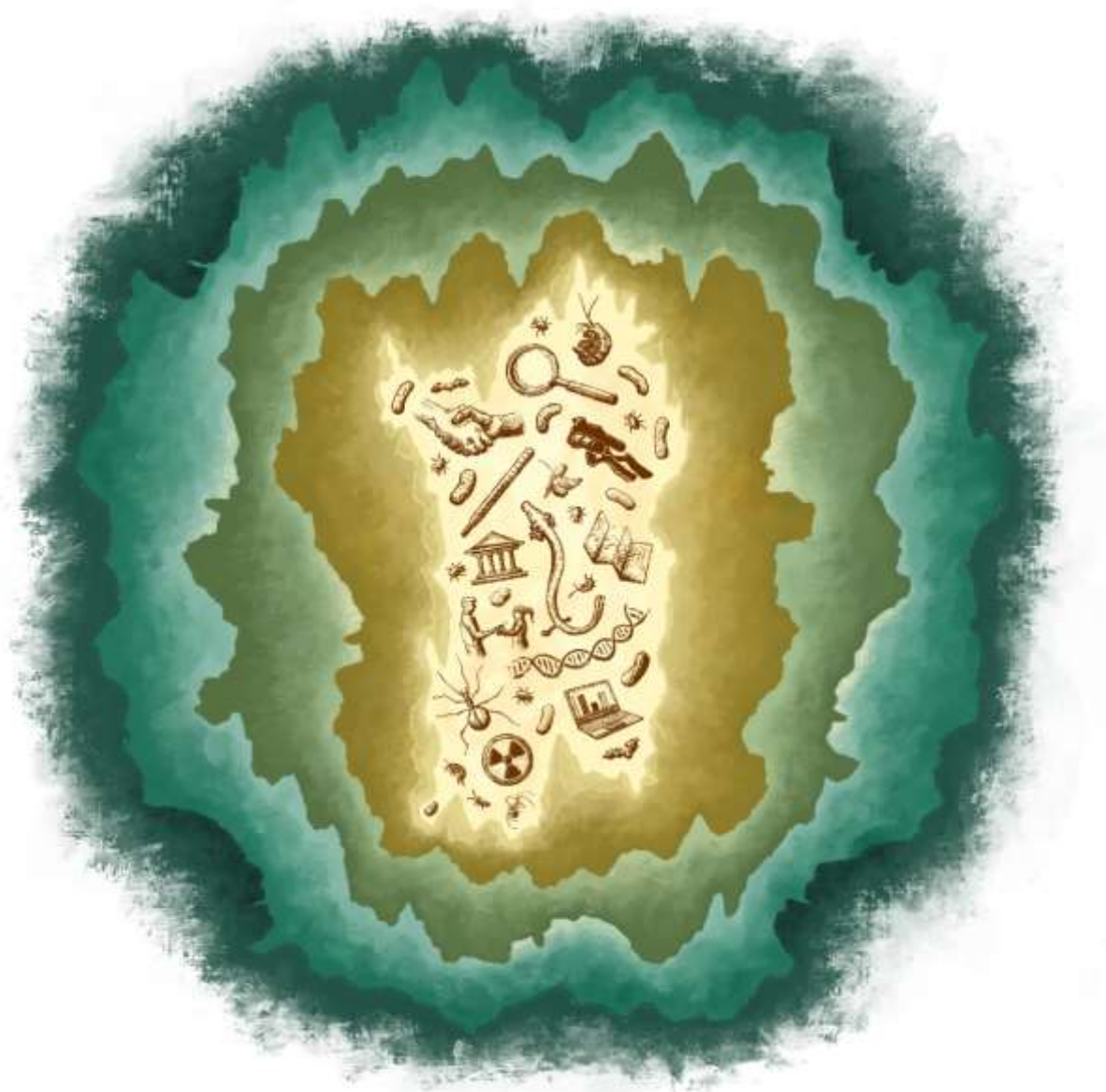




26th International Conference on Subterranean Biology
6th International Symposium on Anchialine Ecosystems

Book of Abstracts



CAGLIARI (SARDINIA)
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Edited by

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Program

Monday, 9 September

8:30 - 14:30	Conference registration and poster preparation * * *
9:00 - 13:00	Workshop DarCO * * *
14:30 - 15:00	Opening of the conference and explanation of the program Presentation of the project METALCAVE. This study received funding from the European Union – Next-GenerationEU – National Recovery and Resilience Plan (NRRP) – MISSION 4 COMPONENT 2, INVESTIMENT N. 1.1., CALL PRIN 2022 PNRR D. .D. 1409 del 14-09-2022 – (METALCAVE) CUP N. E53D23015380001. Presentation of the project Sub-BioMon. This study received funding from the European Union, under the program Biodiversa+, Horizon2020. * * *
15:00 - 19:00	Poster session (exposition of 43 posters) + welcome aperitif

Tuesday, 10 September

8:45 - 9:15	Plenary lectures * * *	
9:15 – 11:00	Oral presentation * * *	Session: Biomonitoring
11:30 -13:00	Coffee Break * * *	
11:30 -13:00	Oral presentation * * *	Session: Distribution
14:30 - 15:00	Lunch Break * * *	
14:30 - 15:00	Plenary lecture * * *	
15:00 - 16:30	Oral presentation * * *	Session: Environmental assessment
17:00 – 18:30	Coffee Break * * *	
17:00 – 18:30	Oral presentation * * *	Session: DNA and genome-based studies



