) and unconsolidated rocks (mostly alluvial deposits, n=21). For occurrence in consolidated rocks, we distinguished between three different types of sites: subterranean river caves (n=20), springs (n=47) and wells (n=51).

Distribution

Mapping of the distribution of *Gallasellus* is based on sampling of 139 sites in the Poitou-Charentes Region. The protocol was described in LEFEBVRE *et al.* (2016). Samples were taken from caves, springs, wells and the hyporheic

zone of streams. Samples were sorted under a stereomicroscope and specimens of *Gallasellus* were identified morphologically. Specimens were preserved in 96 % alcohol and molecular analyses were performed to delimit putative species (see below).

Diversity within Gallasellus

We used the COI gene-based threshold method defined by LEFÉBURE *et al.* (2006) to delimit putative species within *Gallasellus*. This species molecular delimitation method is based on the observation made from 1500 COI sequences belonging to 276 species of crustaceans that two clades diverging by more than 0.16 substitution per site, as measured by patristic distances, have a strong probability (ca 0.99 %) of belonging to different species. Putative species delimited with that molecular method are called molecular operational taxonomic units (MOTU).

Methods for extracting DNA, amplifying the mitochondrial cytochrome oxidase subunit I (COI) gene, building the COI phylogeny and delimiting MOTUs were provided by MORVAN *et al.* (2013). In the present study, the delimitation of MOTUs was performed on a data set containing a total of 2094 COI sequences of Aselloidea, including 53 sequences (specimens) of *Gallasellus* from a total of 28 sites.

3. Results

Biology and ecology

Mean body size in *Gallasellus* is 3.73 ± 0.68 mm (range: 2.16–5.65 mm, n=37 specimens) (Fig. 2). Growth is relatively fast: specimens reach a body size of 2.3 mm two months after hatching. Moulting lasts 24 hours. Females are significantly more numerous (sex ratio 1M:2F, χ^2 test, p=0.02) and larger than males (body size: females: 3.91 ± 0.62 mm, n=27; males: 3.26 ± 0.61 mm; n=8, t-test, p=0.01) (Fig. 3). Life span is not known but exceeds 2 years. Females may produce 2 broods per year; the number of eggs of a 4.8-mm long female was 19 and egg diameter was 0.2 mm.

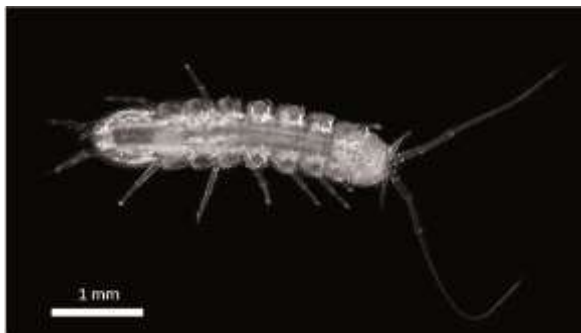


Figure 2: Photo of *Gallasellus* (courtesy of M. Delangle).

Type	Knowledge	References
Sex ratio	Female biased: number of F>M	Lefebvre <i>et al.</i> , 2016
Sexual dimorphism	Female biased: F larger than M	Lefebvre <i>et al.</i> , 2016
Reproduction	First brood at 7 months, 2 broods / year, 6-19 eggs / brood, egg incubation time: 35 days	Henry & Magniez, 1977; Lefebvre <i>et al.</i> , 2016
Chromosomes	26 pairs	Henry & Magniez, 1977
Diet	Primary consumer, organic detritus (50 %), microbial biofilm (38 %)	Ercoli <i>et al.</i> , 2019
Behaviour	Burrower when reared on clay	Henry & Magniez, 1977
Habitat preference	Mostly found in karstic water	Lefebvre <i>et al.</i> , 2016; this study

Figure 3: Biological and ecological knowledge of *Gallasellus*.

Gallasellus is a primary consumer feeding on organic detritus (50 %) and microbial biofilm (38 %). It is preyed upon by *Niphargus ladmiraulti* (Amphipoda). Observations

made during rearing showed that *Gallasellus* dug long galleries in clay. Mean water temperature at 22 occurrence sites was 13.1 °C (from 9.6 to 14.2 °C).

Distribution

Gallasellus was collected from a total of 32 sites belonging to four river catchments: Charente, Loire, Sèvre Niortaise and Dordogne (Fig. 4). Its presence in the Dordogne River is from a single collection which needs to be verified. Its range spans over a maximum linear extent of nearly 200 km. *Gallasellus* occurred at 23 % of sampled sites, but it has not yet been collected in unconsolidated rocks. In consolidated

rocks, it occurred in 40, 30 and 20 % of sampled caves, springs and wells, respectively.

Diversity within *Gallasellus*

The delimitation of MOTUs using the COI gene-based threshold method as defined by LEFÉBURE *et al.* (2006) shows that the genus *Gallasellus* may contain 6 putative species (Fig. 4). Two of them are single-site species, whereas the other four have wider distributions spanning over a maximum linear extent of about 80-100 km. MOTUs were found to co-occur at three sites (Fig. 4).

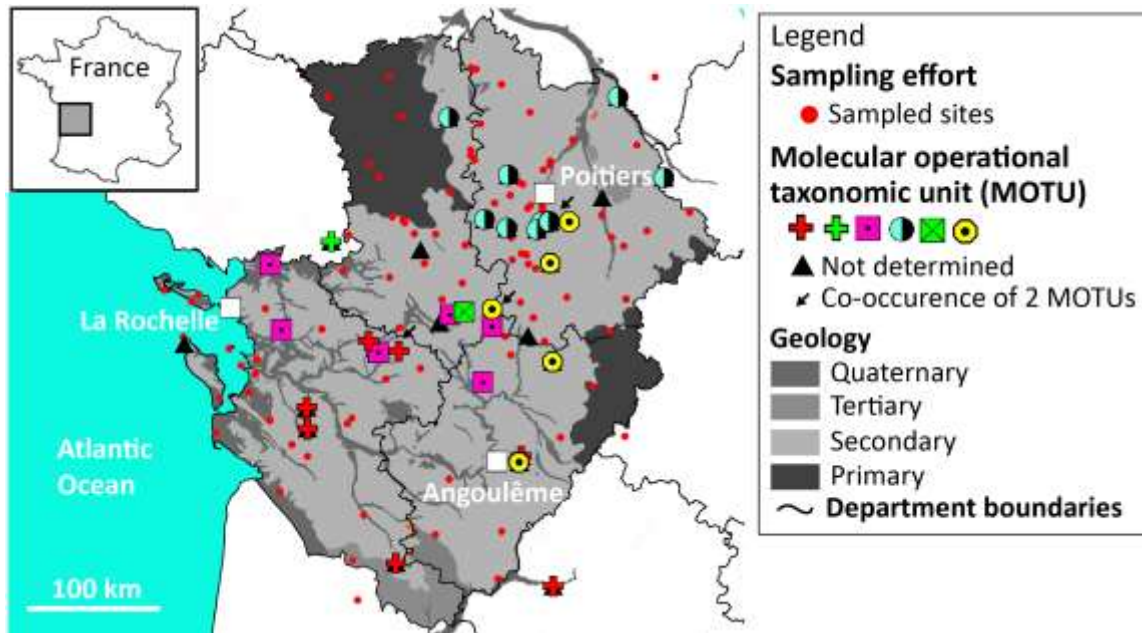


Figure 4: Distribution and diversity of *Gallasellus* in the Poitou-Charentes Region (France).

4. Discussions

Distribution

Increase in sampling effort resulted in a much wider known range of *Gallasellus*. Extensive sampling has indicated the genus is restricted to limestone aquifers. Unless *Gallasellus* has colonized hyporheic corridors of the Loire River catchment, its distribution should be constrained by the Armorican Massif to the North West and by the Central Massif to the South East. In the South, the Pyrenean region has been intensively sampled including by researchers of the CNRS laboratory of Moulis, but *Gallasellus* has never been found. A planned project dedicated to the study of the groundwater fauna of the Nouvelle-Aquitaine Region is intended to sample about 230 sites in south western France on a territory expanding over 58,000 km² (ALEZINE & LEFEBVRE, 2020). This project should help circumscribe the distribution and diversity of *Gallasellus*.

Biology and ecology

Knowledge of the biology and autecology of species is essential for conservation purposes. Contrary to many epigeal asellids, *Gallasellus* is iteroparous, but its life span and life fecundity are not yet known. Obtaining such data would require long-term rearing. Rearing obligate-

groundwater organisms is difficult, but optimal conditions for collecting *Gallasellus* specimens alive, transporting and rearing them were described in HENRY & MAGNIEZ (1977) and LEFEBVRE *et al.* (2016). Also, estimates of effective population size are not yet available, even though DELANGLE (2014) estimated the census population size at the Fountain du Clos des Roches (Roches-Prémarie-Andillé, Vienne) to 79 ± 7 individuals. Subterranean dispersal ways of *Gallasellus* remain to be elucidated. HENRY & MAGNIEZ (1977) suggested that the burrowing behaviour of *Gallasellus* might facilitate its dispersal along interstitial corridors. They further emphasized the importance of former alluvial corridors during Quaternary phases of low sea level for explaining the presence of *Gallasellus* in the Oléron Island (MAGNIEZ & HENRY, 2001). However, the genus has not yet been collected in unconsolidated rocks, suggesting limestone aquifers at greater depths might have contributed to its dispersal.

Organic matter detritus is an important component of the diet of *Gallasellus*. ERCOLI *et al.* (2019) proposed that the decrease in abundance of *Gallasellus* in the subterranean River of Bataillé might be due to wood removal operations. They suggested restoration efforts should be directed

towards reconnecting surface and subterranean ecosystems, including re-opening of sinkholes and traditional wells to promote inputs of decaying organic matter.

Diversity within Gallasellus

Increasing knowledge on the genus *Gallasellus* shows that it contains several species. The COI gene-based threshold method as defined by LEFÉBURE *et al.* (2006) is highly

conservative in that it identifies highly divergent evolutionary units. However, the number of MOTUs is sensitive to sampling effort. Hence, MORVAN *et al.* (2013), EME *et al.* (2018) and the present study successively revealed 3, 4 and 6 MOTUs. A next important step consists in diagnosing and describing these MOTUs based on molecular and morphological criteria.

5. Conclusion

Increasing knowledge on the genus *Gallasellus* has important conservation implications. What has long been considered as a single-site monotypic genus is a potentially species-rich genus containing a mix of wide- and narrow-range species. Future sampling studies (ALEZINE &

LEFÉBVRE, 2020) will not only enable to direct conservation efforts to protect the diversity of *Gallasellus*, but they will also continue to reveal the unexpected diversity of groundwater fauna in south western France.

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An introduction to the World Asellidae Database

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Abstract

The waterlice (Asellidae, Isopoda) are one of the rare family of metazoans containing both a great number of surface aquatic species and subterranean aquatic species. This makes it an ideal case study for understanding eco-evolutionary processes taking place during the transition to groundwater habitats. Comparative studies can be performed on multiple independent pairs of surface and subterranean species which provide true replicates of the ecological transition from surface water to groundwater. However, such studies require new collaborative research tools to assemble taxonomic, phylogenetic, distributional, and biological data for a species-rich family which is distributed across North America, Europe, North Africa and Asia. Here, we introduce the World Asellidae Database, a tool that is being developed to allow multiple laboratories to jointly produce, visualize and query morphology-based and DNA-based species occurrence data and their associated biological materials. Access to metadata and data will be granted to users long before data are publicly released, thereby promoting collaborative projects among laboratories and reducing duplicate sampling and sequencing works.

1. Introduction

The understanding of eco-evolutionary processes taking place during the transition of organisms from surface aquatic habitats to groundwater habitats is one of the pillars of subterranean biology (CULVER *et al.*, 1995). This understanding has long been based on studies conducted at population level, in which the traits of surface aquatic and groundwater populations of the same species are compared. Key comparative studies of that kind include those conducted on populations of the cavefish *Astyanax mexicanus* and the waterlouse *Asellus aquaticus*. (JEFFERY, 2001; PROTAS & JEFFERY, 2012). Another comparative approach consists of comparing traits of multiple surface aquatic and groundwater species while taking into consideration their phylogenetic relationships (LEFÉBURE *et al.*, 2017). This phylogenetic comparative approach can complement intraspecific comparisons by considering multiple independent evolutionary events, for example multiple pairs of sister surface and groundwater species. It also enables consideration of evolutionary processes taking place over long time periods, at least longer than the lifespan of natural populations. However, comparative studies of multiple surface aquatic and groundwater species have remained scarce for two major reasons. First, many groundwater species-rich taxa lack surface relatives,

thereby precluding their use in comparative analyses of surface aquatic and groundwater species. For example, *Niphargus* is a species-rich genus of amphipods, but most of its over 300 described species inhabit groundwater (FISER *et al.*, 2008). Second, species-rich taxa containing both a number of surface aquatic and groundwater species have a wide distribution spanning several continents. Performing comparative analyses using these taxa requires new collaborative research tools to assemble the necessary taxonomic, phylogenetic, distributional and biological data. Here, we introduce the World Asellidae Database (WAD), a tool that is being developed to allow multiple laboratories to jointly manage and query morphology-based and DNA-based species occurrence data and their associated biological vouchers. Asellidae (Isopoda, Pancrustacea) are one of the rare family of metazoans containing both a great number of surface aquatic species and subterranean aquatic species. This makes it an ideal case study for understanding eco-evolutionary processes taking place during the transition from surface aquatic to groundwater habitats. Yet, developing a multiple-user collaborative tool is crucial to facilitate data acquisition and information sharing because the family is distributed across four continents.

2. Materials and methods

The structure of WAD (Figure 1) and its web interface (Figure 2) were first described and released as a free and open-source laboratory information management software in September 2019 (at [https://github.com/GOTIT-](https://github.com/GOTIT-DEV/GOTIT/releases/tag/v1.1.1)

[DEV/GOTIT/releases/tag/v1.1.1](https://github.com/GOTIT-DEV/GOTIT/releases/tag/v1.1.1); MALARD *et al.*, 2020). The database structure and web interface were developed using the system PostgreSQL and PHP Symfony framework, respectively.

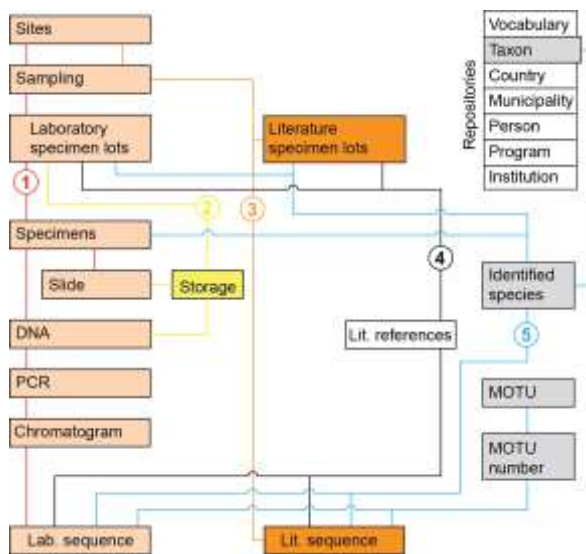


Figure 1: Simplified schematic view of the World Asellidae database (WAD). Numbered and colorized lines show five distinct pathways. (1) red: hosting laboratory data; (2) yellow: storage of biological vouchers; (3) orange: Literature data; (4) black: literature references; (5) blue: species assignment to specimens and DNA sequences. A lot is a collection of specimens of a single species taken at a site and date. MOTU: Molecular taxonomic units; Lab.: laboratory; Lit.: literature. Modified from MALARD *et al.*, 2020.

Briefly (see details in MALARD *et al.*, 2020), the database has five distinct pathways for managing the hosting laboratory data (from sampled site description to DNA sequence metadata), biological vouchers (storage of specimens and DNA), literature data (specimen lots and DNA sequence metadata), literature references, and the assignment of species to specimens and DNA sequences (Figure 1). DNA sequences, either from the hosting laboratory or the

3. Results

The database contains distributional data for all the 23 genera and 428 species and subspecies of asellids known to date. Of importance for studying evolution during transition of organisms from surface aquatic to groundwater habitats, the database contains data and metadata for 150 oculate and pigmented species and 278 depigmented and eyeless species. It also contains distributional data for approximately 40 species and subspecies of Stenasellidae (Isopoda, Pancrustacea), primarily European ones. The literature reference table has over 600 bibliographic references containing information on the original description and distribution of species.

The database dashboard provides a snapshot of the distributional data and their origin in date of November 2020 (Figure 2). The distribution of species occurrence data stored in the database is shown in Figure 3A. Asellids are widespread in North America, Europe, northern Africa and most probably also in Asia, although sampling effort has been less in the latter continent, except in Japan.

literature, can also be assigned to molecular taxonomic units (MOTU). MOTUs are delimited by the users using whatever molecular species delimitation methods and they are linked to taxa. The full database contains 45 tables and 450 fields: taxonomic curation and data quality control are achieved by scientists of LEHNA (Laboratoire d'Ecologie des Hydrosystèmes Naturels et Anthropisés) as part of their every-day laboratory activity.

The web interface has been conceived to ease the every-day management and inputs of data and metadata in the database. A demonstration version of the interface is available at <https://gotit.cnrs.fr> with test data and full documentation. Each heading of the menu contains a number of subheadings which give access to a content form (Figure 2). Content forms list the records contained within tables and provide buttons to delete records and access record forms for creating, reading and updating individual records. The interface also comprises 29 tools for uploading records in batches (Figure 2). They allow the upload of csv file templates containing records. Prior to uploading data in the database, a procedure checks and reports potential errors in the file template, such as identifier duplicates and incorrect data format. A number of ready-to-use queries have been implemented in the 'Species search' add-on of the interface. Among other functionalities, they allow the user to map the distribution of species, to explore the number and geographic distribution of MOTU within species, and to assess the exhaustiveness of molecular sampling within species.

The WAD project was first launched in 2015 by laboratories LEHNA and BBEES (Bases de données Biodiversité, Ecologie, Environnements Sociétés). Benchmark data were first migrated to the database in October 2017 and since August 2018 the web interface has been used as a laboratory information management software to download and manage Asellidae species occurrence data and molecular metadata produced on a day-by-day basis by scientists of LEHNA.

Species occurrence data are from 9403 samples collected at 8114 sites (Figure 2). A total of 7669 species occurrence data are from published literature (Literature data: specimen lots in Figure 2). Another set of 1863 species occurrence data are from sampling of specimen lots carried out by staff of LEHNA and multiple providers who kindly accepted to leg their biological material (Biological material: specimen lots in Figure 2). These specimen lots are labelled and stored in the LEHNA collections at -20°C.

The dashboard also provides a summary of molecular work achieved at LEHNA in date of November 2020 (Figure 2). A total of 5490 individual specimens were extracted from specimen lots for morphological and/or molecular analyses, giving rise to 1702 microscope slides (dissection) and 4811 DNA extracts. Treatment of these DNA extracts resulted in 18560 polymerase chain reactions (PCR), 11529 electropherograms (chromatograms in figure 2) and 8335 DNA sequences. DNA extracts are stored in the LEHNA collections at -80°C. The DNA sequences essentially target

two mitochondrial genes, the mitochondrial cytochrome oxidase subunit I (COI) and the 16S mitochondrial rDNA

genes, and two nuclear genes, the 28S nuclear rDNA and FAST-2 genes (FAST kinase-like protein, subdomain 2).