Wednesday, 11 September

8:45 - 9:15	Plenary lecture * * *	
9:15 - 10:15	Oral presentation * * *	Session: Environmental DNA
10:15 - 13:15	Oral presentation * * *	Session: Biodiversity
	Lunch Break * * *	
14:30 - 15:00	Plenary lecture * * *	
15:00 - 16:30	Oral presentation * * *	Session: Subterranean researches
	Coffee Break * * *	
17:00 - 18:30	Oral presentation	Session: Food-webs

Thursday, 12 September

9:00 - 9:30	Plenary lecture * * *	
9:30 - 10:45	Oral presentation * * *	Session: Microbial diversity
	Coffee Break * * *	
11:15 - 13:00	Oral presentation * * *	Session: Ecology
	Lunch Break * * *	
14:30 - 15:00	Plenary lecture * * *	
15:00 - 16:30	Oral presentation	Session: Conservation



* * *

Coffee Break

* * *

17:00 - 18:30

Oral presentationSession: **Conservation****Friday, 13 September**

9:00 - 9:30

Plenary lecture

* * *

9:30 - 12:45

Oral presentationSession: **Evolution**

* * *

Lunch Break

* * *

13:30 - 15:00

Council and Committees' meetings

* * *

15:00 - 15:15

Oral presentationSession: **Evolution**

* * *

16:30

**Winner announcement and organization of
field trips**

* * *

End of the conference

* * *

20:30 - 00:30

Social dinner**Saturday, 14 September – Social Trips**

Eu policies for the management of the subterranean water bodies

SARA JOHANSSON¹

¹European Environmental Bureau, Belgium

In the year 2000, EU Member States adopted the Water Framework Directive (2000/60/EC, WFD), the main water law to protect water in Europe. The WFD has the overarching objective to achieve good status of Europe's surface and groundwaters and to prevent their deterioration. For a groundwater body to be designated as being in good status, chemical and quantitative parameters need to be fulfilled. This includes compliance with certain pollution thresholds as well as ensuring that abstraction does not exceed available groundwater resources, nor negatively affects connected surface waters or terrestrial ecosystems.

While the principal deadline to reach the environmental objectives of the WFD was in 2015, almost 10 years after this deadline and more than 20 years after the adoption of the Directive, EU Member States are far from delivering on their obligations. More than half of Europe's waters are not in good status and placed under different kinds of exemptions from this objective, such as deadline extensions. After 2027 however, the Member States will have limited scope to justify failures to achieve good status.

The Groundwater Directive (2006/118/EC, GWD) establishes the criteria and procedures for the assessment of chemical status of groundwater. It sets EU-wide groundwater quality standards for nitrates and pesticides and requires Member States to set threshold values for substances of national concern.

The groundwater pollutants regulated under the GWD should be reviewed by the European Commission every 6 years. In October 2022, the Commission presented their proposal, which included two pharmaceuticals (an anticonvulsant and an antibiotic) and three groups of pollutants (PFAS, pharmaceuticals and a group of pesticide degradation products). If the proposal is agreed by the Council and the European Parliament, Member States will be required to take measures to meet the quality standards for these pollutants and to reverse upward concentration trends.

Agriculture is a key pressure on groundwater systems. The Nitrates Directive (91/676/EEC) requires Member States identify waters at risk of nutrient pollution resulting from agricultural activities and to establish codes of good agricultural practices and measures to prevent and reduce water pollution from nitrates. It is therefore a key pillar to the WFD. However, like many other pieces of environmental law, the Nitrates Directive has been poorly implemented. The latest implementation report showed that 14% of groundwater monitoring stations reported nitrate concentrations above the EU drinking water limits.

Significantly increased ambition by the Member States is needed to reach the environmental objectives of the WFD by 2027. This would be aided by swift enforcement action by the European Commission when agreed-upon rules are not followed. Additionally, water protection objectives need to be streamlined into other pieces of legislation, such as agricultural and industrial policies, to avoid conflicting efforts.



Developing the first national monitoring network for groundwater ecology in England

TIM JOHNS¹

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In 2018 the UK Government's Department for Environment, Farming and Rural Affairs (Defra) published its 25 Year Environment Plan, with the ambition to improve the environment within a generation. The plan lists a host of themed goals that include improving the quantity and quality of our waters. It commits to 'taking action to recover threatened, iconic or economically important species and where possible prevent human induced extinction or loss of known threatened species in England', all of which are clearly pertinent to groundwater ecosystems. To realise these ambitions Defra established the Natural Capital Ecosystem Assessment (NCEA) programme to collect long-term data on the extent, condition and change over time of England's ecosystems and natural capital. The programme spans land and water environments, tasking England's Environment Agency, and other regulatory bodies, to deliver this assessment. Objectives include a commitment to increase the spatial scale of data on species distributions and biodiversity and develop methods for assessing ecosystems and reporting status.

Prior to the NCEA programme, in the absence of direct legislative requirements, little regulatory attention was paid to groundwater ecosystems in England. The NCEA provides the opportunity to establish, for the first time, a network dedicated to groundwater ecology. Beginning with an initial pilot study, we are now developing a national monitoring programme, initially focused on stygofauna, which will later expand to include microbial communities and their functioning. The aim of the programme is to collect sufficient data to help us better assess and report on groundwater ecosystem health, ensuring the true value of nature is recognised in applying policy and decision making.