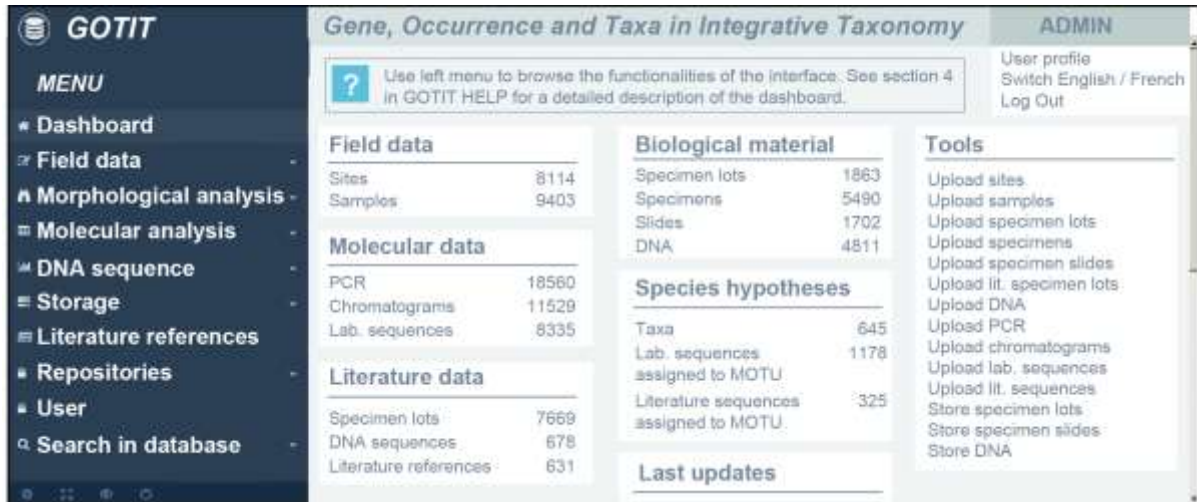


Figure 2: Dashboard of the web interface used for managing data and metadata stored in the World Asellidae database. Headings of the menu give access to lists of records contained in the database tables and to record forms for creating, reading and updating individual records. Tools are for uploading records in batches.

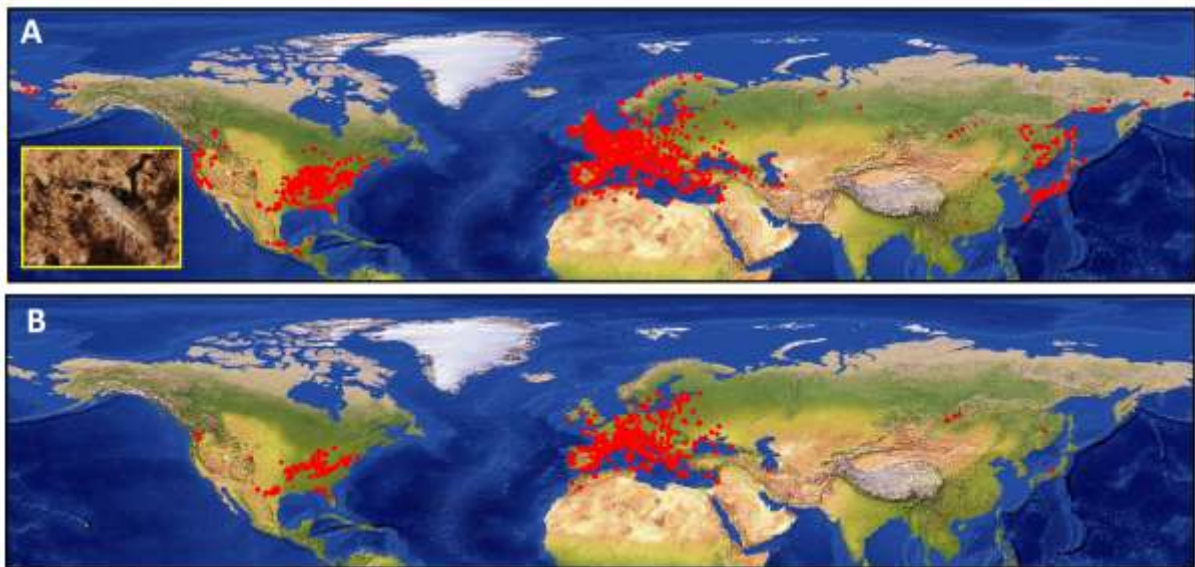


Figure 3: Worldwide distribution of species occurrence (red dots in A) and DNA sequence data (B) contained in the world Asellidae database. Photo of *Proasellus valdensis* (Asellidae, size: 8 mm) in Planches Cave, Jura, France (from R. Le Penneç).

These four genes are used to build phylogenies of Asellidae. Among the available COI sequences, 1178 were assigned to MOTU using four different molecular species delimitation methods. Figure 3B shows the distribution of DNA sequences registered in the database. Comparison with the

map of species occurrence data show that the sequencing effort has focused on Europe and to a lesser extent North America. However, there is a gap in DNA sequences for North-Africa and Asia.

4. Discussion

WAD provides one of the biggest data sets available to date for phylogenetic comparative studies of surface aquatic and groundwater organisms. Recent published studies focused on the comparison of molecular traits, namely genome size (LEFÉBURE *et al.*, 2017) and the rate of molecular evolution

(SACLIER *et al.*, 2019), using a dozen of surface/subterranean species pairs, for which transcriptomic and genomic data were assembled. Yet, comparative studies can potentially be extended to a variety of traits and a much larger number of species by combining

family-wide phylogenies (MORVAN *et al.*, 2013) with morphometric / biological measurements on specimens stored in collections. Candidate traits include body size and shape, number and size of eggs, and the size of species' geographic ranges. Incorporating such traits into the database represents a next important step.

As experienced by a dozen of regular LEHNA users, WAD and its web interface enhance key laboratory activities including research project design, sampling implementation and gap analysis, species identification, DNA sequencing, and contributing information to global biodiversity facilities. Ongoing developments are directed towards opening database access to biodiversity professionals and promoting collaborative projects among laboratories, while promoting the FAIR-data and TRUST principles (WILKINSON *et al.*, 2016;

LIN *et al.*, 2020). User access would be granted either in a read-only mode or in a collaborative mode for data contributors. Meantime, efforts are being made to provide a set of tools to retrieve data and metadata from the database. Expert tools will provide the users with the possibility to retrieve data using SQL queries. Non-expert tools will include the possibility to download data directly from the content forms of the web interface as well as using a user-friendly query builder. Efforts are being made to increase interoperability of WAD with other biodiversity facilities. Links to GenBank are already available for published sequences and developments can easily be implemented to export occurrence data in a Darwin Core format (WIECZOREK *et al.*, 2012).

5. Conclusion

Comparative phylogenetic studies using the Asellidae as a case-study hold much promise for understanding evolution during the ecological transition from surface water to groundwater. These data-intensive studies rely on close cooperation both between research laboratories as well as between laboratories and speleologists who provide many

specimen samples. In the near future, we hope that opening access to WAD strengthens cooperation between research laboratories and speleologists by providing them easier access to biodiversity data to which they continuously contribute.

Acknowledgments

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Search of life in a Mars analogue site - microbes and associated biosignatures in the deep and completely dark salt caves of the Atacama Desert

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Abstract

Salt caves are scarce and poorly investigated mainly because they only exist in extremely remote and arid regions, such as the Atacama Desert (Chile). The Cordillera de la Sal (San Pedro de Atacama) is one of the most important rock salt karst areas in the world forming a Mars-like landscape. The detection of microorganisms and biosignatures preserved in halite speleothems from these hypersaline cave systems is of particular interest for understanding the limits of microbial life, the origin of life on Earth, and predict extraterrestrial habitability. State-of-the-art techniques were used for the accurate characterization of microbial cells and organic molecules from halite speleothems collected in *Cueva Cressi*. This study involved a multi-proxy approach, including metagenomics, FESEM-EDS, Py-GC/MS and stable isotopes (EA-IRMS). We identified and isolated halophilic microorganisms, such as *Salinisphaera* sp. and *Haloparvum* sp., and were able to detect lipid compounds in the speleothems. In this communication, we will present the first data on this fascinating Mars-like ecosystem and introduce novel techniques for the detection of biosignatures and the direct determination of the isotopic composition of environmental samples from extreme and Mars analogue field sites.

1. Introduction

The Atacama Desert (Chile), the driest non-polar place on Earth, is considered an extraterrestrial analogue environment. The soils from this extremely arid region have been widely studied, as they contain organic molecules in very low concentrations as expected on Mars (MARLOW *et al.* 2008). Recently, new explorations were carried out in Cordillera de la Sal (San Pedro de Atacama, Chile), one of the most important rock salt karst areas in the world. Here, over 50 caves and more than 15 km of underground passages, including the longest and deepest cave systems of Chile, have been discovered (DE WAELE & PADOVAN 2016). The Cordillera de la Sal is a 100 km long and 5 km wide NW-SE oriented anticline of Oligo-Miocene age, containing salt layers forming a Mars-like landscape. Scientific research has started a few years ago in these exceptional uncharted caves, mainly focused on geology and mineralogy (DE WAELE & PADOVAN 2016; DE WAELE *et al.* 2017, 2020). Recent mineralogical studies have reported an unexpected mineralogical variety, including very rare halites, sulphates,

and nitrates (DE WAELE *et al.* 2017). Some of these minerals are only found in these extremely arid conditions and are similar to minerals found on Mars.

It is well known that the two main sources of organic matter (OM) in speleothems derive from the overlying soils or from microbial communities thriving in the cave systems (MILLER *et al.* 2016, 2020). The caves in the Cordillera de la Sal are hosted in a completely barren area with no traces of vegetation. Water is almost completely lacking in this area and is mainly found as very salty brines in cave pools, or underneath white salt crusts on the cave floors. Life in this prohibitive area is therefore restricted to some birds of prey (barn owl) and extremely few arthropods. Considering that the soils from Atacama solely contain traces of organic molecules (MARLOW *et al.* 2008) and taking into account our previous results on the presence of halophiles in the halite speleothems of the Atacama salt caves, the assessment of OM in the speleothems from hypersaline Mars analogue cave systems is of particular interest for

astrobiology and for the search of organic molecules (and possibly past or present life) on Mars. Specifically, the isotopic characterization of OM in these speleothems has the potential to provide evidence on its nature and origin, as well as to improve our knowledge on important palaeoclimate alterations (MILLER *et al.* 2016, 2020). In this sense, the use of novel techniques is of utmost importance for the accurate characterization of organic molecules preserved in minerals from planetary field analogue sites, such as in halite speleothems from salt caves.

This study aimed at conducting for the first time an accurate characterization of the microbial communities and biosignatures associated with halite speleothems from the salt caves of Atacama (Fig. 1). Studying these analogues for hypersaline subsurface environments on Mars is thus a reliable way to improve our understanding on the origins of life on Earth, and possibly on other planets, as well as on the environmental conditions where they were developed.

2. Materials and methods

In the frame of a National Geographic funded project (“Reading” the salt caves of Atacama), an expedition was conducted in March 2018 to pursue the exploration of caves in the salt layers of Cordillera de la Sal (San Pedro de Atacama, Chile). Samples of halite speleothems and cave sediments were collected for further laboratory-based analyses (Fig. 2).



Figure 2: Sampling of halite speleothems in Cueva Cressi (Photo by Alessio Romeo, La Venta Esplorazioni Geografiche-National Geographic Society).



Figure 1: Halite speleothems in Cueva Cressi (Photo by Riccardo De Luca, La Venta Esplorazioni Geografiche-National Geographic Society).

During this expedition, 2 new cave areas and 3 km of new underground passages were explored and surveyed with laser scanning and 3D photogrammetric techniques. Intriguing halite speleothems, toothpaste-like halite flowstone, salt crusts on cave floors, yellowish and white crusts in salt-crusted cave pools, halite cottonballs, as well as cave sediments were collected in *Cueva Cressi* and *Cueva del Arco*. A total of 5 replicates were collected from each sampling point in sterile vials for DNA-based analyses, culturing procedures, microscopy techniques, mineralogy and organic geochemistry analyses. An additional replicate sample was used for *in situ* fixation with 2% glutaraldehyde and 1% paraformaldehyde in 0.1M cacodylate buffer (pH 7.4) for further Field Emission Scanning Electron Microscopy (FESEM). Samples were collected using sterile scalpels and stored into sterile 50 ml tubes. The samples were preserved at 4°C until arrival at the laboratory.

In situ fixed samples were washed in cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated by dilution series in ethanol and acetone and dried in a critical point drying device. Finally, the fixed samples were sputter-coated with a thin gold film and observed in a FEI Teneo FESEM using the secondary electron detection mode with an acceleration voltage of 5 kV for ultra high-resolution images and reconnaissance of microbial cells.

After arrival to the laboratory, samples for DNA-based analyses were stored at -80°C for further DNA extraction. Genomic DNA was extracted from approximately 500 mg of sample using the DNeasy PowerLyzer PowerSoil Kit (Qiagen) according to the manufacturer's instructions. Subsequently, DNA concentration was determined using the Qubit 2.0 fluorometer (Thermo-Fisher) and the high-sensitivity DNA assay quantification kit (Thermo-Fisher). DNA samples were then submitted to high-throughput 16S rRNA gene-based amplicon sequencing using the Illumina MiSeq platform at the Medical University of Graz (Austria). The microbial community composition and diversity were determined after bioinformatics processing of the 16S rRNA gene sequences using the Galaxy web-based platform. Classification of the reads were based on the newest release

of the SILVA database and taxonomy information was analysed and visualized using the online web-tool Calypso. The organic geochemical proxy performed to detect the presence of organic compounds preserved in the halite speleothems and cave sediments included the analysis of these samples by analytical pyrolysis (Py-GC/MS) and by elemental and stable isotope analyses (EA-IRMS). The elemental (C and N) and stable isotope analysis ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of the halite speleothems (C3 and C8) and sediment samples (C2 and C4) were determined after carbonates removal as described by MILLER *et al.* (2016). Briefly, an elemental microanalyzer (flash 2000 HT, Thermo Scientific) combined with a continuous flow isotope ratio mass spectrometer (IRMS) (Delta V Advantage, Thermo Scientific) via a ConFlo IV interface unit (Thermo Scientific) were used.

3. Results and Discussion

Field emission scanning electron microscopy (FESEM) examinations of the fixed samples from *Cueva Cressi* revealed the abundance of clay minerals and filamentous structures (Fig. 3A, B). Anhydrite (anhydrous calcium sulphate) crystals were observed for samples of *Cueva del Arco* (Fig. 3C). The presence of salt crystals in some salt crust samples were rarely observed due to their dissolution in the fixative solution. FESEM also showed the presence of microbial-like features, such as square-shaped cells (Fig. 3D) characteristic of extreme halophiles, fusiform-shaped cells (Fig. 3E), as well as coccoid cells (Fig. 3F).

Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) was performed using a 2020i double-shot pyrolyser (Frontier Labs, Fukushima, Japan) attached to a GC/MS system, as described in MILLER *et al.* (2020). Compound identification was attained via single-ion monitoring (SIM) and by comparison with mass spectra libraries (NIST14 and Wiley7). From the structural information provided by the pyrolysis analysis it was possible to construct 3D bubble van Krevelen diagrams as described by JIMENEZ-MORILLO *et al.* (2018). In short, mass spectrometry data were represented by plotting relative intensity for individual compounds calculated as total abundances (bubble size), as 3D bubble in the x,y plane defined by its atomic H/C (y) and O/C (x) ratios.

The first (preliminary) microbiological results derived from the high-throughput 16S rRNA gene-based amplicon sequencing confirmed the presence of halophilic microorganisms, such as *Salinisphaera* sp. and *Haloparvum* sp., as well as other genera commonly found in salt environments, including *Acinetobacter* and *Pseudomonas*. These extremophiles appear to survive and live in these harsh oligotrophic environments without the presence of light and exploiting different metabolic pathways (studies are in progress).

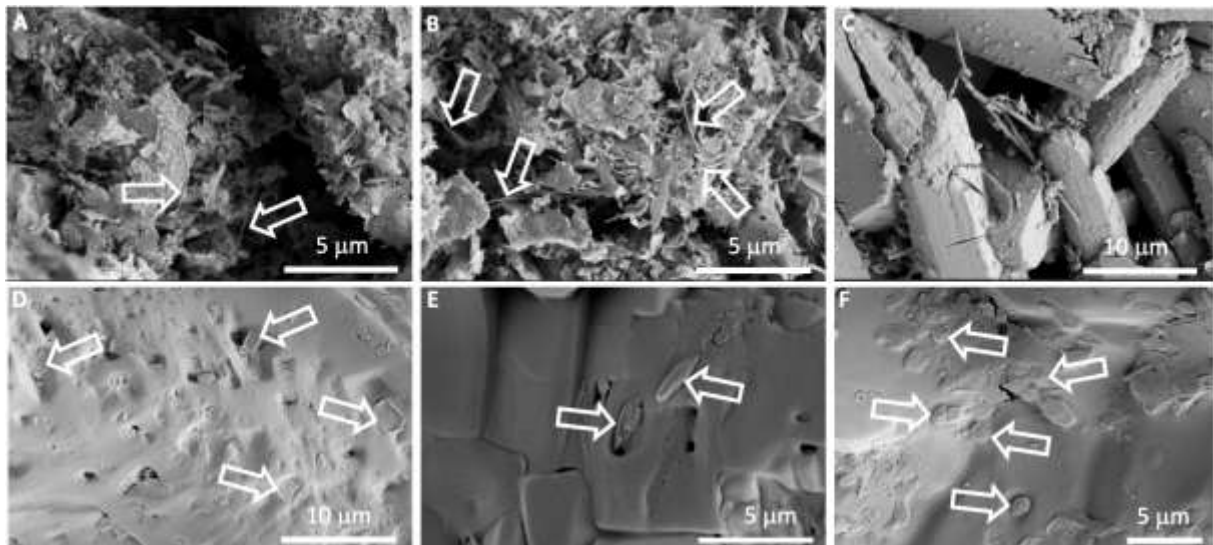


Figure 3: Field emission scanning electron microscopy images of microbial-like structures found associated with salt cave minerals from the Cordillera de la Sal in Atacama. A,B) Clay minerals and filamentous structures (arrows). C) Anhydrite crystals. D) Square-shaped cells (arrows) characteristic of extreme halophiles. E) Fusiform-shaped cells (arrows). F) Coccoid-shaped cells (arrows).

Further metagenomics analyses will allow us to figure out how these living microbes are able to gain energy in these extraterrestrial-analogue conditions.

The elemental and stable isotope analysis showed the existence of significant ($P < 0.05$) differences among the 4 studied samples. The C3 sample showed the highest C content ($> 0.1\%$), while C8 sample is the poorest in C

(0.04%). Considering the C isotope composition, C4 and C8 samples displayed a $\delta^{13}\text{C}$ value of -15 and -25% , respectively. Concerning N analyses, the N abundance and isotope composition were below the detection limit of the EA-IRMS equipment for all the halite speleothems.

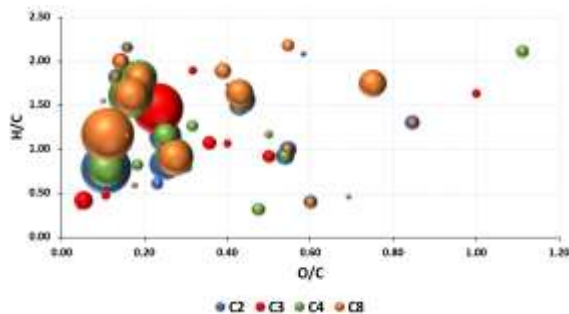


Figure 4: 3D van Krevelen plot obtained by Py-GC/MS.

The presence of organic molecules preserved in the speleothems and sediments was analysed by Py-GC/MS. The 3D van Krevelen plot (Fig. 4), inferred by mass spectrometry data, showed that the OM present in the halite speleothems was mainly made up of lipid compounds ($H/C > 0.5$ and

$0.1 > O/C < 0.3$), which are considered microbial signatures. However, some differences between the samples were found.

Samples C3 and C4 showed the presence of polysaccharides ($O/C > 0.7$). Nevertheless, the organic fraction within C3 sample also displayed the existence of more recalcitrant and aromatic molecules ($H/C < 0.5$ and $O/C < 0.1$), probably derived from microbial activity (JIMENEZ-MORILLO *et al.* 2018). In contrast, the C4 sample showed the presence of furan molecules ($H/C < 0.5$ and $0.3 > O/C < 0.7$), which can be released by microorganisms to form biofilms (EPS). Concerning C2 and C8 samples, they showed a relative high number of peptidic compounds ($H/C > 1.5$ and $0.3 > O/C < 0.5$). However, C8 sample also displayed polysaccharide-like formulas similar to sample C3. The detection of these biomolecules points to the existence of microbial communities thriving under the extreme conditions of the salt caves from the Atacama Desert.