We based the design for our network on a hydrogeological unit approach. We adapted the typology developed for England and Wales by Weitowitz et al (2017), which uses aquifer hydrogeological and hydrochemical characteristics to identify 11 'geohabitats', by recalculating this for England only. We then calculated a practical number of sampling points needed in each unit, weighted by the areal coverage of each geohabitat and a habitat quality score, based on key parameters (e.g. transmissivity). We selected sampling points (mainly boreholes/wells) from our national network of existing groundwater monitoring sites. Selected sites were less than 100m in depth, greater than 5cm in diameter, not pumped or used for supply.

The sampling campaign for this new network started in March 2024, concentrating in the south of England, with 55 sites sampled using a standard netting protocol and water quality samples collected. The network will increase in 2025 to 80 sites to cover the whole country. We have invested in training our staff in sampling and identifying stygobitic macroinvertebrates and later this year will have some initial data on species diversity and local abundances across different geohabitats. We are exploring using eDNA as we expand and build our network to incorporate microbial communities and integrate other data sources that will help us better understand, interpret, and report upon groundwater ecosystems.



Out of sight out of mind in the Plastisphere?

ANNE ROBERTSON¹

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Plastics have been an integral part of human society since the 1950's. Their unsustainable use, poor waste management and durability raise environmental and public health concerns. Our understanding of the fate and transport of microplastics (MPs) in rivers and lakes is improving rapidly. However, few studies have taken place in groundwater even though they represent the largest reservoir of liquid fresh water on earth and their connectivity with the surface means they are often vulnerable to pollutants from surface freshwaters and the land, including plastics. The increasing evidence of MP presence in soils and groundwaters sparks serious concerns that we are at risk of polluting highly valuable and vulnerable water resources and also risk creating a pollution legacy for future generations.

To date, little data on subsurface contamination with MPs is available and their long-term impact on subsurface ecosystems remains largely unknown. Here we present the initial results of two studies designed to redress this imbalance. Our first project aims to identify pathways of microplastic uptake into groundwater food webs (comprising biofilms, microcrustacea and macrofauna), and determine how microplastic properties affect microplastic uptake. Initially we performed controlled feeding experiments to assess rates of microplastic ingestion and the impacts on a model aquatic protist, *Tetrahymena pyriformis* demonstrating the potential for an aquatic ciliate to uptake microplastic across a gradient of environmental concentrations. The ingestion of microplastic beads by *Tetrahymena pyriformis* exhibited a positive but saturating relationship with increasing environmental concentrations. This aligns with a Type II functional response and suggests that *T. pyriformis* actively uptakes microplastic even where relatively rare in the environment, but that significant handling time limits a further increase in the ingestion rate of microplastics. Furthermore, the growth rate of *T. pyriformis* decreased with increasing microplastic concentration suggesting that the ingestion of microplastic has a negative effect on reproduction rates and fitness.

Our second project is a global groundwater sampling effort undertaken by the scientific community. To date, using a specially designed protocol, 30 collaborators from 22 countries ranging from Australia through Europe to the United States have provided 250 samples for microplastic analysis. These samples were collected from 75 different sites, which included springs, wells and open boreholes as well as caves. At each sampling site, about 300 L of groundwater were collected and filtered on-site through a series of metal sieves (123 and 25 μm mesh size). Filtrates and meshes were then collected in glass vials and stored for further analysis.

Sample processing included organic matter digestion over several days using hydrogen peroxide, density separation in glass separation units using zinc chloride, and staining with Nile Red dye, while MP characterisation and polymer identification were carried out using Raman spectroscopy. First results indicate a higher presence of fragments than fibres and of MPs between 25-123 μm than $>123 \mu\text{m}$. We hope that our findings will provide an insight into global levels of groundwater MP contamination and begin to demonstrate the impact of these MP on groundwater ecosystems.



Environmental DNA in groundwater ecosystems – the ultimate complementary biomonitoring tool?

MATTIA SACCÒ¹

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Diversity assessments in subterranean aquatic ecosystems are notoriously complex due to the intrinsically cryptic nature of underground habitats. In combination with other factors, this aspect has triggered a substantial delay in recognising the array and value of subterranean biota when compared to surficial counterparts such as rivers and lakes. As a result, the crucial services provided by groundwater ecosystems are still mostly being overlooked and, when acknowledged, they are often solely related to hydrogeological aspects.

Given the importance of groundwaters as keystone ecosystems, this tendency is not only detrimental for the integrity of subterranean aquatic environments and their living biota, but it is affecting the resilience and sustainability of entire surficial ecosystems depending on underground water reservoirs. Nonetheless, similar to other scientific disciplines, technological advancements are shaping a rapid shift in perspectives and possibilities in assessing and monitoring aquatic diversity.

Particularly, recent developments in the field of molecular biology are opening new exciting opportunities in understanding phylogenetic, biogeographic and ecological patterns in aquatic (and terrestrial) environments. At the forefront of this biomonitoring revolution stands the environmental DNA (eDNA) analysis, a technique that makes use of next-generation sequencing to target fragments of DNA present in the environments as a result of biotic activities of resident communities in the habitat. Since its consolidation in 2012, eDNA has been applied, tested and validated as a complementary tool in a myriad of environments, including, even with a considerable delay, in groundwaters. In this talk we review the (recent) past, (buzzing) present and (promising) future of eDNA in aquatic subterranean environments with the goal of discussing the use, applicability and limitations of this powerful molecular test. We also provide an overview of case studies on novel collection methods (e.g., passive collection), technical aspects (e.g., in-field sequencing) and studies at a regional scale (e.g., participatory science initiatives). Moreover, we outline new field-specific opportunities derived from other eDNA-based investigations in oceans, wetlands and rivers – including combined eDNA-eRNA (environmental RNA) uses, global studies and predictive modelling.