4. Conclusion

Despite the apparent lifeless appearance of the halite caves and the salt speleothems hosted within, and the prohibitive extremely salt environment and lack of light, our first results of the microbiological studies show microorganisms able to thrive even in such harsh, extra-terrestrial analogue environment. Living microbial communities have been found in high-altitude arid Atacama Desert (MAIER *et al.* 2004), and these life forms might have been transported there by wind (AZUA-BUSTOS *et al.* 2019), and adapt to

endolithic life with both phototrophic and heterotrophic organisms (WIERZCHOS *et al.* 2018). This is the first study carried out in this area in the complete darkness of caves. The preliminary results derived from this work provide new insights into the adaptation and evolution of microbial life on Earth, and given that the salt caves from the Atacama Desert are extraterrestrial analogue environments, these data have significance for astrobiology and future planetary explorations.

Acknowledgments

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Biodiversity and conservation status of Divrash cave, Guilan province, Iran

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Abstract

Biodiversity of Divrash cave, located in the western part of the Hyrcanian Forests, Iran, is investigated for the first time. A total number of nine taxa belonging to Mammalia, Arthropoda, and Mollusca were identified at species or genus level in the cave. Furthermore, the flora of the area surrounding the cave was surveyed. Divrash cave is one of the most popular tourist attractions of the Guilan province, but it is vulnerable due to severe human disturbances.

1. Introduction

Located between 36°36'03" to 38°27'07" N and 48°34'25" to 50°26'42" E, Guilan province is bordered by Caspian Sea to the north, and the Alborz mountain range to the south. Very high humidity and annual precipitation, along with a noticeable species richness, are the results of the exceptional location of this region. This area belongs to the southern Caspian Sea geographical unit, and is confined to the western Alborz zone, a Paleozoic platform constituted of Cambrian bedrock. The occurrence of karst in the east and southeast of Guilan, provides a suitable substrate for a considerable number of caves (AGHA-NABATI, 2005). The Hyrcanian or Caspian Forests form a narrow and continuous vegetation belt extending along the northern slopes of the Alborz mountain range. Its total area is 1.85 million ha. It contains more than 3200 species of vascular plants. Three north Iranian provinces (Guilan, Mazandaran, and Golestan)

and a small part of western Azerbaijan, are embedded in Hyrcanian Forests (TOHIDIFAR et al., 2016).

Comprehensive inventories of species diversity in caves of Iran are scarce. MALEK-HOSSEINI (2013) studied the fauna of five caves in southwest Iran and identified 12 mammalian species and 44 arthropods. Although many scientists have studied particular cave taxa including bats, fish, amphibians, insects and crustaceans in recent years (MALEK-HOSSEINI & ZAMANI, 2017; ESMAEILI-RINEH & SARI, 2013; AKMALI et al., 2019; FATEMI et al., 2019), there is no overall picture of Iran cave biological diversity; and all cited surveys remain limited, underestimating species richness. In this study, we surveyed the biological diversity of Divrash cave, from Guilan province for the first time. We discuss in conclusion the anthropogenic threats to the cave and its residents.

2. Materials and methods

Divrash (Noor-Cheshmeh) cave is located in Divrash village, Rudbar, Guilan, Iran. Its geographical coordinates are 36°54'21.3" N and 49°35'41.7" E and its altitude is 373 meters above sea level. The nearest mountain to this cave is Dorfak mountain, which is 2714 meters high (DEHDAR-DARGAHI & FARID-MOJTAHEDI, 2014). We totally visited this cave four times including spring and summer 2019, and summer and autumn 2020. Two researchers, one geologist and one zoologist, were included in each visit; and at least one support fellow accompanied the main group. The basis of our work was geology and karst studies along with terrestrial fauna investigations. Not all the results of our research are included here, because some topics need further reviews. Faunal and speleological studies took about one hour of investigation in each visit. In spring and summer 2019, the mammalian fauna of the cave was checked. During 2020, the surveys were more comprehensive. Cave fauna included three species of bats, three arachnids, one cricket, one lepidopteran, and one gastropod and lichens, mosses and mold were collected as well. Bats were caught by a mist-net set up at the cave entrance and released after photographing (Fig. 1). One specimen of each group of

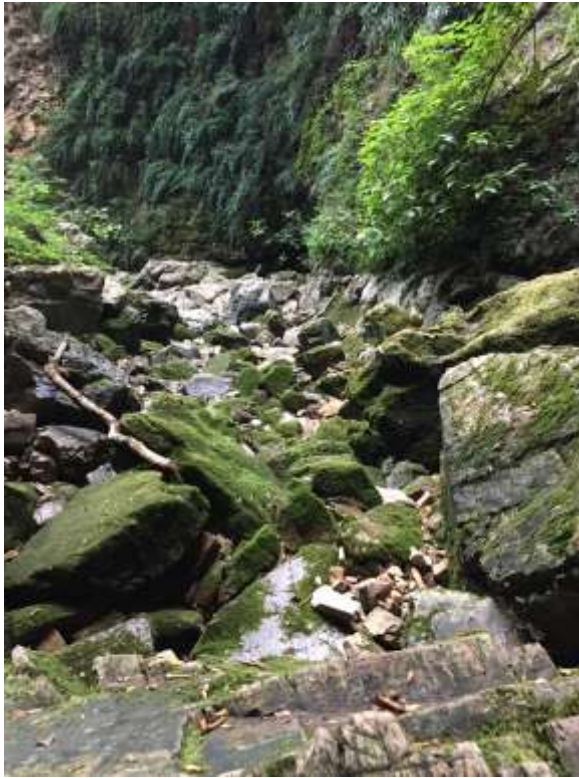
arthropod and mollusk was collected by hand or net to use for further identification. Bats, spiders, cricket, moth and snail species were identified using the identification keys of DIETZ & HELVERSEN (2004), Jacque and DIPPENAAR-SCHOEMAN (2007), DI RUSSO et al. (2007), DOMBROSKIE (2011), and EVERSHAM (2018), respectively. Additionally, we received generous assistance from experienced zoologists for accurate identification of the specimens.



Figure 1: Mist-net set up at one of the cave openings with bat individuals.

3. Results

In this study, three species of bats (*Miniopterus pallidus* Thomas, 1907, *Rhinolophus hipposideros* (Borkhausen, 1797), and *Rhinolophus* sp.), one cave cricket of the genus *Dolichopoda* Bolivar, 1880, the lepidopteran *Scoliopteryx libatrix* (Linnaeus, 1758), a glass snail of the genus *Oxychilus* Fitzinger, 1833, and three spiders of the genera *Tegenaria*, *Pholcus*, and *Metellina* were identified (Fig. 6). The surrounding vegetation of the cave is typical of Hyrcanian forests. *Ficus* and *Acer* grow around the cave entrance (Fig. 2). Other plant species which cover the walls of the valley, are *Hedera pastuchovii* and *Luzula pilosa* (TALEBI et al., 2014). Lichens are seen inside the cave where no light can penetrate. Distribution of the mosses is confined to the twilight area of the cave. Mold and some roots from unknown plant were also observed inside the cave.



Representatives from three different spider families were observed in the studied cave. All three families have been listed in Iran spider fauna. In the current study, we identified the spiders in their natural habitat and no specimen was captured. So, we cautiously place the observed individuals among *Tegenaria*, *Pholcus*, and *Metellina* belonging to families Agelenidae, Pholcidae, and Tetragnathidae, respectively (Fig. 4). Previous records on three above genera, show their northern distribution in Hyrcanian forests.



Figure 4: *Metellina* sp. from the cave.



Figure 5: left: glass snail; Right: *Scoliopteryx libatrix*.

Another arthropod observed in the cave was a cricket of the genus *Dolichopoda* (Fig. 3), which was present on the walls at an accessible height, as well as on the ground of the cave. About 20 specimens of this species were counted during all visits at different times of the year. A few individuals of *Scoliopteryx libatrix* (Linnaeus, 1758) (Fig. 5) from the family Erebidae were observed on the roof of the cave. Another invertebrate of the cave is the glass snail genus *Oxychilus* (Fig. 5). A few individuals of this snail were observed in and out of the cave.

Group	Genus or species	Nb spec	Nb obs
Mollusca	<i>Oxychilus</i> sp.	0	2
Arthropoda	<i>Scoliopteryx libatrix</i>	1	1
	<i>Dolichopoda</i> sp.	1	4
	<i>Tegenaria</i> sp.	0	1
	<i>Pholcus</i> sp.	0	2
	<i>Metellina</i> sp.	0	2
Mammalia	<i>Miniopterus pallidus</i>	2	4
	<i>Rhinolophus hipposideros</i>	2	4
	<i>Rhinolophus</i> sp.	0	1

Figure 6: the summary of results on terrestrial fauna. Nb spec, Number of collected specimens; Nb obs, Number of observation times.

4. Discussion

Caves of Hyrcanian Forests have been rarely explored for their biodiversity. This study revealed that cave fauna in this region is diverse and the occurrence of new species is to be expected. New records on some species are presented in the current work.

Pale bent-winged bats are reported for the first time from western parts of Hyrcanian forests. *Miniopterus pallidus* is a common bat species in Iran. This species has been recorded in at least 60 sites throughout the country (BENDA *et al.*, 2012; FATHIPOUR *et al.*, 2016; SHAHABI *et al.*, 2017; AKMALI *et al.*, 2019). Although there are only three records from Hyrcanian forests, in Golestan province located in eastern parts of the forests, the occurrence of this species in the other regions of the forests (Mazandaran and Guilan provinces) needs to be confirmed.

Rhinolophus hipposideros (Fig. 3) is also a common bat species in Iran. It has a rather continuous western distribution, and also a few scattered records from the eastern part of the country. In Guilan province, the records on this species are limited to three sites, none of them caves (BENDA *et al.*, 2012; NADERI *et al.*, 2017).

Spiders of Iran are diverse. MIRSHAMSI *et al.* (2015) have provided the checklist of spiders for the country which is regularly updated. Additionally, MALEK-HOSSEINI & ZAMANI (2017) listed the subterranean arthropods of Iran including cave spiders. So far, 763 arachnid species have been recorded in Iran.

Cave cricket, genus *Dolichopoda*, is mainly found in caves. In Iran, *Dolichopoda hyrcana* Bey-Bienko 1969 was reported from the northern slopes of the Alborz mountains in Chalus region (DI RUSSO & RAMPINI, 2013). Other cave crickets including the subfamily Bothriophylacinae Miram, 1934 is recently studied from Zagros mountain ranges (TAHAMI *et al.*, 2017).

Herald moth was previously reported from Arasbaran forest in East Azerbaijan Province at northeast of the country (LEHMANN & ZAHIRI, 2007).

Glass snail was only observed on our autumn visit to the cave. Some previous studies revealed the occurrence of this genus in the Hyrcanian forest. ELIAZIAN *et al.* (1979) recorded three species of this genus including *O. filicum*, *O. duboisi*, and *O. herzi* from northern Iran (Caspian Sea area). Additionally, MANSOORIAN (2005) studied the terrestrial mollusk fauna of Mazandaran and Golestan provinces (east

and central parts of Hyrcanian forest); and reported *O. persicus* and *O. herzi*.



Figure 7: The opening of the middle room of Divrash cave.

Understanding the biotic and abiotic conditions of the caves is a prerequisite to conservation management. Caves are a very attractive destination for tourism activities. But without appropriate knowledge about the value of the caves and their biodiversity, anthropogenic disturbances would not be avoided. Around 500 to 800 tourists visit Divrash cave during the warm seasons every week. The way to the cave is through the ancient Hyrcanian Forests and a river flows in the valley on the way to the cave.

Divrash cave has three separate openings with separate rooms. The connection between these three main rooms remains unexplored (Fig. 7). There is a permanent spring in the middle cave whose water level rises during the rainy season. The temporary river emerging from the cave entrance joins the Divrash river on the way to the village (NATEGEH, 2016). Due to the large number of visitors including tourists and mountaineers, illegal excavations, and vandalism, the destruction process of this cave is continuously accelerating. Noise pollution, swimming in the cave spring, physical destruction of cave speleothem, dumping garbage, and smoking are the most destructive behaviors of visitors in this cave which may lead to biodiversity loss.

5. Conclusion

Subterranean ecosystems harbor the most complicated kinds of life. The way cave organisms interact, and the survival of the whole cave biological network have been the subjects of many studies. Investigations on cave biodiversity itself and taxonomic identifications, are the first step to understand subterranean life complexity.

Divrash is the first cave in the western part of Hyrcanian Forests of which the biodiversity has been significantly investigated.

Altogether, nine species or genera from three main taxa including Mammalia, Arthropoda, and Mollusca were

identified in Divrash cave. *Miniopterus pallidus* is reported from Guilan province for the first time. *Scoliopteryx libatrix* is recorded from a hypogean habitat for the first time in the country.

Unfortunately, due to various anthropogenic interferences, the degree of destruction in this cave is high. Therefore, the existence of an official institution for the protection and sustainable operation of caves through organized tourism activities is necessary. This may help to get closer to sustainable development.

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Taxonomic and metabolic profiles show different structures and energetic metabolism into the microbial communities in a tropical cave (Brazil).

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Abstract

Microorganisms play an important ecological role in caves, but most of the studies used inferences through 16S rRNA sequences. Nowadays it would be better to gain a deeper insight into community's functional capabilities from unamplified environmental DNA and metagenomic sequencing. This is the first study to explore microbial community structure and metabolic potential in a Brazilian cave. Environmental DNA was extracted from composed samples of sediment (surface and subterranean) from Angelica cave and it was sequenced in an Illumina HiSeq2500 instrument. Functional annotation of metagenomic sequences and taxonomic diversity were performed by the metagenomics RAST server. Bacteria was the main domain in both sites and Archaea was more present in subterranean (1.89%) than surface (0.22%) samples. A small fraction was assigned to Eukaryota (0.62%) and Viruses (0.17%). Functional genes involved in microbial metabolisms are highly represented in that communities. Microbial genes related to oxidative phosphorylation, carbon fixation in prokaryotes and nitrogen metabolism were more prominent in subterranean than on surface. Behind the information on microbial diversity, we also reveal insights into microbial adaptations and strategies under cave conditions without photosynthetic organisms. The metabolic profile highlights the diversity of microbial processes to get energy and sustain the trophic network.

1. Introduction

Microbial communities in caves are under-explored habitats and scientific studies were performed to understand the taxonomic distribution since 1900 (TOMCZYK-ZAK & ZIELENKIEWICZ, 2016). However, most of those studies were carried out using culture-dependent techniques, but microorganisms generally have highly specific growth conditions, and the growth of some microorganisms is also dependent on specific interactions with their neighbors. The "metagenomics" techniques come to light in 1998 and since then the total genomic DNA from microorganisms can be extracted, which means the genetic materials of cultivable and uncultivable microorganisms will be assessed for molecular analysis (HUGERTH & ANDERSSON, 2017).

The whole metagenome sequencing (WMS) is used to know the information on genetic sequences about all environmental DNA. In WMS, not only the taxonomic profiling (who are there) but also their functional possibility (what are they doing) of microorganisms can also be predicted. The WMS approach has scarcely been used to explore cave microbiomes around the world. One of the pioneering research was applied to explore the Kartchner Caverns (USA) (ORTIZ *et al.*, 2014). The results indicated that bacteria were dominant in those communities, followed by archaea, eukaryota, and viruses. Regarding the functional genes, that microbiome showed an alternative primary

production strategy (e.g. CO₂ fixation) and genes encoding DNA repair enzymes. These metabolic pathways might be one of the adaptive strategies of cave microorganisms for growth and development under low-nutrient conditions and stress caused by high calcium concentrations.

In general, few studies are carried out in tropical caves, even those using PCR-dependent approach. Only two tropical caves have been studied using WMS (TETU *et al.*, 2013; WISESCHAT *et al.*, 2019). Although the authors found interesting results regarding energy metabolism, these studies were carried out on slime mats from a submerged cave, without associated aquatic macrofauna and no detectable organic carbon, and dark zone sediment without comparison with the surface microbiome. Therefore, the aims of this study was to examine (1) the taxonomic and functional composition of a cave sediment metagenome and (2) comparisons of the cave genomic profiles to surface environments to identify important strategies developed by cave microbes to survive in this oligotrophic environment. These analyses were driven by the hypothesis that although tropical caves show mostly heterotrophic microorganisms, it is possible to find a greater abundance of genes involved with chemoautotrophic activities than the surface environment.

2. Materials and methods

Terra Ronca State Park (PETeR) (46°100' - 46°300'S; 13°300' - 13°500' W), located at São Domingos city (Goiás State, central Brazil), has a large subterranean system formed by rivers arriving from the Serra Geral Plateau, a morphologic feature originated in the sandstones of the Urucuia Formation (Cretaceous age). PETeR is a karst area crossed by parallel streams running westwards to join the Paraná River, a tributary of the Upper Tocantins River, in Amazonas Basin. The study area is inserted at the Cerrado phytogeographical domain (the savannah-like Brazilian vegetation) and the climate is tropical semi-humid (PAULA *et al.*, 2020). Angélica Cave, one of the many caves in PETeR, is crossed by the Angélica River and is one of the largest caves of Brazil, with an extension of approximately 14 km and an entrance approximately 10.0 m height (BICHUETTE *et al.*, 2015). Samplings were conducted in April/2017 (license n° 28992-11 (ICMBio/SISBIO) and n° 14886/2010 (Secima, Goiás)). Soil or cave sediment, composed of squares of approximately 0.25 m², were collected in two areas: surface (outside of the cave) and subterranean (dark zone). The samples were transported to the laboratory in coolers, homogenized, sieved (2 mm mesh), and stored in a refrigerator at 4°C. DNA was extracted from each sample using a MoBio PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad,

CA, USA) following manufacturer's instructions. The metagenomic libraries were prepared using the Nextera XT DNA Sample Preparation kit (Illumina, Inc., San Diego, CA, United States), according to the manufacturer's protocol. The metagenomic libraries were sequenced using the Illumina HiSeq2500 and the sequencing reads were deposited in the Metagenome Rapid Annotation (MG-RAST) server with accession numbers 404466 (surface) and 404464 (subterranean). MG-RAST server version 4.0.3 (MEYER *et al.*, 2008) was also used for the assembly of paired-end reads, pre-processing and annotation of the sequences according to the instructions provided by MG-RAST. The default parameters were used for all analyses. For taxonomic profiles, the best hit classification at a maximum evalue of 1e⁻⁵, a minimum identity of 60% against the RefSeq database (O'LEARY *et al.*, 2016) were used. To identify genes and their functions, the reads were annotated against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (KANEHISA *et al.*, 2016) using default parameters and the KEGG web service. Analysis of variance and Student's t tests with a 5% probability threshold were also applied to verify the significance of the differences among the functional genes composition.

3. Results

A total of 39,187,976 and 47,908,961 sequences with an average length of 105 bp were generated from metagenomic sequencing of cave and surface samples, respectively. Taxonomic analysis showed the Bacteria domain as a dominant group in both environments (97.26% cave / 98.56% surface), but Archaea (1.89%) was the second dominant group in cave sample, while Eukaryota (0.62%) was the second dominant group in surface sample. Viruses were the smallest fraction of the sequences (0.17% cave / 0.14% surface). Within the bacteria communities, cave sample was dominated by *Proteobacteria* (59.19%) and *Actinobacteria* (41.92%) was the dominant group on surface sample. Most abundant bacterial taxa in cave and surface are shown in Figure 1. Archaea communities were composed mostly by *Thaumarchaeota* and within Eukaryota 10.69% and 44.12% of the sequences were fungi in cave and surface samples, respectively.

The assembled reads from cave and surface metagenomes resulted in 35,291,146 and 44,030,240 predicted sequences, respectively. Predicted known functions were 26.08% in cave and 23.85% in surface samples. Based on the KEGG classification system the functional profile was distributed in metabolism genes (60.59% cave / 61.17% surface), genetic information (18.44% cave / 17.59% surface), environmental information (15.07% cave / 15.01% surface), cellular processes (4.04% cave / 4.31% surface), human diseases (1.45% cave / 1.54% surface) and organismal systems (0.38% cave / 0.35% surface). Within metabolism function, a total of 11 categories were assigned, but only categories related to energetic pathways will be shown here. A total of eight energetic pathways were predicted based on gene

sequences. Cave microbiome stood out with the largest group of genes related to oxidative phosphorylation, carbon fixation in prokaryotes and nitrogen metabolism.



Figure 1. Taxonomic profile at phylum level for bacterial communities from dark zone (cave) and surface environments. Bar plot showing the dominant taxa into cave environment.