Despite the limitations affecting its use in groundwater ecosystems, eDNA hosts great potential in unveiling neglected diversity patterns. This new era of subterranean biomonitoring will play a key role in increasing the spotlight on the importance of subterranean biota for the maintenance of ecological dynamics within the global water cycle and beyond.



The Octopipi, Osmosamplers and Echosounders: Innovations for conducting safe and effective hydro-biogeochemical research in karst subterranean estuaries

JOHN W. POHLMAN¹, DAVID BRANKOVITS², MICHAEL CASSO¹, LEE-GRAY BOZE¹, NEIL GANJU¹, LAURA LAPHAM³, JAKE EMMERT⁴, EMILE BERGERON¹

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Karst subterranean estuaries (KSEs) are globally dispersed and comparable in size to many surface estuaries, yet mechanisms of carbon and nitrogen cycling and the environmental factors regulating chemical and biological distributions are poorly constrained. This is, in part, because conducting safe and representative sampling within KSEs is a unique challenge. Cave diving — the only practical way to directly investigate KSEs — is intrusive, time-limited and requires attention to life-sustaining details. Hence, physicochemical changes and biogeochemical processes that vary over small spatial and large temporal scales are difficult to document.

Inspired by our experiences as estuarine, coastal, and cold seep oceanographers, our team has been developing and adapting devices for biogeochemical and hydrogeochemical research in KSEs over the past decade. Our goal is to sample and record environmental variability with sufficient accuracy and precision to construct hydrologically-based biogeochemical models defining KSE ecosystem function and health. In this presentation, I will introduce the remotely-operated Octopipi (OPP) sampling system that collects pristine water column and sediment pore fluids in situ at centimeter-scale spatial resolution, the range over which important physical, chemical and biological processes regulating the carbon and nitrogen cycle occur. I will also describe adaptations of osmotically-driven pumps (Osmosamplers) that collect a continuous aqueous sample stream from a set location to create long-term (months to year) chemical records for constituents not measurable with in-situ sensors. In combination with sensor-based records, we have determined how meteorological events influence the cycling and distribution of carbon species such as methane, dissolved organic carbon, and CO₂. Laboratory innovations utilizing laser spectrometers add to these studies by providing stable carbon isotopic and concentration data for methane and CO₂. Our investigations of KSE eco-hydrology also include the deployment of hydroacoustic instrumentation to image halocline dynamics between stratified layers with unprecedented clarity. From these observations, we have conceived a lateral mixing model based on differential movement of haloclines rather than the widely embraced vertical mixing model.

Each tool presented will be structured within the framework of the scientific question that motivated its development and/or adaption. Our goal is to introduce the scientific community (including non-trogloodytes) to these sampling and data acquisition tools with the hope of promoting their broader application.



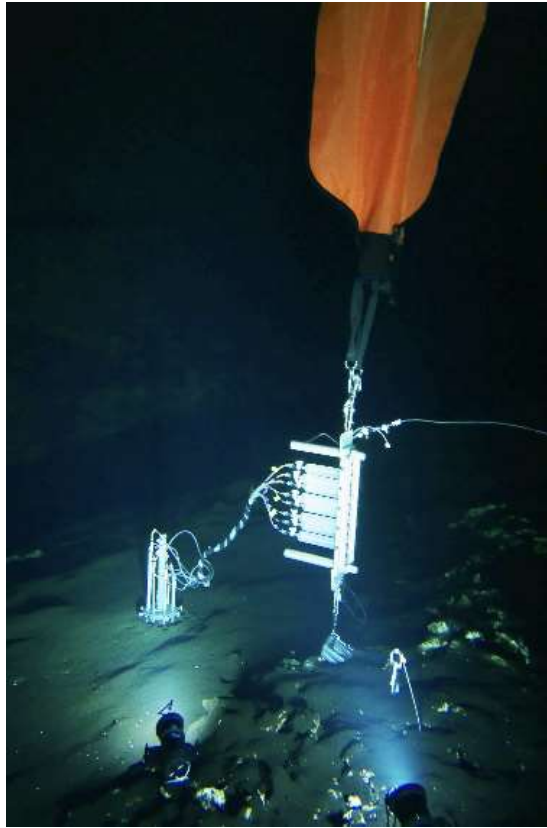


Photo: *The PoWerOPP (PW-OPP) configuration of Octopipi (OPP) in situ remote sampling system during a deployment within the Ox Bel Ha karst subterranean estuary*

Biofilms in caves and mines as a window into exciting and novel microbial ecophysiologicals

TILLMANN LUEDERS¹

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The terrestrial subsurface is mostly considered as an oligotrophic, low biomass habitat for prokaryotes and also for higher organisms. Still, hot-spots of microbial growth and activity in the subsurface can be found at oxic-anoxic interfaces, where reduced waters, solutes and gases become exposed to atmospheric oxygen, giving rise to rich chemolithoautotrophic microbial growth. This can lead to the formation of massive microbial biofilms, covering the walls of caves and mines. Here, we report on two different case studies, where we are currently combining advanced biogeochemistry, microbiology and 'omics technologies to unravel the fascinating, partially unfathomed physiology and ecology of the microbiota within such biofilms.

In the cavern of an iodine-rich former medicinal spring in prealpine Southern Germany, we have previously described methane-oxidizing microbial biofilms forming massive snottites at the ceiling. Up to 3000 ppm of geogenic methane in the cavern explain this rich chemolithoautotrophic growth, but the spring water can also contain up to 23 mM of iodine. Since reactive iodine species can be toxic for microbes, the massive microbial growth in this cave is a fascinating phenomenon. Potentially, microbes capable of utilizing different iodine species could be prevalent in the biofilms. Indeed, we have now identified distinct biofilm bacteria capable of oxidizing iodide coupled to oxygen as electron acceptor. Moreover, evidence for an iodate-dependent methane oxidation is suggested via metagenomic data. A metagenome-assembled genome (MAG) of a member of the recently discovered Methylospirillum, *Candidatus Methylospirillum iodofontis*, hosted genetic signatures of oxygenic denitrification, aerobic methane oxidation, and also of iodate respiration.

Further, we investigate intriguing biofilms discovered in a man-made subsurface ecosystem, a historic copper mine in Northern Bavaria. Extensive asphalt-like biofilms have been found on the walls, which were highly enriched in Mn(IV)-oxides (up to 40% dw) and teeming with bacterial cells. Identification of the labile Mn(III) around cells via STXM-NEXAFS spectroscopy suggested active biological Mn-oxidation in situ. We reconstructed 132 MAGs, of which, intriguingly, nearly half encoded characteristic multi-heme cytochromes indicative of extracellular electron transfer, a necessary capacity for utilizing extracellular mineral phases. Many MAGs with a capacity for autotrophic CO₂ fixation contained such multi-heme cytochromes and constituted up to 23% of the total community. Metal-oxidizers within uncultured families of the *Gammaproteobacteria*, *Nitrospirillum*, and *Acidobacteriota* were identified as major primary producers in these biofilms. We continue to explore and aim to isolate such novel microbial ecotypes from the biofilms, to complete our perspective of the fascinating microbes hosted in such peculiar subsurface habitats.



Assessing the vulnerability of subterranean biodiversity to climate change from an experimental perspective

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Research into the impacts of climate change on subterranean biodiversity has made significant progress in recent years, revealing profound effects occurring at gene to community levels, with varying strength and direction. However, our knowledge of how subterranean organisms, often constrained by a reduced capacity for dispersal or microhabitat selection, cope with environmental variation is still very limited. In this context, basic aspects of their thermal biology remain largely unexplored, such as their capacity to adjust thermal tolerance through physiological plasticity and evolutionary change, or the factors that affect such thermal tolerance and hence, their sensitivity to climate change. Experimental approaches exploring relevant physiological traits for coping with environmental stress are essential for providing insights into these questions. Indeed, subterranean ecosystems ideally exemplify how experimental data can provide more realistic predictions of species responses to climate change than traditional approaches based on correlative species distribution models.

I will give an overview of recent advances in climate change research on subterranean fauna based on such an experimental perspective. I will especially focus on the work conducted by my research group using mainly insects as study models. Our early studies revealed that specialization to the climatically stable deep subterranean environment involved a reduction of the physiological capacity and plasticity to withstand thermal variability. Surprisingly, a wide gap between upper thermal limits and mean habitat temperature has been found across different and disparate arthropod groups, suggesting a lack of adjustment of heat tolerance to current local temperature in subterranean ectotherms. These findings point to a key role of thermal variability in shaping thermal tolerance, apparently irrespective of mean temperature. Prediction exercises of species responses to climate change that account for experimentally measured thermal tolerance draw a different and less dramatic fate for subterranean species than those based only on projected changes in habitat availability modelled from distribution data. I will discuss future perspectives on how such predictions might be further refined by different complementary approaches. For example, exploring thermally sensitive processes at the molecular level to identify early signs of sublethal stress, as well as accounting for other limiting factors for subterranean species, which may potentially interact with temperature, could provide a more ecologically relevant approach to organismal physiological tolerance. Our ongoing research is also directed towards estimating rates of evolutionary change from experimentally measured thermal tolerance traits to predict the margin for evolutionary rescue of subterranean species under a rapidly changing climate.

Despite the technical obstacles that experimental work entails in these habitats, the subterranean environment provides unique opportunities for advancing the field of conservation physiology. Integrating physiological knowledge into vulnerability assessments should be a priority to design informed conservation strategies that effectively protect subterranean biodiversity amidst the challenges posed by global change.



Convergent evolution and genetic mechanisms of cave adaptation in the olm (*Proteus anguinus*)

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Convergent evolution, the independent evolution of analogous phenotypes under similar environmental conditions, is perhaps the most convincing and powerful process through which the effect of natural selection becomes evident. The outcome is highly predictable, leading to evolutionary independent and sometimes tens of millions of years divergent, yet phenotypically indistinguishable species.

Cave animals in general, including the olm (*Proteus anguinus*), are textbook examples showcasing convergent evolution. While the phenotypic consequences of convergent evolution are well-known, little is known on what happens on the level of the genome. Are the same mutations, genes, pathways and genomic regions involved each time and again, or are these completely different? In short, how many ways are there to build similar, but evolutionarily independent phenotypes?