KEGG analysis of the metagenome identified putative genes from four known CO₂-fixation pathway in prokaryotes (Figure 2.1). Small fraction of the genes was associated with 3-hydroxypropionate bi-cycle, while the most putative genes were assigned to reductive acetyl-CoA pathway (Wood-Ljungdahl pathway), the 3-hydroxypropionate /

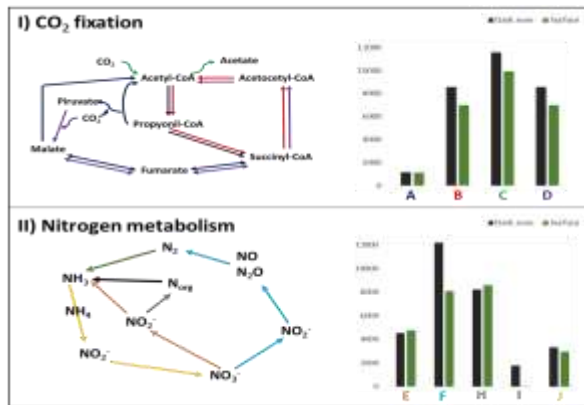


Figure 2. Overview of the identified genes related to CO₂-fixation (I) and nitrogen metabolism (II) from cave (black bars) and surface (green bars) samples. Bar plots show the number of sequences assigned to each pathway, where each colored letter represents a specific pathway. CO₂-fixation pathways show the 3-hydroxypropionate bi-cycle (dark blue - A), HP/HP cycle (red - B), Wood-Ljungdahl pathway (green

- C) and DC/HB cycle (purple - D). Nitrogen pathway stood out the nitrate reduction (orange - E), denitrification (blue - F), nitrate assimilation (gray - H), nitrogen fixation (dark green - I) and nitrification (yellow - J).

4-hydroxybutyrate (HP/HP) cycle and the dicarboxylate-4-HB (DC/HB) cycle. Genes assigned to carbonic anhydrase, responsible for reversible hydration of gaseous CO₂ to carbonic acid, were also detected in cave and surface samples.

An extensive analysis of nitrogen cycling genes was performed revealing genes related to nitrate reduction, denitrification, nitrate assimilation, nitrogen fixation and nitrification (Figure 2.II). Cave sample stood out regarding denitrification (35.40% cave / 27.64% surface) and nitrogen fixation (5.14% cave / 0.12% surface) genes in relation to surface sample. Finally, key genes required for ammonia oxidation by bacteria, such as ammonia monooxygenase (*amoA*) and hydroxylamine oxidase, were more abundant than ammonia-oxidizing archaea genes.

4. Discussions

Angélica cave showed a clear difference in the taxonomic composition of the cave and surface microbial communities, similarly to other cave metagenomes (ORTIZ *et al.*, 2014; MENDONZA *et al.*, 2016). *Proteobacteria* has been reported able to degrade many kinds of complex organic substrates, as a guano and plant debris (D'AURIA *et al.*, 2018). The core bacterial community also shows organisms able to use several resources, such as hydrocarbon (*Pseudomonas stutzeri*), nitroaromatic compounds (*Acidovorax sp. JS42*), toluene and ethylbenzene (*Aromatoleum aromaticum*). *Actinobacteria*, known for its high heterotrophic activity, play also an important biological role in biomineralization processes that lead to the formation of various secondary mineral deposits in caves (TOMCZYK-ŻAK & ZIELENKIEWICZ, 2016). In particular, carbonic anhydrase plays an important role in calcium carbonate precipitation and it is considered one of the key enzymes promoting carbonate mineralization (LU *et al.*, 2019). Thus, the presence of genes assigned to carbonic anhydrase enzyme suggests that the microbes from Angélica cave may be one of the drivers responsible for the calcium carbonate precipitation and cave lithology. This study showed the cave as a good host environment for functional genes involved in energy metabolism. The oxidative phosphorylation is recognized as one of the most important processes in all organisms, since it provides energy through heterotrophic pathways (GEL'MAN *et al.*, 1967). Unlike photosynthetic and glycolytic phosphorylation, the oxidative phosphorylation of ADP to ATP occurs through a series of electron transfer reactions by specific compounds (flavins, cytochrome) that form the respiratory chain. Angélica cave also showed the importance of chemoautotrophic microorganisms with different CO₂-fixation pathways. The Wood-Ljungdahl pathway is the only carbon fixation pathway that produces ATP rather than consumes it, making it the most efficient route for carbon assimilation used by bacteria and archaea. The Wood-

Ljungdahl pathway serves as a way for CO₂ fixation and energy conservation, which allows microorganisms to grow autotrophically on H₂+CO₂ (LEVER, 2016). The HP/HP cycle and the DC/HB cycle are considered key pathways in archaeal CO₂-fixation mechanisms (BERG *et al.*, 2010). These pathways are a marker for CO₂ fixation by *Thaumarchaeota*, the most abundant group of archaea in Angélica cave. Therefore, the abundance of archaeal genes for HP/HP cycle and DC/HB cycle shows that autotrophic archaea may contribute significantly to carbon fixation in Angélica cave. Higher number of denitrification genes into the Angélica cave than on the surface may show more insights into the energy metabolism, since these microorganisms can conserve energy by reducing nitrate. If denitrification is occurring, nitrate must be rapidly scavenged and cycled, or, alternatively, nitrate may be periodically available in specific microbial niches (HAMILTON *et al.*, 2015). Published research usually focuses on genes required for ammonia oxidation, which show to be more related to archaeas in cave environments (ORTIZ *et al.*, 2014; WISESCHAT *et al.*, 2019). Nevertheless, Angélica Cave showed more genes required for ammonia oxidation by bacteria. *Nitrosomonas*, *Nitrospira*, *Nitrosospira* - showed in the core bacterial community, also reinforce the result that bacteria are key agents in nitrogen metabolism. Previous studies in cave microbiomes attempt to identify the *nifH* gene as a biomarker for nitrogen-fixing bacteria (MARQUES *et al.*, 2018). However, five other genes are required for nitrogen fixation, and they were all detected in Angélica cave. The high abundance of nitrogenases genes within the cave compared to surface indicates that nitrogen-fixing bacteria should be favored with the availability of energy, from the external environment and chemoautotrophic activity, and low availability of organic matter inside the cave, following less competitiveness by resource and energy.

5. Conclusion

Angelica cave showed a high diversity of organisms with a wide range of genes related to metabolic functions. Although sequence information does not directly imply that the detected genes will be expressed, it is likely that the microorganisms have an inherent capability to perform such functions according to environmental conditions. Bacteria, the dominant group, has organisms related to heterotrophic and autotrophic activities. Wood – Ljungdahl pathway was the main process to CO₂-fixation in bacterial communities. Bacterial communities were also strongly linked to nitrogen metabolism, mainly nitrogen-fixation, ammonia oxidation and denitrification. Archaea domain, dominated by

Thaumarchaeota, was the main drivers in CO₂-fixation through the HP/HB cycle and DC/HB cycle. Therefore bacteria and archaea occupy different functional niches according to the genes detected. Then, Angelica cave is supported by heterotrophic, and the potential chemioautotrophic activities may be a supplementary pathway. It clarifies the different survival strategies in subterranean microbiomes in relation to surface environment. This study may support future research that intends to go deeply into the energy metabolism of cave microbiomes.

Acknowledgments

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The Moulis Cave (Ariège, S. France): from the CNRS Subterranean Laboratory to a Karst-&-Cave scientific platform

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Abstract

Located north of the Pyrenean range, Moulis Cave is known worldwide for its cave laboratory founded in 1948 and dedicated to the study of cave organisms and their karst environment. Geologically, this zone belongs to the Mesozoic cover of the Arize Massif. The 2.4 km²-karst system of Moulis Cave is developed in Jurassic limestones and dolostones.

The deep and upper parts of the cave remain in their natural state with a temporary stream and a variety of aragonite and calcite speleothems. The front part is formed by a fossil meandering corridor which has been partly artificialized to build the cave laboratory.

For almost 60 years, the CNRS Subterranean Laboratory of Moulis undertook research focussed on evolution and adaptation of species to subterranean life and on the genesis and dynamics of karst systems. In 2007, it was converted to a general experimental ecology station conducting research on both epigean and hypogean species and their ecosystems. Today, the Karst & Cave platform encompasses four caves near Moulis and supports research work on subterranean life, on karst deposits and a test lab for cave instrumentation. Subterranean laboratories are set up in two of these caves for breeding cave salamanders including *Proteus anguinus* and the Pyrenean *Calotriton asper*.

Résumé

La Grotte de Moulis (Ariège, S. France): du Laboratoire Souterrain du CNRS à une plateforme scientifique Karsts & Grottes

Située sur le piémont nord-pyrénéen, la Grotte de Moulis est mondialement connue pour sa grotte-laboratoire fondée en 1948 et dédiée à l'étude des organismes cavernicoles et de leur environnement karstique. Géologiquement, cette zone appartient à la couverture mésozoïque du Massif de l'Arize. Étendu sur 2.4 km², le système karstique de la Grotte de Moulis est développé dans les dolomies jurassiques.

Les parties profondes et supérieures de la grotte sont conservées dans leur état naturel et comportent un ruisseau temporaire et une variété de spéléothèmes aragonitiques et calcitiques. La partie frontale correspond à une galerie fossile partiellement artificialisée pour constituer la grotte-laboratoire.

Pendant près de 60 ans, le Laboratoire Souterrain du CNRS a développé des recherches sur l'évolution et l'adaptation des espèces à la vie souterraine et sur la genèse et la dynamique des systèmes karstiques. En 2007, il a été converti en une station d'écologie expérimentale qui conduit des recherches sur des espèces épigées et hypogées et sur les écosystèmes. La plateforme Karsts & Grottes comprend quatre grottes et développe des travaux sur la vie souterraine et les dépôts endokarstiques. Des laboratoires souterrains sont installés dans deux de ces grottes pour l'élevage des salamandres hypogées *Proteus anguinus* et *Calotriton asper*.

1. Introduction

The Moulis cave was selected at the end of the 1940's to develop the CNRS subterranean laboratory. In 2007, this research lab was then converted into a field and research ecological station of the CNRS (Centre National de la Recherche Scientifique). Gradually, this new station developed and evolved into a theoretical and experimental ecology station (SETE). The present-day CNRS-Toulouse

University structure, SETE includes both a research and scientific-service dimension. During the last ten years, several experimental scientific platforms have been developed on the site of Moulis and its area. The aim of this paper is to present the newly re-emerging Karsts & Caves platform.

2. Moulis Cave : the natural setting

Moulis cave is located in the northern zone of the Pyrenean range, in the western Mesozoic cover of the Arize Massif.

The karst system of the Moulis cave is developed in Jurassic limestones and dolostones in the Sourroque-Cap de la Pène synclinal and extends over 2.4 square kilometres.

Located at 787 metres altitude about 2.5 kilometres southeast from the entrance of Moulis cave, the Portillou cave contains the most upstream part of the Moulis system (CARRERE 1954). The stream from Portillou then flows through the lower unattainable part of Moulis cave and finally reaches the base level of the Lez River in the Moulis village at 426 metres of altitude (Fig. 1). The front part of

Moulis cave is formed by a fossil, weakly meandering corridor, the floor of which has been partly artificialized over the first few hundreds of metres, when building the cave laboratory. The natural entrance of the cave is rather narrow. Therefore, an artificial tunnel was dug to connect the main gallery to the outside. The deep part of this corridor remained in its natural state with a temporary stream and a variety of aragonite and calcite speleothems. The upper part of the cave is formed by upward galleries and large collapse rooms.

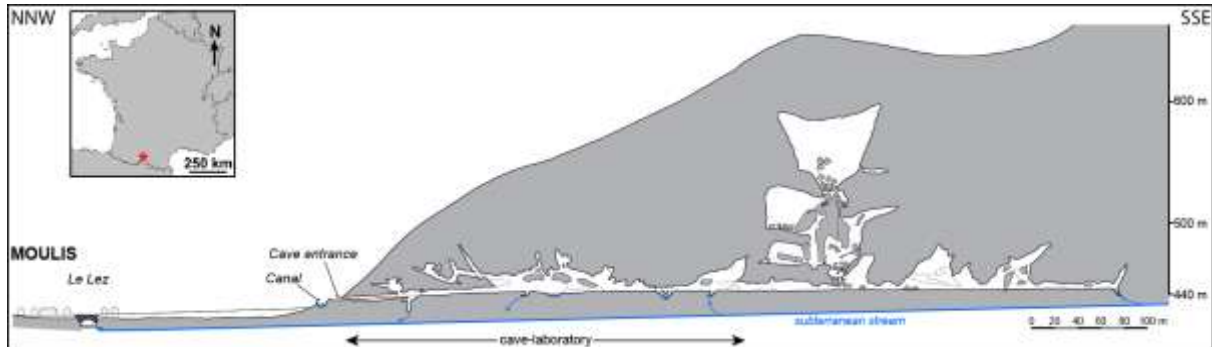


Figure 1: Location and schematic section of the Moulis Cave.

3. The CNRS Subterranean Laboratory history

After the World War II, under the leadership of René Jeannel and Louis Fage, both professors at the National Museum of Natural History, a commission of speleology was created at the CNRS, the main aim of which was the creation of an underground laboratory. The site of Moulis was selected because of its proximity to both the small city of Saint-Girons and the numerous extensive karst systems, and the occurrence of a large horizontal cave. In 1948, the CNRS Subterranean Laboratory was formally established. Albert Vandiel, professor at the University of Toulouse, was its first director.

For almost 60 years, the Subterranean Laboratory of Moulis developed multidisciplinary research focused on the evolution and adaptation of species to subterranean life and

on the genesis and dynamics of karst systems (e.g., ANDRIEUX 1969, GÈZE & RENAULT 1955, JUBERTHIE & GERS 1992, JUBERTHIE *et al.* 1996).

In 2007, the Moulis laboratory was converted into an experimental ecology station conducting research on both epigeal and hypogean species and ecosystems, beyond biospeleology and the biology of Pyrenean endemic species, which were its privileged research fields in the past. In the last decade, the ecological station gradually developed becoming the Theoretical and Experimental Ecology Station (SETE), and it has been equipped with several innovative experimental devices. Beside the research unit, SETE has built a suite of scientific platform services, including the Karst & Caves platform.

4. The Moulis Cave today

Seismological Station: The first device was installed in 1964. Set up in early 1986, the current seismograph is operated by the OMP (Observatoire Midi-Pyrénées, CNRS & Toulouse University) and integrated into the Permanent Broadband Network of the French seismological and geodesic network RESIF, launched in 2009 to federate, modernize and develop the means of geophysical observation of the internal Earth.

Study of subterranean life (Fig. 2): Research focusses on the ecology, behavior and physiology of cave salamanders in particular *Proteus anguinus*, which has been raised in the cave since the 50s from individuals imported from Slovenia at this time. Breeding of a population of about 100

individuals is maintained today in the cave in aquariums and cement ponds.

Study of karst deposits (Fig. 2): Aim of this research is to better understand the relationship between speleothem growth and climate, and to generate reliable information on past climatic changes in the Pyrenees. For this purpose, several climate parameters are monitored in the cave and outside, crystal farming stations have been set up in the cave since 2012 and studies of speleothems undertaken.

Test lab for cave instrumentation: Recently, a technical device for testing loggers used to monitor cave parameters has been set up in the cave room dedicated to

measurements and experimentation on karst deposits and cave climate.



Figure 2: The Moulis Cave today. Central image, the slab at the entrance of the cave-laboratory is made of the famous 'Grand Antique' marble, an Urgonian-Aptian tectonic breccia, from the neighboring Aubert quarry. 1. Entrance to the cave laboratory. 2. *Proteus anguinus* in an aquarium. 3. Artificial lake for water storage in the Vandel room. 4. Giant stalagmites in the Totem room in the upper parts of the cave.

5. The Karst & Caves scientific platform

This platform includes four karst caves located in the vicinity of the village of Moulis (Fig. 3): the Cave of Moulis, the cave of Aulignac (Les Bordes-sur-Lez), the cave of Sainte-Catherine (Balaguères), the cave of Aliou (Cazavet). The two first caves have been set up for the breeding of *Proteus anguinus* and *Calotriton asper*, a Pyrenean endemic salamander. Local invertebrate species can also be studied and the cave lab can host in captivity subterranean species from other caves from the temperate zone, for experimental research. More generally, all fourth caves can be instrumented and used for new projects.



Figure 3: The four karst caves of SETE.

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Low-cost temperature monitoring system in the Salnitro Cave: a bat roost cave in Southern Sicily

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Abstract

Salnitro Cave is a small cavity located on the right slope of the Carboj River canyon, in Southern Sicily. This cave is carved in Lower Jurassic limestone and is composed of three large chambers connected through short galleries. Its name derives from the presence of a huge bat guano pile which in the past was wrongly named as saltpetre. The guano is from different bat species, including: *Myotis myotis*, *Myotis capaccinii*, *Miniopterus schreibersii*, and occasionally single individuals of *Pipistrellus kuhlii*, *Rhinolophus euryale* and *Rhinolophus hipposideros*. The bats occur in the cave during the whole year, yet species and their phenology change, as well as the area they occupy. We observed periodic changes between the use of two cave rooms. In order to better understand the cave climate, a network of low-cost dataloggers was set to measure continuously underground air temperature and relative humidity every hour. The monitoring was conducted from June 2015 to June 2017, in the framework of the BART (BAt Roosts Temperature) project. In this paper we present the design of the measuring points and preliminary data of the features monitored.

1. Introduction

A fair number of Sicilian caves host large colonies of bats. Several studies about species, their distribution and abundance were carried out (RAGONESI, 1968; ZAVA *et al.*, 1986; MUCEDDA *et al.*, 2019). Recently, bat fossils have been studied for their palaeoecological and paleoenvironmental importance (SALARI *et al.*, 2019) and for their minerogenetic role (AUDRA *et al.*, 2019). More systematic studies on the Sicilian bats would be needed to update knowledge on the underground bat fauna, especially in relation to the continuous threats to their habitats and increasing anthropization. In Sicily, only a few studies concerned the climate of the caves where bats occur (FULCO *et al.*, 2014).

A first attempt to link the cave climate to the presence of bats was made in the framework of the BATs Roosts Temperature (BART) project. This project was conceived and

carried out by researchers from the Palermo University and from Le Taddarite speleological association of Palermo, from June 2015 to June 2017. It was focused mainly on the measurements of air temperature in bat roost caves using low-cost devices (FULCO *et al.*, 2015).

In order to understand the relationship between the cave climate and the bat communities, an environmental monitoring network composed of over 30 measurement points, has been set up in four caves in south-west Sicily (Salnitro Cave, Acqua Fitusa Cave, Personaggi Cave, Barone Cave). Three of these cavities host large bat colonies, while the fourth cavity (Barone Cave) is not used and serves as a control.

In this paper we present design criteria of the measurement network and preliminary data of environmental features in the Salnitro Cave.

2. Material and methods

The Salnitro Cave is a small cavity located on the right slope of the Carboj River canyon, in Southern Sicily (Fig. 1). This canyon, up to 150 m deep, is cut by the homonymous river which is blocked by a dam, roughly 1 km upstream of the cave, to form an artificial reservoir: the Arancio Lake. Around this lake a large number of vineyards are present, so widespread as to represent a monoculture. The Salnitro Cave is 260 m long and 18 m deep, and is composed of three large chambers connected through short galleries carved in

Lower Jurassic limestone (Fig. 1). Each room shows impressive sizes (length x width x height) for a so small cave. The southern chamber near the entrance (n. 1 in figure 1) is 22x22x10 m; the northernmost one is 32x20x15 m (n. 2 in figure 1); the eastern room (n. 3 in figure 1) reaches dimensions of 40x20x15 m. Condensation-corrosion morphologies such as mega-scallops, cupolas, chimineas and differential corrosion forms are widespread on the roof and walls of the entire cave (Fig. 2).