Using genomic approaches, we first reconstructed the evolutionary history of olms. Several distinct olm lineages diverged from around 17 to 5 million years ago, and probably repeatedly colonized caves. Except for one of these lineages, they share a set of derived phenotypic traits typical for species living in caves, including the loss of eyes and pigmentation, and elongation of their appendages and the snout. With the help of the olm's sequenced genome – one of the largest within tetrapods – and comparative genomics across subterranean and surface vertebrates, we investigated the genetic mechanisms by which their characteristic cave phenotype has evolved.

We show that subterranean vertebrates share a significant number of expanded and contracted gene families, and a significant number of shared genes under selection. Moreover, the rate of gene expansion and positive selection is larger than expected compared to surface species, suggesting that cave animals undergo major genomic changes when adapting to the subterranean realm. This challenges the view that animals which transitioned to the subterranean realm were primarily under the influence of neutral evolutionary processes, but rather undergo large changes in their gene use and function. Finally, we show that at least part of these genomic changes evolved independently in different cave-adapted species.



Ecosystem controls on an anchialine pool shrimp species, *Halocaridina rubra*, presence and behaviors in North Kona, Hawai'i

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Loko wai'ōpae (anchialine pool) habitats are tidally influenced, land-locked aquatic habitats with subterranean connections to the sea that are home to unique assemblages of organisms, such as the endemic shrimp, 'ōpae'ula (*Halocaridina rubra*). 'Ōpae'ula abundance is intimately linked to perpetuation of Native Hawaiian fishing practices along the North Kona coast as these shrimps are highly valued as superior palu (chum/bait) to catch 'ōpelu (mackerel sp.). However, the stability of anchialine environments is threatened by sea level rise, pollution due to groundwater contamination and runoff from anthropogenic sources, as well as disturbance by invasive species. Decrease in 'ōpae'ula populations is of critical concern to lineal descendants, customary practitioners and loko wai'ōpae caretakers. In this study, we are working in partnership with community groups and organizations to understand how ecosystem controls, such as water quality and predators, impact the presence and behaviors of 'ōpae'ula. We are sampling a total of 30 loko wai'ōpae habitats and 'ōpae'ula populations during wet and dry seasons across North Kona, Hawai'i. Here we aim to: 1) measure how geology and land use affect the water quality of different loko wai'ōpae, 2) characterize 'ōpae'ula behaviors in response to predators, food availability, and water quality shifts. Extensive behavioral studies on wild populations of 'ōpae'ula are lacking. Therefore, our study will help to illuminate behaviors that will aid in the protection of 'ōpae'ula populations and will assist in identifying where conservation efforts will be best utilized for the preservation of their unique habitats.



The current state of cave fauna and its conservation in Georgia

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Studies of invertebrates in Georgian karst caves, Western Caucasus started at the end of 19th century and have dealt primarily with taxonomic, faunistic and zoogeographic topics, with little or no information on species/community ecology and phylo-/biogeography. Ecological investigations of cave fauna are usually hindered by limited accessibility of cave habitats and frequently require special climbing and diving skills and equipment. In Georgia, the pace of biodiversity research is generally slow, and the knowledge on cave fauna is far from comprehensive. The biodiversity of caves of north-western Georgia (Apkhazeti (=Abkhazia) region) is relatively better investigated than in other Georgian karst areas. This is probably due to the fact that Apkhazeti was the most attractive and visited touristic region during the former Soviet period. In spite of recent advances in faunistic investigations, data available so far probably present just a fraction of diversity in Georgian caves. The cave fauna of Georgia has been recognized as unique on a global scale considering 583 invertebrate species belonging to 58 orders in 17 classes and 7 phyla have been sampled in 181 surveyed caves. 131 species are exclusive subterranean, known from 97 caves: 60 are troglobionts and 71 species are stygobionts. Sixty-five species are single cave endemics. Family Zenkevitchiidae (Crustacea: Amphipoda) [12 species] and 19 genera of cave-adapted invertebrates: *Adaugammarus* Sidorov, Gontcharov & Sharina, 2015 [4 species], *Kruberia* Sidorov & Samokhin, 2016 [2 species] and *Zenkevitchia* Birstein, 1940 [6 species] (Amphipoda); *Caucasogeyeria* Grego & Mumladze, 2020 [5 species], *Hausdorfenia* Grego & Mumladze, 2020 [1 species], *Imeretiopsis* Grego & Mumladze, 2020 [3 species], *Kartvelobia* Grego & Mumladze, 2020 [3 species], *Lesticulus* Schileyko, 1988 [1 species], *Motsametia* Vinarski, Palatov & Glöer, 2014 [1 species], *Pontohoratia* Vinarski, Palatov & Glöer, 2014 [3 species] and *Sitnikovia* Chertoprud, Palatov, Vinarski, 2020 [2 species] (Gastropoda); *Borutzkyella* Tabacaru, 1993 [1 species], *Colchidoniscus* Borutzky, 1974 [1 species] and *Mingrelloniscus* Borutzky, 1974 [1 species] (Isopoda); *Martvilia* Antić & Reip, 2020 [1 species] (Diplopoda); *Troglaphorura* Vargovitsh, 2019 [1 species] and *Troglopalites* Vargovitsh, 2012 [1 species] (Collembola); *Inotrechus* Dolzhansky & Ljovuschkin, 1989 [2 species] and *Taniatrechus* Belousov & Dolzhanskij, 1994 [2 species] (Coleoptera) are Georgia endemics. The most widespread troglobiont is the isopod *Trichoniscus aphoniscus* Borutzky, 1977 (Trichoniscidae), recorded in 13 caves. The springtail, *Troglaphorura gladiator* Vargovitsh, 2019 (Onychiuridae), is the most troglomorphic animal in Georgian caves. The cave with highest troglobiotic species richness (18 species) is Kveda Shakurani Cave. The largest number of troglobiotic species (73) is known from Apkhazeti region. The limited knowledge on biodiversity and lack of general awareness on caves of Georgia is reflected in the critical underrepresentation of cave species/ecosystems in the regional conservation efforts. There are only two cave restricted species listed in the IUCN Red List of Threatened Species from Georgia, both are snails: *Tschernomorica caucasica* (Starobogatov, 1962) and *Caucasopsis schakuranica* (Starobogatov, 1962) (Hydrobiidae), both listed as Data Deficient. Two species of troglobiotic carabid beetles - *Inotrechusinjaevae* Dolranski & Ljovuschkin, 1989 and *I. kurnakovi* Dolranski & Ljovuschkin, 1989 have Critically Endangered status in the Georgian Red List.