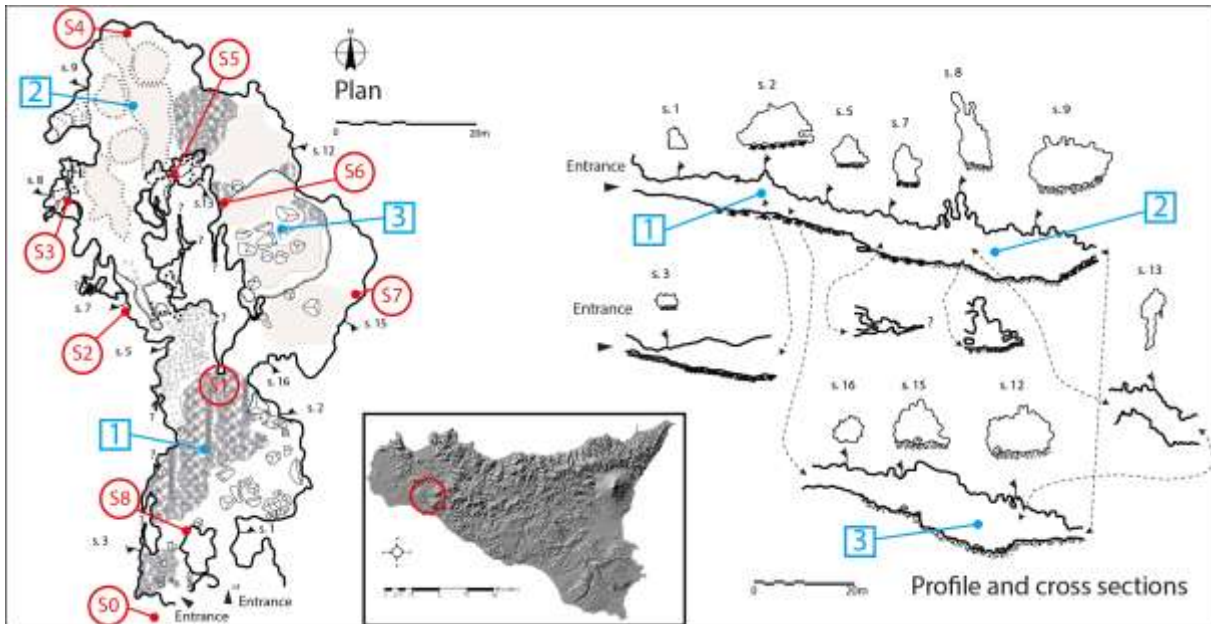


Figure 1: Salnitro Cave survey (by: A. Fulco, P. Valenti and M. Vattano). On the plan view the datalogger position is shown in the red circles. Blue squared labels indicate the different chambers of the cave. In the black frame the cave location in Sicily is highlighted.

Great amounts of bat guano cover the floor of the chambers n. 2 and n. 3 (Fig. 2). In the last one breakdown deposits are present too. The cave name really derives from the presence of the huge bat guano deposit wrongly indicated as saltpeter, which in the past was intensely excavated. The excavation marks still present in the cave, and the signs of displacement and stacking of the rock debris permit to assume that about 800 m³ of guano was extracted from this cavity.

In the Salnitro Cave a monitoring network composed of 8 measuring points has been created choosing the most representative cave environments (Fig. 1). Air temperature (T) and relative air humidity (RH) low-cost dataloggers (Lascar Electronic EN-EL-USB1 and EN-EL-USB2, respectively) were used from June 2015 to June 2017. For each sensor, measurable *T range* is -35 to + 80 °C with a resolution of 0.5 °C, the measurable *RH range* is 0 to 100%. The dataloggers are protected against moisture to IP67 standard (complete protection against any dust entering and submersion for 30 minutes up to 1 meter depth) and are equipped with a long-life replaceable lithium battery. At each point a couple of dataloggers were positioned at a distance of about 50 cm to each other and at a height of 2-3 m above the floor. In particular, at five points the pair consisted of two T dataloggers (S1, S2, S3, S5, S7, S8), and at three of T and RH dataloggers (S0, S4, S6). Unfortunately, RH data were recorded only by one sensor (S4) as one instrument was burned by an outer wildfire (S0) and the other malfunctioned (S6). Each instrument was set up to register data hourly, and data download was done every three months. For each pair of temperature dataloggers the collected data were averaged.

The cave hosts a large plurispecific colony of bats occurring mainly in the ceilings of the largest rooms (chambers n. 2 and n. 3 in figure 1). From June 2015 to June 2017 bat counting and species recognition were carried out. Due to the large number of individuals in the colonies, we used a camera to conduct the census.

For bats in multi-layered groups this method brings only one value, which should be considered minimal (AGNELLI *et al.*, 2004).



Figure 2: View of the northern chamber in the cave (chamber n. 2 in figure 1). On the roof, several corrosion-condensation cupolas are visible as well as the differential corrosion forms of the walls. The cupolas in the center of the photo, characterized by the classic black "spots", are one of the main sites chosen by bats. Below cupolas the current guano deposits can be seen (Photo by M. Vattano).

3. Results

Our studies identified different species living in the cavity, such as: *Myotis myotis*, *Myotis capaccinii*, *Miniopterus schreibersii* and, occasionally, single specimens of *Pipistrellus kuhlii*, *Rhinolophus euryale* and *Rhinolophus hipposideros*. *M. capaccinii* and *M. schreibersii* roost in small clusters of a few specimens, while *M. myotis* in multi-layered groups. Some individuals of *M. schreibersii*, though, prefer to temporarily inhabit the passage connecting the chambers n. 2 and n. 3 (Fig. 1), and remain more isolated from the main colony. For this reason, we decided to place dataloggers also in this area.

Bat colony occurs in the cave during the whole year, yet species composition and phenology change, as well as the areas they occupy. Indeed, we observed periodic shifts between the ceilings of the two largest cave rooms (chambers n. 2 and n. 3 in figure 1).

The picture used for the census, taken during breeding, showed multi-layered groups with a true minimum consistency of 480 individuals, so we expect the colony to be of at least one thousand individuals.

Breeding is considered to be the period with the highest presence of both adults and young individuals throughout the year.

Preliminary results from the monitoring activity are shown in figure 3 where RH and T monthly averages are reported. The external temperature (S0) shows higher values during the spring-summer semester and lower values in the autumn-winter seasons.

A higher positive peak is visible between July and October 2016, due to a huge wildfire that took place in the whole canyon affecting also the cave entrance area.

The air cave T has a similar trend to that of the external air T, even if the variations are recorded with a delay of variable time depending on the distance from the entrance. This time delay ranges from 0-1 month for the sensors nearest to the entrance area (S1, S7, S8) up to 3 months for the inner points (S3, S4, S5, S6).

The dataloggers nearest to the cave opening (S8) recorded the more extreme values for both Tmax (22.9 °C) and Tmin (13.5 °C). The 2016 Tmax value is most alike the external one, compared to 2015, due to the wildfire mentioned before. The innermost dataloggers show, as expected, a lower oscillation between Tmax and Tmin which in position S4 is between 16.5 and 13.7 °C.

RH in the innermost part of the cave (S4) ranges between 80 and 100%.

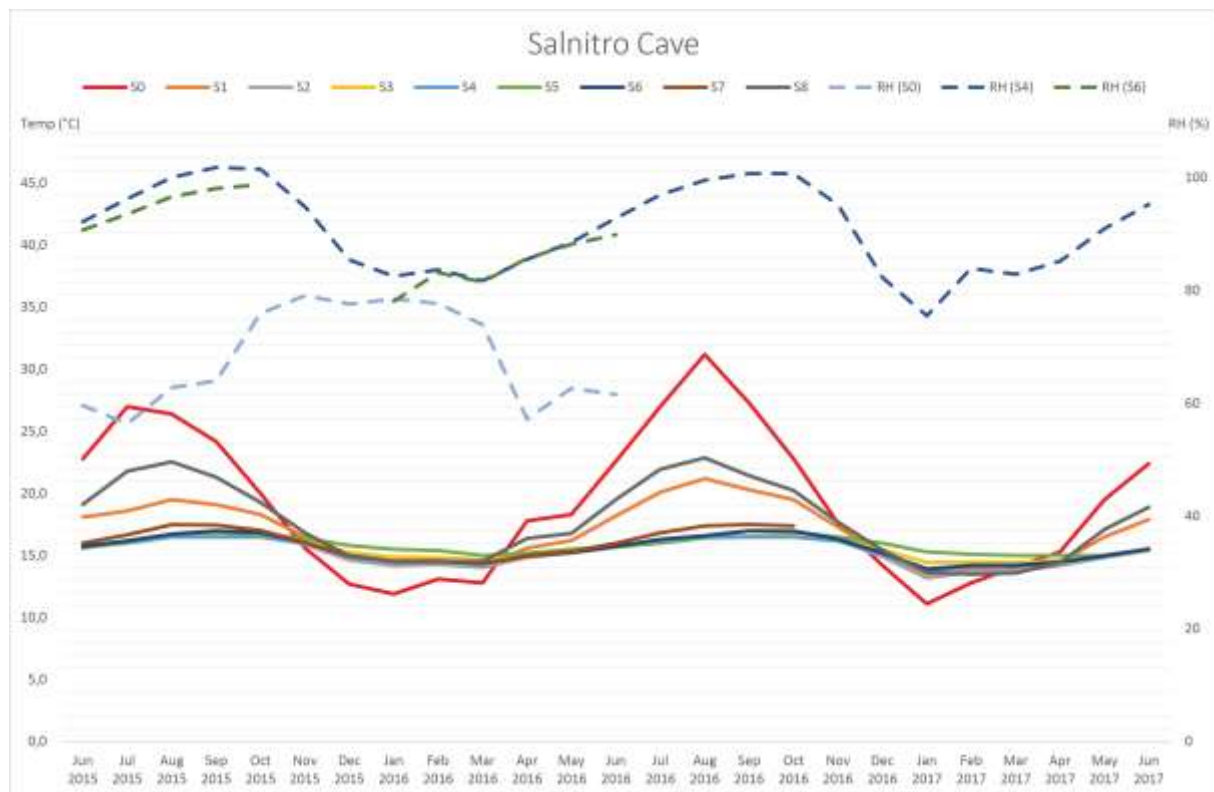


Figure 3: Graph showing the monthly averages of the pairs of dataloggers positioned in the Salnitro Cave from June 2015 to June 2017. The location of the data loggers is indicated in figure 1. Dashed lines indicate the relative humidity values.

5. Discussion and Conclusions

The monitoring activity allowed to define a preliminary characterization of the air temperature in the Salnitro Cave. The cave environment shows a different response to external thermometric variations, according to the distance from the outside, as it is easy to predict. In general, the innermost passages (chambers n. 2 and n. 3 in figure 1) have up to three-month phase shift with external temperature variations and are the least affected by external climate events (Fig. 3). In these passages, indeed, air temperature has variations less than 3 °C in the considered timeframe. This is a positive factor for the presence of the colony thus developing its phenology in this very environment. Another favorable condition is the presence of droppings and urine, confirming the significant size of the colony and its long-lasting loyalty to the site. The accumulation of excrements stabilizes temperature and humidity (McWILLIAM, 1988) or increases the temperature within the breeding colonies (McCRACKEN & GUSTIN, 1991; BONACCORSO *et al.*, 1992).

Our data and observation allowed us to suppose that there is a strong correlation between climate stability conditions and the occurrence of a colony at microclimate level.

Actually, according to the first results there is evidence that the great reproductive colony of *M. myotis* permanently occupies only the deepest rooms which are also those with a more stable climate.

Other species, in smaller clusters or solitary individuals, occupy both chambers 2 and 3 and a small passage connecting the two rooms. At the current state of the art, dynamics of movements, which are to be monthly correlated with the annual phenology, are not clear yet. Moreover, the climate conditions needed by these species are extremely variable due to a phenology characterized by swarming, hibernation and mating phases.

The detailed analysis of the data, currently underway, will allow us to better define the relationships between the cave temperature and the activity of the bat colony.

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GIS Modeling of the Ranges of Endangered Troglobites

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Abstract

Cavernous outcrops along the Balcones Fault Zone in Texas, USA, contain troglobitic terrestrial invertebrate species federally listed as endangered by the US Fish and Wildlife Service to insure their survival. Previous studies used simple and broad statistical methods to define 10 karst fauna regions (KFRs), distinct ecological regions used to manage and protect the species. This investigation uses hydrogeologic and biological data to create a Geographic Information System model that maps the ranges of the 39 troglobites limited to the study area from 479 localities.

Following extensive evaluation of modeling methods, the best analyzes the range of distribution for each species, instead of modeling the effects of physical conditions on species distribution. The clustering of multiple range margins is interpreted to reflect the presence of a barrier or restriction to species distributions *a posteriori*. Geologic contacts, faults, streams, soils, and other factors are examined carefully in areas where the range margins cluster to determine if they may create a barrier or restriction. Where no hydrogeological explanation is found for a cluster of range margins, subsurface ecological conditions are assumed as the likely cause. Results from this technique generally support the earlier KFR boundaries but provide important revisions and biological insights.

1. Introduction

The eastern margin of the Edwards Plateau, along the Balcones Fault Zone in Texas, USA, is a bio-geologically complex region. Species living in its caves and related voids have become physically isolated from each other through time, resulting in genetic isolation that has produced new species known to occur only within small geographic areas. The expansion of Austin and neighboring communities onto the karst where these species occur poses a threat to their survival due to the destruction and sealing of caves and karst features, changes in nutrient and moisture input into the karst ecosystem, contaminants introduced into the karst ecosystem, and competition with and predation by non-native species introduced by urbanization (ELLIOTT, 1993, 2000).

Seven terrestrial troglobitic species of karst invertebrates in the Austin, Texas, region are federally listed as endangered by the US Fish and Wildlife Service (USFWS) to insure their

survival (USFWS, 1988; 1993). In 1992, GEORGE VENI & ASSOCIATES examined the stratigraphic, structural, and hydrological controls on cave development in the Austin area, coupled with an evaluation of the distribution of troglobitic species. They subdivided the area into major ecological communities called Karst Fauna Regions (KFRs), and into four Karst Zones for habitat management, which USFWS has used to guide species recovery efforts. VENI & MARTINEZ (2007) digitized the original 1992 paper maps in a Geographic Information System (GIS) and updated the KFRs and zones.

The current investigation further updates the biological database and combines it with hydrogeological data to construct a GIS model for a robust, detailed, objective, statistical analysis of factors that might influence troglobite distribution, and use those results to modify KFR and zone boundaries as appropriate to the results.

2. Methodology

A total of 479 localities are identified for the seven endangered species as well as 32 non-endangered terrestrial troglobites. They occur along a 160-km north-south reach of the Balcones Fault Zone, with an east-west extent of up to 50 km. The 39 species include seven genera (one genus of carabid beetle, mold beetle, harvestman, millipede, pseudoscorpion, and two spider genera) but do not include all troglobites known in the study area, only those restricted to the study area which would provide analytical insights to identify barriers or restrictions to species distribution that would promote speciation. The 39 species include invertebrates that occupy the same ecological niches as the endangered species, to better

constrain the distribution of the endangered species. The locations and names of the localities were verified to the extent possible, significantly improving the precision of their geographic coordinates and eliminating duplication and other problems resulting from naming errors.

These biological data were entered into a GIS along with layers of other potentially relevant data. We attempted multiple methods to identify the most accurate means of evaluating the distribution of the troglobite species. Using models of species richness, endemicity, clustering, spatial autocorrelation, occurrence omission, average and prediction for species and caves, and with a variety of GIS tools such as DIVA (HIJMANS et al., 2012), Maxent (PHILLIPS

et al., 2020), and SMD (BROWN, 2017), we tested factors involving geology, hydrology, cave microclimates, surface climate, vegetation, and soils for their potential effects on species distribution. Most didn't have sufficient data, enough details, or the requested resolution. Other data varied in quality and resolution over the study area in ways that might bias the results.

Following this extensive evaluation, we determined the best modeling method was to reverse the GIS analysis. Rather than model the effects of various physical conditions on the species' distribution, we determined and analyzed the distribution range of each species. Range margins were established by combining a standard range for the species based on sampling data, with the ranges for localities beyond those standard ranges based on a set of more complex criteria. The clustering of multiple range margins within a limited area was then interpreted to reflect the presence of a barrier or restriction to species distributions *a posteriori*. Geologic contacts, faults, streams, soils, and other factors were then examined carefully in areas where

the range margins cluster to determine if they may create a barrier or restriction to the species' distribution. Where no hydrogeological explanation is found for a cluster of range margins, subsurface ecological conditions beyond the scope of this investigation are assumed as the likely cause.

A detailed series of modeling steps determined the areas each of the 39 species likely occurs in based on its known localities, with 3-km radius defining minimum areas for single-site endemic species, assuming the presence of cavernous rock. Critical to this model was the fact that most troglobites in the study area are allopatric in their genera, thus where the range of one species ends, another will usually start. Where clusters of range margins occurred within an objectively pre-defined set of parameters, they suggest potential barriers or restrictions to species distribution. Criteria were also established to determine if range margin clusters reflected a KFR boundary. Additionally, previously established criteria for delineating karst zone boundaries (VENI & MARTINEZ, 2007) were expanded to include the modeling results.

3. Results

The GIS modeling identified 15 range margin clusters that were studied as potential KFR boundaries. Figure 1 focuses on one area as an example of the results. The south end of the figure shows nine species ranges occur at and north of Brushy Creek. Two are excluded from further consideration because only small portions of their modeled range margins extend into the cluster. Also, their nearest known localities are about 11 km and 15 km north of Brushy Creek and are probably not reliable indicators of boundary conditions along the creek. The area of the seven remaining range margins exceeds the pre-defined span for a cluster, but excluding the most distant margin, produces a six-species cluster within the defined limits. Three other species occur within the cluster area, but their margins are not part of the cluster. With six of the ten species in the area (60%) occurring within the cluster and no other modeling artifacts observed, the cluster suggests a potential KFR boundary.

Examination of geologic maps of the Brushy Creek area shows faults are not an influence in species distribution since the range margins are perpendicular to the faults and two of the species in the area occur on each side of the faults; the rest of the species are located away from the one, small faulted area near Brushy Creek. There were also no differences in lithology

to account for the cluster; all the caves are in the Edwards Limestone.

The only remaining hydrogeologic constraint is Brushy Creek which narrows the cavernous unit to 2.8 km and reduces its thickness. While the available Edwards Limestone increases eastward to nearly its full thickness, the almost perennial flow of Brushy Creek keeps the Edwards saturated and inaccessible to terrestrial troglobites except during drought conditions. The presence of a KFR boundary along the creek is also supported by the locations of the six species in the cluster. While the modeled margins of the cluster extend over 5 km north of Brushy Creek, none of the species are found north of the creek. This cluster supports the presence of a KFR boundary along Brushy Creek, which is a restriction to species distribution because it limits but does not absolutely prevent troglobitic species from crossing the boundary.

In contrast, the previous KFR studies identified the South Fork of the San Gabriel River, near the north end of Figure 1, as a KFR boundary. However, the few range margins in that area did not qualify as a cluster, and that KFR boundary was removed. Similar model analysis of the other 13 range clusters, with comparison to hydrogeologic features, supported, modified, and created new KFR boundaries in the study area.

4. Discussion and Conclusions

The GIS modeling analysis of species distribution across the varied hydrogeological landscape of the study area proves a useful tool to objectively quantify the potential presence of KFR boundaries, especially with the substantial limits on available data and types of data for the model. The results support the initial hypothesis from GEORGE VENI & ASSOCIATES (1992), used conceptually to define Karst Zone 2, that if cavernous rock is present and appropriate habitat conditions exist, then its caves will contain troglobitic species. KFR boundaries occur where cavernous rock becomes absent, thin and/or narrow, and filled at least

periodically filled with water. Faults create boundaries only where they juxtapose cavernous and non-cavernous rock. While some faults and other geologic factors may have local effects, no effects are seen at the scale of the KFRs.

Most of the KFR boundaries are restrictions, not barriers to species distribution. Some boundaries may not have restricted species in the past, as indicated by certain species occurring on each side of a boundary, but the boundaries are based on current conditions which dictate management needs. Whether or not the endangered troglobites are present in an area also depends on biogeographical factors

beyond the scope of this investigation to assess, such as competition with other species and microclimatic conditions.

Of the eight original KFR boundaries, five were supported by this GIS analysis, three were modified, and one was eliminated. Additionally, two new boundaries were established, one of which divided an existing KFR into two. Further, eight informal KFRs were established for the regions that constrain the distribution of the endangered species. Lastly, an undesignated KFR was established, comprised of small, isolated areas of the cavernous rock unit outside the formal and informal KFR boundaries. These areas have not been studied for caves or karst fauna and cannot be included nor excluded from adjacent KFRs until they are investigated.

The karst zones were modified in expected and new ways. With more localities reported for endangered species since the revisions in 2007, Zone 1, where endangered species are known to occur, grew to fill many of the former Zone 2 areas, which had a high probability for their presence. The number of zones expanded from the four previous to include two subzones for Zone 3 and 4 to better identify their biological status and manage their ecosystems, based on their potential for different ecological communities vs. potential cave and karst occurrence. In addition to the new localities, the GIS troglobite distribution modeling proved a valuable tool in revising the karst zones, along with the improvement of cave location precisions, and associated cave and species updates and information.

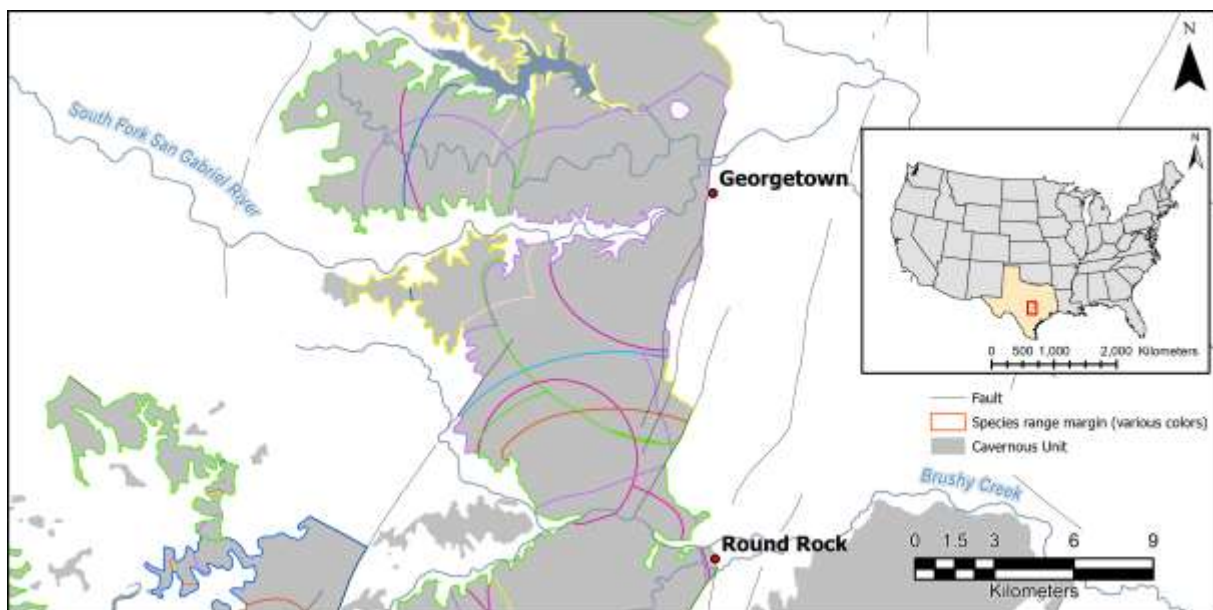


Figure 1: Troglobite species ranges in a portion of the study area, showing clustered range margins along Brushy Creek but no clustering along the South Fork of the San Gabriel River. The red rectangle in the US map outlines the entire study area; the detailed range map above shows only a small section of that area.

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Can an artificial cave be a hotspot for mollusc diversity? Overview of land snail biodiversity underneath Paris and surrounding area

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Abstract

Since the birth of biospeleology in the 19th century, researchers have studied all groups of invertebrates living in subterranean ecosystems. Similarly to natural caves, artificial cavities provide suitable habitats for a distinct underground fauna, a big part of which remains poorly understood. Mollusc investigations in underground quarries are indeed limited because of their lower malacological biodiversity. Some 10–40 m underneath Paris, kilometers of passageways are the result of previous exploitation of limestone, chalk and gypsum, establishing an underground network used and visited by underground workers and explorers for centuries. This work presents the first large-scale inventory of the malacological biodiversity in the Parisian underground quarries. Seven eutroglophiles, one endogean and seven troglonexes taxa were identified. *Oxychilus* species are the most common in quarries and the occurrence of *Zonitoides arboreus* is a new mention for the Paris region. The inventory of molluscan taxa in the Parisian quarries considers the relationship between human activities and the environment in this particular ecosystem to better understand quarries' biodiversity. The organic allochthonous material introduced by man and the molluscan carnivorous occasional feeding behavior seem to be two conditions that favor the presence of eutroglophile molluscs.

Résumé

Une grotte artificielle peut-elle être un hotspot pour la diversité des mollusques ? Aperçu de la biodiversité des escargots terrestres sous Paris et sa région. Depuis l'avènement de la biospéléologie au XIX^e siècle, les chercheurs ont étudié tous les groupes d'invertébrés vivant dans les écosystèmes souterrains. Tout comme les grottes naturelles, les cavités artificielles sont des habitats propices à une faune souterraine particulière, dont une grande partie demeure mal connue. En effet, elles sont peu étudiées en raison de leur plus faible biodiversité malacologique. Entre 10 et 40 mètres de profondeur sous Paris et dans les environs, des kilomètres de galeries sont le résultat de l'exploitation antérieure du calcaire, de la craie et du gypse. Pendant des siècles, l'ensemble du réseau souterrain a été utilisé et fréquenté par les travailleurs et les explorateurs. Cette étude présente le premier inventaire à large échelle de la diversité malacologique des carrières parisiennes. Sept taxons eutroglophiles, un endogé et sept troglonexes ont été identifiés. Les espèces d'*Oxychilus* sont les plus courantes dans les carrières et la présence de *Zonitoides arboreus* est une mention inédite pour la région parisienne. L'inventaire des mollusques dans les carrières parisiennes met en évidence l'importance d'appréhender les relations entre l'homme et l'environnement dans cet écosystème particulier pour mieux comprendre sa biodiversité. La matière organique allochtone apportée par l'homme et le régime carnivore occasionnel de certains mollusques semblent être deux conditions favorables au développement des mollusques eutroglophiles.

1. Introduction

Caves and cavities are suitable environments for the penetration and development of non-marine mollusc populations (GERMAIN 1911; JEANNEL 1943; WEIGAND 2014). However, the community of malacologists still lack an updated and complete synthesis of the land snail species living in the French subterranean environments. In mainland France, they comprise one troglobiont species (*Zospeum bellesi* Gittenberger, 1973; Ellobiidae), several eutroglophiles (such as Agriolimacidae, Arionidae, Discidae, Gastrodontidae, Limacidae, Oxychilidae), some endogean (e.g. Helicodiscidae, Ferussaciidae, Testacellidae), a wide diversity of stygobionts (e.g. Bythinellidae, Hydrobiidae,

Moitessieridae), and some bivalves (e.g. Dreissenidae, Sphaeridae) (GERMAIN 1911; JEANNEL 1943; VANDEL 1964; BERNASCONI & RIEDEL 1994; KERNEY et al. 1999; PRIÉ 2019).

Like natural caves, former underground quarries provide a relevant interface to observe and study subterranean life. Although a review of the literature shows that some mollusc species are known to be occasionally found in different types of underground habitats (VANDEL 1964; BERNASCONI & RIEDEL 1994; WEIGAND 2014; PRIÉ 2019), no specific work has been carried out on the malacofauna of artificial cavities. The pioneering biospeleological work of VIRÉ

(1896) in the catacombs of Paris did not mention any occurrence of molluscs (GÉRARDS 1908). BALAZUC et al. (1951) were the first authors citing malacofauna from the ancient Parisian quarries but only few taxa have been recorded. Unlike natural caves, underground Parisian quarries represent a peculiar anthropogenically modified ecosystem. Since the time of their exploitation, quarries have been regularly visited and transformed. In natural cavities, the vulnerability of subterranean habitats and their fauna to disturbance or pollution is generally accepted (MAMMOLA et al. 2019). We wonder whether the latter factors lead to malacofauna impoverishment through the degradation of habitats. Our objectives were to i) study the extant malacological biodiversity and its distribution, and ii) assess the potential impact of human activities on this taxonomic diversity.

Initiated by Marina FERRAND (second author of this article) in 2016, an extensive and generalist biospeleological inventory has greatly increased our knowledge on the Parisian subterranean invertebrates. Many more locations have been investigated since, allowing the list of identified taxa, including occurrence information, to be significantly extended (e.g., GEOFFROY & FERRAND 2020; DELFOSSE et al. 2020). We note that particular emphasis has been put on molluscs since 2018. We present here the first overview focusing on the land snails living in the former Parisian underground quarries. Our aim was to provide a list of the taxa that commonly dwell in artificial caves and to discuss their distribution in this unique subterranean environment.

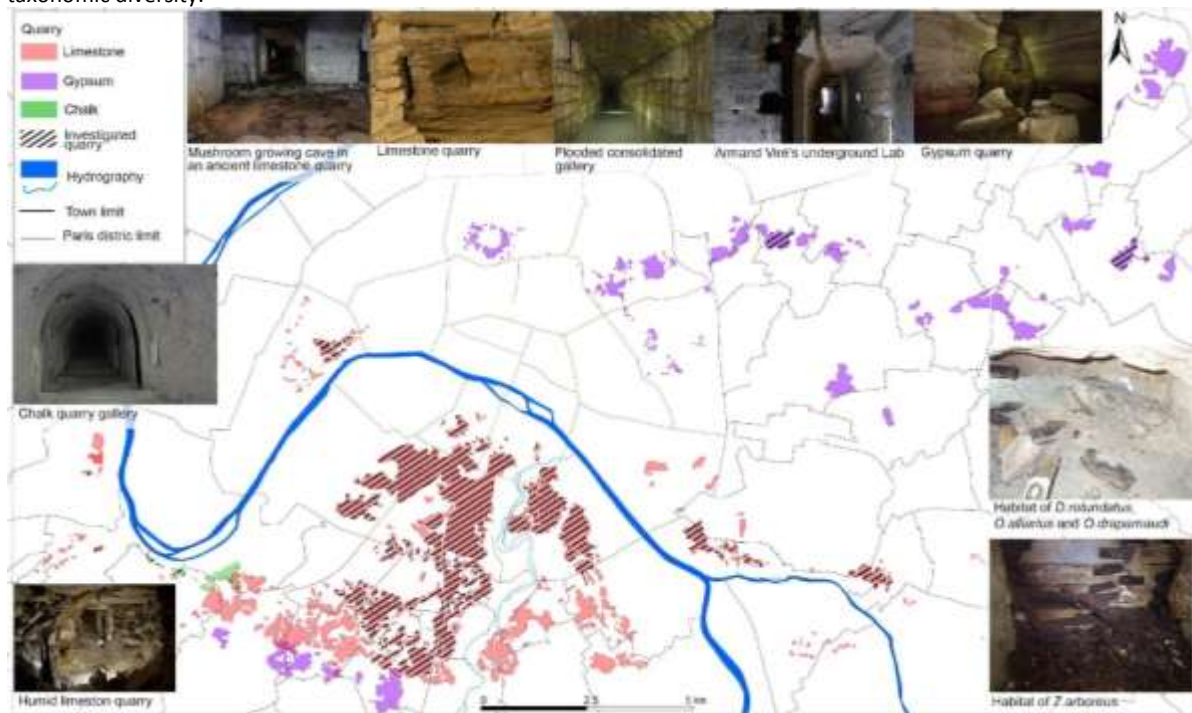


Figure 1: Map of the Parisian quarries (limestone, gypsum and chalk) presenting the context of the study, several types of subterranean environments and molluscs habitats

2. Materials and methods

Tertiary beds in the center of Paris Basin provide many geological resources. Today, at a 10 to 40-meters depth beneath the surface, kilometers-long galleries result from the former exploitation of underground quarries. In Paris and surrounding area, artificial caves of limestone, gypsum, and chalk represent a complex network with a total surface area of than 3000 hectares (limestone: 2349 ha; gypsum 651 ha; chalk: 65 ha; source: IGC <https://www.paris.fr/pages/tout-savoir-sur-les-sous-sols-2317>, last consultation:12.20) (Fig. 1). Some of them comprise kilometers of galleries whereas others are only hundreds of meters long. In the subterranean environment, physical conditions commonly fluctuate but obscurity is complete. The temperature varies between 12°C and 19°C (c. 14°C on average) and the constant humidity reaches 80%. Water is present in the form of puddles and basins, and in some galleries, water level reaches 1.5 m. Because

limestone and chalk are exploited, this environment is commonly calcareous. Most of the organic matter is of human origin (e.g., wood consolidation, underground explorer waste). Indeed, this underground network has been visited for centuries. Since the eighties, cataphiles (i.e., underground explorers) transform parts of the catacombs of Paris for events and activities (e.g., parties, meetings, construction, restoration, art). Knowing that mollusc density is generally low in subterranean ecosystems, our sampling protocol was designed to observe as many species and individuals as possible. To do so, we investigated mollusc diversity mostly focusing on organic-rich and humid areas (Fig. 2A) by favoring a reasoned sampling plan (CUCHERAT & DEMUYNCK 2008). Occasionally, random areas were also investigated (Fig. 2B). We carried out a direct method of collection by hand picking land snails and slugs (Fig.2C).



Figure 2: Biospeleological on A) humid, decayed wood, B) at a random sampling location and C) handpicking specimens.

Live specimens collected were systematically preserved in 96% alcohol for possible future genetic analysis. Concurrently, we developed a citizen science approach involving the cataphile community on social networks (Forums, Facebook, Discord). Observations and pictures done by underground explorers have been gathered and data was checked by the authors. Specimens were identified based on conchological characteristics, using malacological guides (KERNEY et al. 1999; WELTER-SCHULTES 2012). When identification was challenging (for Oxychilidae for instance), specimens were determined after dissection. Dry material and material in alcohol, fully labelled, are curated by Quentin WACKENHEIM and kept in his personal collection.

3. Results

Since 2016, 37 underground quarries from different exploitation types have been investigated (gypsum: 2, chalk: 2, limestone: 33) (Fig.1). They are spread over 15 towns, in Hauts-de-Seine (Bagneux, Châtillon, Malakoff, Meudon, Montrouge, Saint-Cloud, Sèvres), Val-de-Marne (Arcueil, Cachan, Charenton-le-Pont, Saint-Maurice), Seine-Saint-Denis (Gagny, Romainville), and Paris (5th, 6th, 13th, 14th, 15th, 16th city districts).

Altogether 15 molluscan taxa were recovered. They are classified into three groups: the eutroglophiles (7 taxa), the troglloxenes (7 taxa), and the endogean (1 taxon). We consider a gastropod species to be a troglloxene when it was only found occurring close to the entrance (and not deep in the quarry), whereas a eutroglophile snail is commonly found inside the cave (SKET 2008).

List of the taxa occurring in the former Parisian quarries

Eutroglophile

Discidae

Discus rotundatus (Müller, 1774)

Gastrodontiidae

Zonitoides arboreus (Say, 1816)

Oxychilidae

Oxychilus alliarus (Miller, 1822)

Oxychilus cellarius (Müller, 1774)

Oxychilus draparnaudi (Beck, 1837)

Limacidae

Limax maximus Linnaeus, 1758

Arionidae

Arion distinctus Mabille, 1868

Endogean

Ferussaciidae

Cecilioides acicula (Müller, 1774)

Troglloxene

Lauriidae

Lauria cylindracea (Da Costa, 1778)

Valloniidae

Acanthinula aculeata (Müller, 1774)

Clausiliidae

Cochlodina laminata (Montagu, 1803)

Hygromiidae

Trachulus hispidus (Linnaeus, 1758)

Helicidae

Cepaea nemoralis (Linnaeus, 1758)

Cornu aspersum (Müller, 1774)

Helix lucorum (Linnaeus, 1758)

4. Discussions

The considerable surface of former quarries makes it difficult to investigate each gallery extensively. Implementing a citizen science approach by encouraging cataphiles to share their observations of underground invertebrates has improved and enlarged our work. Overall, by listing 15 molluscan taxa, the present study completes the seminal work of BALAZUC et al. (1951) who provided only little information about the malacofauna living in the former Parisian quarries. Mollusc populations appear to be slightly isolated from each other; only one or two species are generally found in the same observation site, more rarely three. However, in some cases, the input of troglloxene species near entrances provides a higher diversity. Most of the molluscs recorded were sampled from rotten wood or humid gallery walls. According to our observations, moisture and the presence of organic matter (mostly dead wood) (Fig.

1) seem to be two ecological factors that determine the presence of eutroglophiles snails and slugs.

Despite its low density, the underground malacological community is characterized by a relative abundance of *Oxychilus* species which appear to be the most common land snails in the Parisian quarries. *Oxychilus draparnaudi* (Beck, 1837) is one of the most widespread and generally inhabits places rearranged by cataphiles or deadwood-rich habitats. The introduced Gastrodonti *Zonitoides arboreus* (Say, 1816) is still poorly known in France. The identification of several populations of *Zonitoides arboreus* under Paris is a new occurrence for this taxon in the north of France and the Parisian region. The discovery in May 2020 of several specimens living on rotten wood used for consolidating an isolated gallery (Fig. 1) supports the link between human activities in quarries and the introduction of allochthonous mollusc species.

The eutroglophiles identified are tolerant and require organic matter. In this peculiar environment, organic matter is provided by human allochthonous input and by the subterranean biomass itself. The occasional carnivorous feeding behaviour of Gastrodontidae, Oxychilidae and slugs favor their adaptation underground.

Furthermore, the anthropic organic input contributes to maintain suitable ecological conditions and to support the food chain. Presence and diversity of surface and subsurface

fauna are a prerequisite for the penetration of eutroglophiles molluscs (WEIGAND 2014). Since Paris and surrounding areas are largely urbanized, molluscs' local habitats are restricted and fragmented. Consequently, the Parisian urban malacological diversity tends to be homogeneous and characterized by tolerant species (CLERGEAU et al. 2011; WACKENHEIM 2017). The molluscan underground inventory we compiled is consistent with the latter observation.

5. Conclusions

The mollusc inventory in the Parisian quarries highlights the importance to consider the relations between human activities and the environment in this peculiar ecosystem to better understand its biodiversity. This study leads to the following main conclusions:

- this first overview focused on mollusc diversity in subterranean quarries completes the few data published by BALAZUC et al. (1951): the malacological diversity of the former Parisian underground quarries comprise 7 eutroglophiles, 1 endogean and 7 troglonexes species.

- involving cataphiles in a citizen science approach improves and expands our investigations.

- *Oxychilus* species are the most common land snails in the Parisian quarries and the occurrence of *Zonitoides arboreus* is a new record for Paris area.

- organic matter provided by human allochthonous input and the molluscs' occasional carnivorous feeding behavior seem to be two conditions that favor the development of eutroglophiles molluscs.

Acknowledgments

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Microniphargus, first results of phylogenetic analysis

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Abstract

The 1.2 – 1.5 mm long *Microniphargus* was collected from Ireland, England and Belgium. The specimens do not show significant morphological differences. Two genetic markers, Folmer fragment of the cytochrome *c* oxidase subunit 1 (COI) and Verovnik's fragment of the nuclear 28S ribosomal gene (28S) indicate that the status of *Microniphargus* as a genus is justified, and that it is a sister clade to *Pseudoniphargus*. It must therefore be included in the family Pseudoniphargidae. Species delimitation models indicate that *Microniphargus* comprises several species. A new project at Université libre de Bruxelles aims to study *Microniphargus* in greater detail.

Résumé

Microniphargus, premiers résultats de l'analyse phylogénétique. Des exemplaires de *Microniphargus* mesurant 1,2 à 1,5 mm de long ont été collectés à la fois en Irlande, en Angleterre et en Belgique. Les spécimens ne présentent pas de différences morphologiques significatives. Les deux marqueurs génétiques utilisés, le fragment de Folmer de la sous-unité 1 du cytochrome *c* oxydase (COI) et le fragment de Verovnik du gène ribosomal nucléaire 28S (28S), indiquent que le statut de *Microniphargus* en tant que genre est justifié et qu'il s'agit d'un clade frère de *Pseudoniphargus*. Il devrait donc être inclus dans la famille des Pseudoniphargidae. Les modèles de délimitation espèces indiquent que *Microniphargus* comprend plusieurs espèces. Un nouveau projet visant à étudier plus en détail le genre *Microniphargus* a vu le jour à l'Université libre de Bruxelles.