Ice cave entrances as a unique natural laboratory: Eco-physiological traits of Collembola from contrasting habitats

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The pronounced inverted microclimatic gradients at ice cave entrances offer ideal model environments to understand ecophysiological adaptations of the soil and subterranean fauna to these contrasting habitats. The ability to survive low temperatures as an ecophysiological trait, helps to explain the distribution patterns of individual taxa and to specify their ecological niches. Despite the very attractive topic, there is still very little data on resistance adaptations of organisms, and life history strategies in environments with strong microclimatic gradients. The supercooling point (SCP) may be used as an appropriate tool for physiological comparisons between species and their individual populations. In our study, the cold tolerance of selected Collembola species was examined by differential scanning calorimetry (DSC). A total of four species and nine populations from microclimatically contrasting sites of entrances of the ice caves (Western Carpathians Mts, Slovakia) served for this study. Significant differences in SCPs between *Folsomia manlachei* and *Folsomia penicula* and *Folsomia* sp. and *Ceratophysella sigillata* were confirmed. Moreover, significant intraspecific differences in the cold tolerance of *F. penicula* and *C. sigillata* were found. The population of *F. manlachei* that inhabited a cold and relatively high-elevation site with a steep north-facing scree slope at the entrance of the Ľadová pivnica in the Belianske Tatry Mts was the most cold resistant (mean supercooling point, SCP of $-22.20 \pm 2.77^\circ\text{C}$). On the contrary, the population of *F. penicula* from thermophilous and low-elevation site in front of the cave entrance of the Silická ľadnica Ice Cave was the most cold sensitive (SCP of $-10.72 \pm 4.58^\circ\text{C}$), thus congruent with the environmental characteristics of sites. In all tested species, the SCP values were far lower than the monthly and annual temperature mean and minimum at their corresponding sites, showing some capacity for temperature acclimatisation. Moreover, body size in relationship with habitat temperature was examined in six populations of two springtail species, showing that populations of the given species were larger in colder environments. This is consistent with “the temperature-size rule in ectotherms” (Atkinson theory). Specifically, *F. manlachei* from the entrance of the Dobšinská Ice Cave was larger (0.809 ± 0.1 mm, $T_{\text{mean}} = 0.01 \pm 2.08$) compared to populations from Ľadová pivnica and Ľadová jama on the Muránska planina plateau (0.745 ± 0.1 mm, $T_{\text{mean}} = 2.63 \pm 3.6^\circ\text{C}$ and 0.733 ± 0.1 mm, $T_{\text{mean}} = 3.51 \pm 4.0^\circ\text{C}$, respectively). Similarly, *F. penicula* from entrance of the Dobšinská Ice Cave was larger (1.288 ± 0.2 mm, $T_{\text{mean}} = 2.45 \pm 2.3^\circ\text{C}$) compared to populations from Ľadová jama and Silická ľadnica Ice Cave (1.243 ± 0.2 mm, $T_{\text{mean}} = 5.93 \pm 4.5^\circ\text{C}$ and 1.163 ± 0.2 mm, $T_{\text{mean}} = 8.3 \pm 5.9^\circ\text{C}$, respectively). We conclude that microclimatic gradients in ice cave entrances constitute ideal experimental settings for studying functional traits of soil invertebrates, especially their thermal resistance as an adaptation in the context of increasing temperatures under recent climate change.

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Environmental drivers of variation in hyporheic invertebrate community composition