Zusammenfassung

Microniphargus, erste Ergebnisse einer phylogenetischen Analyse. Der 1,2 - 1,5 mm lange *Microniphargus* wurde in Irland, England und Belgien gesammelt. Die Tiere zeigen keine signifikanten morphologischen Unterschiede. Zwei genetische Marker, das Folmer-Fragment der Cytochrom-*c*-Oxidase-Untereinheit 1 (COI) und das Verovnik-Fragment des ribosomalen 28S-nuclearen Gens (28S), weisen darauf hin, dass der Status von *Microniphargus* als Gattung gerechtfertigt ist und dass es sich um eine Schwesterklade von *Pseudoniphargus* handelt. Er muss daher in die Familie der Pseudoniphargidae überführt werden. Artenabgrenzungsmodelle weisen darauf hin, dass *Microniphargus* mehrere Arten umfasst. Ein neues Projekt an der Université libre de Bruxelles zielt darauf ab, *Microniphargus* genauer zu untersuchen.

1. Introduction

Subterranean biotopes are – at least by volume – much larger than epigeal ones. Caves represent just one of many subterranean biotopes, the part accessible by humans. The aquatic habitats within, known to cavers as cave rivers, cave lakes or sumps, represent just a fraction of all groundwater. Although groundwater due to the absence of sun light appears hostile to life, it hosts a diverse range of animals. Before we identify the species occurring in groundwater, we must first ask how a species is defined. The biological species concept (specimens belong to the same species if they can produce fertile offspring) does not help if only dead specimens are available. As this is normally the case with invertebrates, for more than 200 years species have been defined by their morphology: If two specimens are indistinguishable by morphology, they are considered to belong to the same species. This method is questionable for

some groups, as species within them can have very similar morphology, resulting in cryptic species (morphologically identical but genetically distinct). Thus, several molecular methods based on DNA have been developed to delimit species (PUILLANDRE et al., 2012; SPORI and FLOT, 2020; ZHANG et al., 2013).

DNA segments of different specimens with usually 500 to 2000 base pairs are sequenced and compared (figure 3). The more similar the base segments are, the closer related are the specimens.

In some cases, DNA sequencing results in much better species delimitation than morphology alone. The quality of the delimitation increases if two or more segments are sequenced and both achieve congruent results.

One problem, similar to the delimitation by morphology, remains: how big must the genetic difference be to call two clades two different species?

A team at the Université libre de Bruxelles under the head of Prof. Jean-François Flot studies subterranean amphipods (*Niphargus*, *Microniphargus*, *Niphargellus*, *Crangonyx* etc.) and their DNA sequences.

Here we provide a summary of the results of a study conducted on the phylogenetic position and molecular diversity of the genus *Microniphargus*, full details of which will be published in WEBER et al. (2021).



Figure 1: Scanning electron micrograph of *Microniphargus*
Photo: Julian Carter

have seen *Niphargus virei* (CHEVREUX, 1896). With its body length up to 40 mm, it is one of the giants of the subterranean fauna. Conversely, *Microniphargus leruthi* (SCHELLENBERG, 1934) at 1.2 to 1.5 mm long, counts as a dwarf species (figures 1 & 2). It is no surprise that such a small species is often overlooked.

Microniphargus leruthi, up to now placed within a monospecific genus in the family Niphargidae, has been reported from Ireland, southern Britain, Wallonia (Belgium), Luxembourg, and Germany. The type locality is Engihoul Cave (= Grotte Lyell; Wallonia, Belgium, SCHELLENBERG, 1934).

Three questions arise: Is *Microniphargus* a single species? Is it a separate genus or does it belong to *Niphargus*? Is its classification within Niphargidae correct?

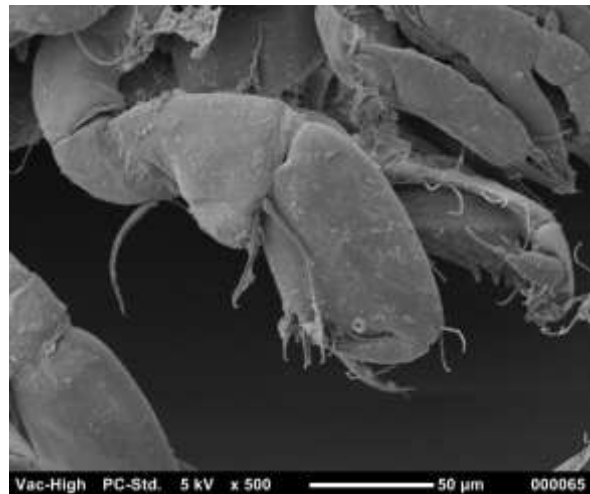


Figure 2: Scanning electron micrograph of *Microniphargus*' gnathopods. Photo: Julian Carter

With over 400 species (HORTON *et al.*, 2019, accessed March 2019), the family Niphargidae is one of the best-known groups of subterranean organisms. Cavers, especially if they are active in the southern half of France, may already



Figure 3: Example of DNA showing 100 base pairs of the COI marker, not sufficient for species delimitation but sufficient to explain the principle: line 1-3: *Niphargus schellenbergi* with similar, but not identical sequences; lines 4-7: *Microniphargus* with similar, but not identical sequences. Downloaded from Genbank 30th Jan 2021

2. Materials and methods



Figure 4: Collecting sites in Ireland, England and Belgium

We carried out intensive targeted sampling for *Microniphargus*, in three geographically separated regions from which it has been reported, using a combination of manual searching and netting with a 250 µm mesh.

We failed to find *Microniphargus* in Engihoul Cave which was completely dry during our trip. However, we successfully collected specimens (figure 4) from: the Grotte de Comblain (Wallonia, Belgium), which is 20 km away from the type locality (9 specimens, of which 3 were sequenced); Pollidubh Cave (County Clare, Ireland, 5 specimens, 3 sequenced); Sweetwater Pot (South Devon, England, 11 specimens, 2 sequenced, figure 5); and Swildon's Hole (Somerset, England, 7 specimens, 2 sequenced).

Morphological examination under a stereoscopic microscope identified the collected specimens as *Microniphargus leruthi*. No significant morphological differences between specimens from the four collecting sites or among specimens from the same collecting site were observed.



All specimens were preserved in pure 96% ethanol and kept at -20°C to avoid damaging the DNA.

We isolated the DNA using NucleoSpin® Tissue Kits (Macherey-Nagel), and stored the isolates at -20°C in the collection of the Evolutionary Biology & Ecology research unit at the Université libre de Bruxelles (ULB). We sequenced two DNA markers via polymerase chain reaction (PCR), the fast mutating Folmer fragment of the cytochrome c oxidase subunit 1 (COI) (FOLMER et al., 1994) as well as the slow mutating Verovnik's fragment of the nuclear 28S ribosomal gene (VEROVNIK et al., 2005). Details can be found in WEBER *et al.* (2021).

Figure 5: Collecting in the sump at the bottom of Sweetwater Pot (South Devon, England) with a net. Photo: John Boulton

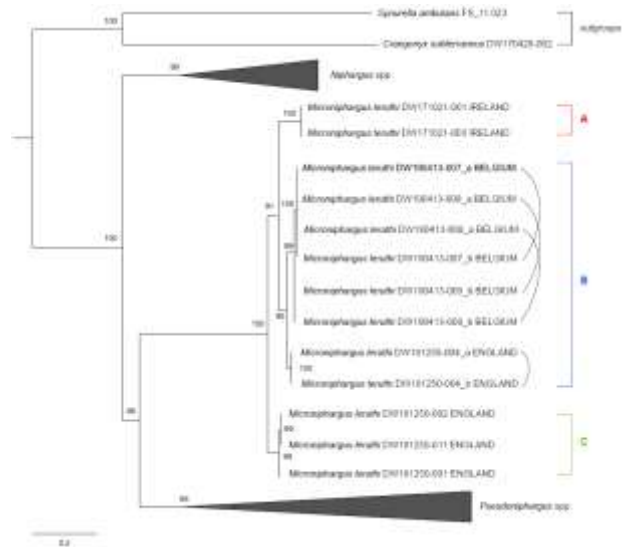
3. Results

Both markers (28S, data not shown and COI, figure 6) show similar results with high support, indicating that they are plausible.

In both cases, *Microniphargus* is the sister clade to *Pseudoniphargus* (in the family Pseudoniphargidae), is recovered monophyletic (includes all the descendants of one common ancestor), and belongs in neither *Pseudoniphargus* or *Niphargus*.

Microniphargus comprises several narrow clades. The most common species delimitation models (ABGD, Automatic Barcode Gap Discovery, available online at <https://bioinfo.mnhn.fr/abi/public/abgd/>; PTP available online at <https://mptp.h-its.org/#/tree>) divide it into three to four different species.

Figure 6: COI maximum-likelihood phylogeny of *Microniphargus* and *Pseudoniphargus* (with two *Crangonyctids* as outgroups). The tree was turned into a haploweb by adding connections between haplotypes found co-occurring in the same individual. Source: WEBER *et al.* (2021)



4. Discussion

Both the COI and 28S phylogenetic trees show that *Microniphargus* is monophyletic and clearly distinct from *Niphargus* and *Pseudoniphargus*. Its status as a separate genus established using morphological characters (SCHELLENBERG, 1934) is confirmed using molecular markers.

The conclusions reached by MOSKRIK and VEROVNIK (2019) and COPILAS-CIOCIANU *et al.* (2020) of the close affinity between *Microniphargus* and *Pseudoniphargus* is confirmed by the present study.

To avoid paraphyly (splitting one monophyletic group into several taxonomic units) of Niphargidae the results of our study suggest that the genus *Microniphargus* must be included within the family Pseudoniphargidae.

Even though we could not definitively determine how many species *Microniphargus* actually includes, it seems obvious that it comprises several cryptic species, at least two of which appear to be endemic to Ireland and south west England.

5. Conclusion

We have learned that with niphargids, species delimitation and allocation to a particular taxonomic group based on morphology alone can be difficult and potentially inaccurate.

Species delimitation based on DNA sequencing seems to be more efficient but still could not confirm exactly how many species of *Microniphargus* actually exist. These initial results are based on a limited number of specimens and sites, and do not include data from Germany (North Rhine-Westphalia, Harz, Württemberg) where *Microniphargus leruthi* has been reported several times but we were unable to find any specimens for sequencing.

To address these issues, we intend to establish a project to study *Microniphargus* in greater detail, starting with more sampling sites in all regions where *Microniphargus* has been recorded.

For each specimen we will extract DNA which will then be passed on to be prepared and sequenced. From each

resulting set of reads the complete mitochondrial genome will be assembled, as well as the ribosomal DNA. In case some of the mitochondrial genomes turn out to be difficult to close (for instance because of a long, highly repetitive control region), we will use Nanopore to generate long reads.

Microniphargus species appear to be very difficult, or impossible, to determine using traditional morphological characters. Using DNA-based approaches, we can add far more information on the status of *Microniphargus*. However, further collecting and sequencing is required to resolve outstanding phylogenetic questions. To study its morphology in greater detail, we plan to use a more efficient and modern microscopic technique, called confocal laser scanning microscopy, which has almost never been used in amphipod taxonomy till now. This may reveal novel characters that can distinguish closely related *Microniphargus* species that would otherwise be considered cryptic.

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Availability of DNA barcodes in subterranean amphipods of Europe

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Abstract

Groundwater presents the main source of potable water in Europe, but it is also a home to many specialized species. Current monitoring schemes of groundwater do not include the biotic component. Studies and conservation of groundwater fauna are challenging. Sampling techniques are demanding and due to low species detectability, multiple visits are necessary. Shortage of taxonomists, combined with morphologically indistinguishable (cryptic) species, hamper the studies of groundwater biodiversity. Recent advances in DNA barcoding, using DNA sequences as a tool in species identification, may present an opportunity to improve knowledge on species distributions, as well as accelerate their potential use in biomonitoring of groundwater quality. We analysed DNA barcode availability in the extensive dataset of subterranean amphipods of the Niphargidae family in Europe. We included data on spatial distribution of 328 morphologically diagnosed species (MDS), and 820 CO1 haplotypes, and compared their number per 100 x 100 km grid cells. Even though the proportion of MDS with at least one CO1 haplotype is substantial, the spatial coverage of CO1 haplotypes within MDS is very incomplete. DNA sequences need to be gathered within the whole MDS distribution range – especially in taxa with high endemism and cryptic diversity, such as in subterranean species.

1. Introduction

Groundwater is freshwater in the subterranean realm, found at different depths across various geological substrates. It can be accessed in caves, wells, springs, or by sampling water from unconsolidated sediments near or under riverbeds (CULVER & PIPAN 2019). It is a home to many animal species, including the ones that live exclusively in subterranean habitats, which are called aquatic troglobionts or stygobionts. Stygobionts present about 8% of all freshwater species in Europe (SKET 1999). Even though local species richness in groundwater is generally lower than in surface habitats, the share of stygobionts in some animal groups exceeds the share of surface ones. For example, crustaceans from the order Bathynellacea have only subterranean representatives (SKET 1999). Stygobionts are often narrow endemics and represent some of the most valuable parts of natural heritage of Europe. Moreover, maintenance of intact groundwater communities is a prerequisite for maintenance of groundwater ecosystem functioning and services (GRIEBLER & AVRAMOV 2015). Groundwater is of vital importance for human existence, as it presents the main source of potable water (GRIEBLER & AVRAMOV 2015). In Europe, it is protected under the European Groundwater Directive (Directive 2006/118/EC). In the 1st Article it states that "groundwater is a valuable natural resource and as such should be protected from deterioration and chemical pollution. This is particularly important for groundwater dependent ecosystems, and for the use of groundwater in water supply for human

consumption". Therefore it is subject to regular monitoring. However, current monitoring of groundwater controls only the quantity and chemical quality of the water. Inclusion of biotic components into monitoring would substantially improve the quality of groundwater monitoring in the long term.

The use of organisms in groundwater monitoring needs some preconditions to be met: a large body of data on species distribution needs to be gathered, species need to be routinely sampled and accurately identified, their biology needs to be known, and their abundance needs to be evaluated. All these steps are challenging. Subterranean habitats are difficult to access. Sampling sites accessible to humans present only a fraction of available habitats. Subterranean species are typically rare, and difficult to detect. Assessment of species richness of a certain site/area requires multiple sampling visits, special sampling gear and techniques including time demanding use of baited traps. The next serious hurdle is identification of species, mainly due to shortage of taxonomists with an expertise to identify the collected individuals. On the top of it, identification using morphological traits has limitations that became obvious with the widespread use of molecular tools. Subterranean species are morphologically often similar to such an extent that their identity can be revealed only with help of molecular techniques. This slows down gathering of knowledge on species distribution and biodiversity assessment at selected subterranean sites.

Recent advances in molecular methods are an opportunity to improve knowledge on species distributions, as well as accelerate their potential use in biomonitoring of groundwater quality. In this study, we limited ourselves to only one of the aforementioned challenges, i.e. species identification. In order to use DNA sequences in monitoring of species presence and biodiversity, DNA sequence libraries need to be set for numerous species. We specifically asked whether the state-of-art of our knowledge of DNA taxonomy is sufficient to use DNA sequences as a tool for routine species identification.

Here, we investigated the availability and completeness of DNA sequences for the family of subterranean amphipods

Niphargidae in Europe. Amphipods are an important part of groundwater macroinvertebrates. With over 400 species, Niphargidae represent an important part of this diversity and could be one of the taxa that would be included into groundwater monitoring. Niphargidae are a family where a large amount of distributional data has been recently gathered at European level (ZAGMAJSTER et al. 2014), and a set of CO1 haplotypes was gathered at the same scale (EME et al. 2018).

So, having two large datasets in hand – distributional database, and a database on CO1 haplotypes, we can ask – how complete is the coverage of DNA barcodes of Niphargidae in Europe?

2. Materials and methods

Our study area included continental Europe, up to the border with Russia (Fig. 1). The area was overlaid with a grid of 100 x 100 km cells, which served as the basic spatial unit for the study.

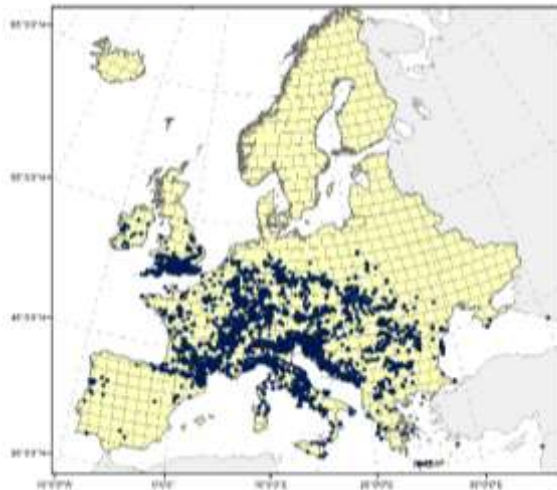


Figure 1: The point occurrence data on morphologically diagnosed species of Niphargidae in Europe, covered with 100 x 100 km grid cells.

We used the information on Niphargidae distribution, based on morphologically diagnosed species (referred to as MDS further in the text). Distributional records were taken from the European Groundwater Crustacean Dataset (ZAGMAJSTER et al. 2014) and included 10.721 records (species – locality – source), and 6614 localities with spatial coordinates, spread across Europe.

Data on presence of CO1 haplotypes for MDS were taken from EME et al. (2018), and unpublished haplotypes from the SubBioDB (database on subterranean species distribution, managed by SubBioLab; state in August 2019). This included 328 MDS, and 820 CO1 haplotypes to be used in the analyses.

First, we analysed the proportion of MDS that had at least one CO1 haplotype known, and the frequency distribution of CO1 haplotypes per species.

In the second set of analyses, we mapped the proportion of MDS with known CO1 haplotypes per 100x100 km grid cell. First, we mapped the proportion of MDS with at least one known CO1 haplotype per grid cell, even if CO1 sequence was not sampled in this particular grid cell. In the second approach, we took into consideration possible geographic variation within species' haplotype diversity. Thus, we mapped the proportion of MDS represented with CO1 sequences from this particular grid cell.

3. Results

Out of 328 Niphargidae MDS analysed in this study, 188 MDS (57.3%) had at least one CO1 haplotype sequenced. Of the latter, 46.8% (88 MDSs) had only 1 CO1 haplotype known, 30.9% (58 MDSs) 2–4 CO1 haplotypes, 11.0% (22 MDSs) 5–10 CO1 haplotypes, and 10.6% (20 MDSs) 11–52 CO1 haplotypes known.

Spatial representation of the number of MDS per grid cell and number of different CO1 haplotypes per grid cell showed an uneven pattern in Europe (Fig. 2A and B). Cells with the highest numbers in both measures were situated in the north-western Balkans, with most of these cells covering Slovenia and parts of Northern Italy, and one grid cell in the border zone of Southeast Croatia (Fig. 2A). There were only three grid cells with more than 46 CO1 haplotypes per cell, situated in both fore-mentioned regions with a large number of MDSs (Fig. 2B).

When we mapped the MDS with at least one CO1 haplotype per grid cell, the proportion of such species exceeded 75% of species within individual grid cells in 58.2% cells with at least one MDS (182 out of 313 cells; Fig. 2C). Only 12.4% of grid cells were with MDS without information on CO1 haplotype (39 out of 313 cells; Fig.2C). This implies a good coverage per grid cell with Niphargidae in Europe.

The results were very different when the spatial position of CO1 haplotypes was considered, i.e. when MDS with actually sequenced CO1 genes in a particular grid cell were considered. In this case, only 14% of cells (44 out of 313; Fig. 2D) had 75% of MDS with at least one CO1 haplotype, and 56.8% of grid cells had no CO1 haplotype (178 out of 313; Fig. 2D).

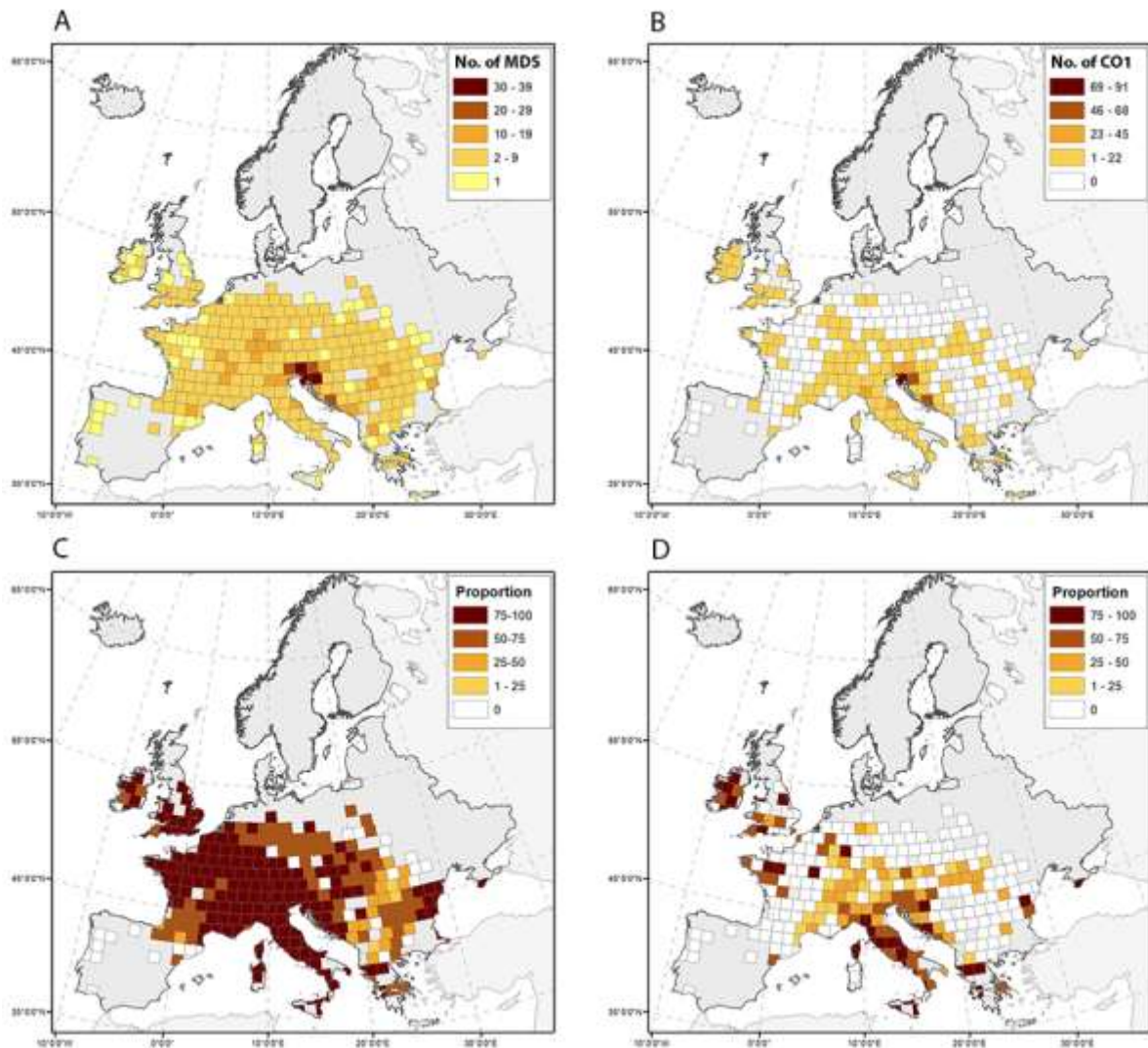


Figure 2: Maps showing the numbers and proportions of *Niphargidae* in Europe, per 100 x 100 km grid cells : number of morphologically diagnosed species (MDS) (A) and known CO1 haplotypes (B); proportion of MDS per cell, having at least one known CO1 haplotype (C) and having at least one known CO1 haplotype in that exact cell (D).

4. Discussion

The interpretation of results is challenging. The share of barcode-defined MDS in our case is roughly similar to shares reported from surface taxa, which are arguably easier to study (WEIGAND et al. 2019). When geographic origin of CO1 haplotypes is not taken into consideration, the coverage of MDS with CO1 is high. This view is overly naïve, given that EME et al. (2018) showed that each MDS on average contains 2-3 putative species based on molecular delimitation methods. Thus, the geographic origin of CO1 haplotype needs to be taken into account. Given that most of the MDS are represented with only one CO1 haplotype, it seems we are far away from an accurate DNA barcode database. This problem, however, could not be of the same extent all over Europe. Species' ranges increase in size with increasing latitude (ZAGMAJSTER et al. 2014, EME et al. 2018). Northern species have on average larger ranges,

which was noted also in molecularly delimited taxonomic units (EME et al. 2018). For this reason, here the MDS may have less variability in CO1 haplotypes. But, there are also differences in geographic coverage of CO1 haplotypes, which is better in the south. When global hotspots in groundwater biodiversity are checked (ZAGMAJSTER et al. 2014), at least some of them seem to be relatively well covered with CO1 haplotypes, opening the possibility that DNA-based monitoring could be implemented at least in some of the most species rich regions. Still, DNA sequences need to be gathered within the whole MDS distribution range, especially in regions with high endemism and cryptic diversity.

There was substantial progress in understanding how DNA could be implemented into monitoring of European surface freshwater (PAWLOWSKI et al. 2018). We believe that a

similar implementation for groundwater should not be delayed, and that DNA techniques should be applied to groundwater ecosystems and groundwater communities at a higher pace. Herein presented results offer a rough priority list of tasks. Our study is a single taxon and is likely biased. There is a need to complete DNA barcodes for Niphargidae, but also to implement similar analyses using other taxa. Promotion of such studies are of prime importance for development of DNA barcodes. While DNA sequences can be obtained rather easily in the laboratory, the taxonomic

evaluation of individuals studied should not be left behind. This is why the development of groundwater barcoding needs taxonomists that would taxonomically evaluate the data. This would lead to a network of laboratories specialized for different groups of subterranean taxa, which would add to a common high quality shared DNA barcode database. It is only with such background, that DNA sequences could be routinely used in monitoring groundwater all over Europe.

5. Conclusion

This study is the first quantitative evaluation of the completeness of DNA barcodes for species identifications within a groundwater taxon in Europe. We show that it is not enough to evaluate DNA sequences per MDS. Spatial information on CO1 haplotype origin should also be included: this is very important especially in taxa with high cryptic diversity and endemism. Currently, DNA barcodes

could potentially be used for monitoring in some regions only. More work in completing the DNA barcode databases for Niphargidae and especially other taxonomic groups, is needed to support their potential use in biomonitoring of groundwater.

Acknowledgments

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Cave of the Adaouste (France, Provence): a major regional roosting for the populations of Mediterranean bats. Study for a concerted management of the site

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Abstract

The *Adaouste* cave opens 15 km north of the *Sainte-Victoire* mountain. It has been known since the late 1950s as a major regional roosting for many species of Mediterranean bats. Indeed, its geographical position located on a hill 200 m above the lower Durance river is an ideal hunting area. Today, the low urbanization of the region means that the cavity is still used by populations of bats. However, the *Adaouste* cave, very well known locally and easily accessible, undergoes some human occupations which can be very harmful for the bats. As the previous studies were limited, there was an urgent need to better understand the cave uses by bats. To this end, a study was recently proposed by the *Fédération Française de Spéléologie*. It includes ultrasonic listening, field visits and climate monitoring of the different areas of occupation of the cave. We present the first results of this study. At the same time, a cavity management plan is being considered. We thus hope to preserve the cavity for bats while maintaining access to the cavity for cavers during periods of low attendance. Indeed, the cavity, still partially described, is of strong karstological interest.

Résumé

La grotte de l'Adaouste (France, Provence) : un gîte régional majeur pour les populations de chiroptères méditerranéens. Étude pour une gestion concertée du site. La grotte de l'*Adaouste* s'ouvre à 15 km au nord de la montagne *Sainte-Victoire*. Elle est connue depuis la fin des années 1950 comme un gîte régional majeur pour de nombreuses espèces de chauves-souris méditerranéennes. En effet, sa position géographique située sur une colline à 200 m au-dessus de la vallée de la basse Durance est idéale comme zone de chasse. Aujourd'hui, la faible urbanisation de la région fait que la cavité est toujours utilisée par les populations de chiroptères. Cependant, la grotte, très connue localement et facilement accessible, subit une fréquentation et parfois des dégradations humaines qui peuvent être préjudiciables. Les études précédentes étant partielles, il était urgent de mieux comprendre les utilisations de la cavité par les chauves-souris. À cette fin, une étude a été récemment proposée par la *Fédération française de spéléologie*. Elle comprend une écoute par ultrasons, des visites de terrain et un suivi climatique des différentes zones d'occupation de la grotte. Nous présentons ici les premiers résultats. Dans le même temps, un plan de gestion de la grotte de l'*Adaouste* est en cours de réflexion. Nous espérons ainsi préserver la cavité pour les populations de chiroptères tout en maintenant l'accès à la cavité pour les spéléologues pendant les périodes de faible fréquentation. En effet, la cavité encore partiellement décrite, présente un fort intérêt karstologique.

1. Introduction

The *Adaouste* cave (also called Davouste) is located in Provence, in the Durance lower valley, 15 km north of the Sainte-Victoire Mountain. It is perched on a hill, 400 m above sea level, overlooking the Durance valley from nearly 200 m. Its pedestrian access is easy and attendance to the upper room does not require any specific equipment. It has always been known locally. It has been the support for several studies on various themes (archaeology, karstology, biospeology).

Concerning bats, it is mentioned as an important roost in the study conducted by Gallocher in 1959 (GALLOCHER, 1959) and by Védovini, an entomologist who in 1961 observed significant deposits of guano and the presence of specific parasites (VÉDOVINI, 1967). Since then, numerous occasional observations or net captures have been made, in particular by the *Groupe des chiroptères de Provence* (GCP). But there was a lack of systematic annual observations to determine the

periods of occupation and use of the cavity (transit roost, reproduction, nursery, etc.). In addition, speleologists and the owner of the cavity have noted many degradations and occupations harmful to bats (rave party, bivouacs, fires). It was therefore urgent to better understand the dynamics of populations in order to protect this site in a reasoned manner.

The *Comité départemental de spéléologie et de Canyonisme des Bouches-du-Rhône* (CDSC13, local part of FFS) thus proposed a study program. It is part of the animation policy of the Natura 2000 *Montagne Sainte-Victoire* area and has been completed by the GCP with a continuous ultrasound listening program over one year.

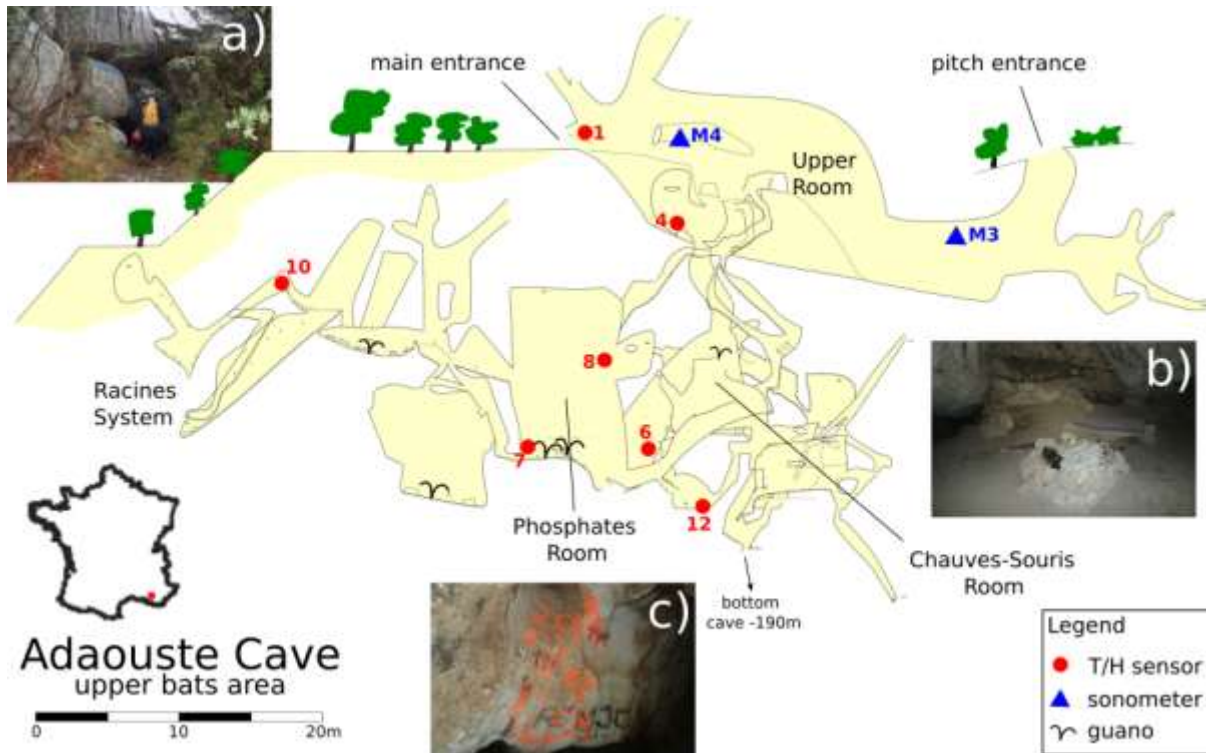


Figure 1: Topographic extended section of upper entrance area (EGELS et CORE 2020). Photos : a) Cave main entrance (L. Core). b) Upper Room, bivouac and campfire (M. Egels). c) Chauve-souris Room, one of the many paint tags (M. Egels).

2. Cave description and various degradations

The cavity begins with a large room with multiple entrances. The main entrance is 2x2 metres in size. At the other end of the room is a large pitch never used by cavers. Other small entrances give access to this room. A pitch gives access to the *Chauves-souris Room*, a vast sloping room where presence of bats, blackened cupolas and piles of guano are regularly observed. At the bottom of this room, a narrow opening gives access to the *Phosphates Room* also widely used by bats. It has a vertical development of around 20 metres. Subsequently, a complex network of small connected rooms developed: the

Racines System. Here ends the description of the upper network. The rest of the cavity develops from the *Chauves-souris Room* through a series of pitches, the presence of bats is much rarer. The upper entrance room shows numerous deteriorations and traces of various occupations, including a bivouac and campfires area (Figure 1, photo b). Very many wild inscriptions are regularly inscribed in the painting (Figure 1, photos a and c). The owner of the premises also testifies to occasional occupations for rave parties.

3. Materials and methods

Due to its hypogenic nature, the Adaouste cave presents a complex and labyrinthine path. Since 2016, a team of

speleologists under the leadership of the *Association Sport et Nature* speleo club has taken an exhaustive topography of the

cavity. This is a fundamental element which is a prerequisite for any scientific study.

Twelve temperature and humidity probes were distributed in the cavity between August 2018 and November 2019. The acquisition step was set at one hour. A probe was deployed near the entrance as a reference for outdoor conditions. The others were placed in areas frequented by bats with the aim of measuring the thermal gradients between the low and high parts. Only seven probes lasted a year of measurement in underground conditions. Figure 2 shows the distribution of the probes in the cavity.

The objective of this study being to acquire knowledge in order to propose a management plan for the cavity with possibly

closure of the main entrance, we chose to place two ultrasound passive batboxes (Peersonic RPA3) in the upper room. The first near the main entrance between the latter and the wells leading to the bat rooms. The second near the base of the access shaft to the main hall in order to check if the bats were making this entrance and could readjust if the main entrance was closed (see figure 1). The main settings of our batboxes are as follows: maximum listening time: 2 minutes, detection threshold: -50 db; gain: 0 db. The good quality sound recordings made it possible to determine the species. They have been analyzed by *Asellia Ecologie* using the *Tadarida* software (BAS et al., 2017).

4. Results and discussion

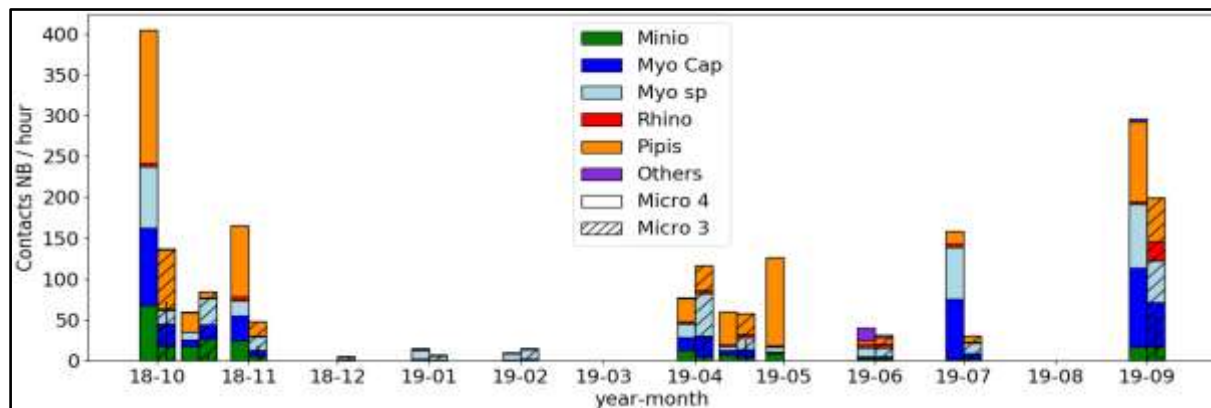


Figure 2: Cumulative bars plot of bats species or groups of species. Unit is number of contacts per hours.

The annual average temperature measured outside is 12.3° C. The top of the Phosphate room is a remarkable hot spot in the cavity (14.3° C on average) with very low variations in temperature and humidity. Figure 2 shows the cumulative species (or groups of species) histograms of the number of ultrasound contacts per hour for each listening campaign. Each listening includes between 2 and 4 nights. The empty bars show the recordings of batbox 4, the hatched bars those of batbox 3. The green bars represent *Miniopterus schreibersii*, the dark blue *Myotis capaccinii*, the light blue the other *Myotis* species (*M. nattereri*, *M. daubentonii*, *M. myotis*, *M. sp.*), red, *Rhinolophus* (*R. hipposideros* and *R. ferrumequinum*), yellow, *Pipistrellus* (*P. pipistrellus* and *P. pygmaeus*) and violets all other species detected (*Plecotus sp.*, *Eptesicus serotinus*, *Hypsugo savii*). Considering all species, the maximum peak in attendance at the cave is very concentrated in the summer-autumn period. The period of lowest attendance is in winter. Spring also shows strong activity although weaker than in autumn. In the remainder of the analysis, we will focus on two species: *M. schreibersii* and *M. capaccinii*. Indeed, they are targeted as species with a very high conservation stake for the PACA region (DENTZ et al., 2018), the preservation of underground deposits for these two cave-dwelling species is

therefore a major regional concern. *M. schreibersii* is very present in late summer / early fall, little present in spring and absent in winter and mid-summer. The catch data made by the GCP shows the joint presence of males and females in autumn. This is a strong indication for the use of the cave as a breeding ground. Spring attendance may be associated with transit. Is the cavity used as a nursery place? Although pregnant females were identified during the June 2001 captures and the temperatures measured are compatible, the low attendance observed in June/July would tend towards a negative response. For *M. capaccinii*, we note a strong autumn activity with the presence of males and females during the captures suggesting a phase of reproduction. The species is also present in the spring for a transit occupation. Finally, the heavy occupation of July 2019 could mean the presence of a nursery. As pointed out, the climatic conditions in the Phosphate Room are favorable, moreover, it is the area where the most important piles of guano are located. Based on our results, the cavity is not used for hibernation in winter. The temperatures measured between 12 and 14.5° C on average in the different parts of the cave support this finding (DODELIN, 2012). The high fall attendance and the diversity of species present (males and females) could be the sign of a swarming cavity. Bats

populations can exhibit significant annual variability and occupy various caves of the Durance area. The preservation of a single roost cave is necessary but will remain insufficient.

Larger environmental actions will also be necessary (pesticides, urbanization, preservation of rivers, etc.).

5. Conclusions

The *Adaouste* site cave is an important cave for the preservation of bats. Its geographical position, its speleological development and its climatology make it a roost very frequented by many species, among which *M. schreibersii* and *M. capaccinii*, both identified as endangered. The features associated with the cave are autumn and spring transit, most likely reproduction, potentially a nursery for *M. capaccinii*.

Given the human pressure on this site, it is urgent to set in place protective measures. A collective action associating the *Grand site Sainte-Victoire*, the GCP, the CDSC13 and the owner is in progress and will make it possible to finance a reasoned closure of the cavity. Access for speleological and scientific activity will be possible during periods of less crowds of bats.

Acknowledgments

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