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The hyporheic zone is an interstitial ecotone in saturated sediments beneath and adjacent to stream channels. In the hyporheic zone, groundwater-surface water interactions drive metabolic processes important to the overall health of the river system. The zone also provides habitat for epigeal (surface water) and groundwater obligate invertebrates, both of which help to maintain hyporheic function and facilitate essential ecological processes. Despite their contributions to river health, hyporheic invertebrates continue to be overlooked as a component of whole-river ecology, and little is understood about how hyporheic communities respond to variations in hydrology, geochemistry, and land use. To investigate relationships between hyporheic invertebrates and environmental drivers, we examined hyporheic community composition, abundance, and distribution at 34 sample sites along four rivers in Central Texas, USA. Three to five hyporheic samples were collected at each site via Bou-Rouch pump, sorted at 10x magnification, and invertebrates were identified to the lowest reasonable taxonomic resolution. Geologic, hydrologic, and land use parameters were collected for the watershed upstream of each site. Geochemistry and dissolved nutrient concentrations were measured in the lab using filtered water samples, and field parameters (DO, T, pH, SC) were measured in the field at each sample point. Redundancy analysis and multiple multivariate regression were then used to explore relationships between hyporheic invertebrate communities and collected environmental parameters. Both intra- and inter-river variations were observed in hyporheic invertebrate abundance, distribution, and community composition. Abundance and relative proportions of groundwater obligate vs. drought-tolerant benthic invertebrates also varied between rivers. Several environmental parameters were correlated with variations in community composition. Geologic, land use, ionic, and hydrologic parameters were the most consistently predictive. The abundance of invertebrate taxa often associated with groundwater, including Cyclopoida, Harpacticoida, Amphipoda, Isopoda, and Bathynellidae, was found to be highly correlated with geology and land use. For example, percentages of dolostone and igneous geology in upstream watersheds accounted for 74% and 70% of variation in Bathynellidae and Cyclopoida abundance, respectively. Dissolved ammonium concentration explained 35% and 61% of variation in Ostracoda and Amphipoda abundance, respectively. The influence of environmental parameters on Insect abundance varied by taxa, with Diptera most strongly influenced by land use ($R^2=0.50$), Ephemeroptera by geology ($R^2=0.45$), Trichoptera by water chemistry (pH, conductivity, and sulfide concentration ($R^2=0.31$), and Coleoptera by hydrology ($R^2=0.50$). Understanding how hyporheic communities respond to environmental influences will facilitate improved management and conservation in the face of continued anthropogenic disturbances, especially if changes in hyporheic community structure result in changes in ecosystem function.



Temperature-independent telomere lengthening with age in the long-lived human fish (*Proteus anguinus*)

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Generally, in the absence of active enzymatic repair by telomerase, telomere ends universally and inevitably erode over time in the adult soma, with however a strong effect of the thermal history of the individual. We investigated the age-related changes in relative telomere length in the skin of the cave dweller *Proteus anguinus* who lives naturally in a stable thermal environment over its entire long life (possibly up to 80 years), allowing comparison with other homeothermic animals like birds and mammals. Data showed a positive relation between telomere length and individual age, independent of sex and body size. A segmented analysis highlighted a breakpoint in the telomere length–age relationship, suggesting that telomere length reached a plateau at the age of 25 years. Further studies are engaged to investigate whether the telomere really lengthens thorough the life or individuals with longer telomere get a selective advantage.



The DarCo project: A cost-effective plan to incorporate subterranean ecosystems in post-2020 biodiversity and climate change agendas

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Subterranean ecosystems host a broad diversity of specialized and endemic organisms that account for a unique fraction of the global taxonomic, phylogenetic, and functional diversity. Furthermore, they deliver crucial ecosystem services—especially the provisioning of potable water to more than half of the world’s population. Yet, these out-of-sight ecosystems are systematically overlooked in post-2020 biodiversity and climate change targets. Only 6.9% of known subterranean ecosystems overlap with the global network of protected areas, with just a few of these areas designed to account for their vertical dimension. Two main impediments are responsible for this lack of protection. First, subterranean biodiversity patterns remain largely unmapped, even in areas with a long speleological tradition such as Europe. Second, we lack a mechanistic understanding of subterranean species' response to human-induced perturbations. The project DarCo (2023–2026; biodiversa+ funding scheme) aims to map subterranean biodiversity patterns across Europe and develop an explicit plan to incorporate subterranean ecosystems in the European Union (EU) Biodiversity Strategy for 2030. To this end, we have established a multidisciplinary team of leading scientists in subterranean biology, macroecology, and conservation science from a broad range of European countries. The project is articulated in three interconnected work packages devoted to direct research (WP2–4), plus a fourth package (WP5) aimed at maximizing the dissemination of results and engagement of stakeholders to implement practical conservation. First, by compiling existing databases and leveraging a capillary network of international collaborators, we will gather distribution data, traits, and phylogenies for all major subterranean animal groups, including crustaceans, mollusks, insects, and vertebrates (WP2). These data will serve to predict species responses to human threats using Hierarchical Modelling of Species Communities (WP3). Models' predictions of biodiversity change will provide the basis for a dynamic mapping of European subterranean life. By intersecting maps of diversity patterns, threats, and protected areas, we will design a plan to protect subterranean biodiversity complementing the current EU network of protected areas (Natura 2000), while taking into account climate-driven shifts in subterranean ecoregions (WP4). Finally, through target activities in WP5, we seek to raise societal awareness about subterranean ecosystems and invite stakeholders to incorporate subterranean biodiversity in multilateral agreements. In compliance with the European Plan S, we will make all data open and re-usable by the development of a centralized and open database on subterranean life—the Subterranean Biodiversity Platform. This will ensure that future generations will be able to build upon knowledge accumulated on subterranean biodiversity and monitor the effectiveness of today’s protection measures in the years ahead.



Revealing cryptic diversity of the cave populations of *Heteromurus nitidus* (Templeton, 1835) using an integrative taxonomical method

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Collembola are hexapods with a global distribution, occurring from great depths of caves to the mountain tops and in latitudinal gradient from the High Arctic to Antarctica. *Heteromurus nitidus* (Templeton, 1835) is a cosmopolitan collembolan species associated with high accumulation of organic material, commonly found in agricultural fields, forest soils, and inside the caves. This species is mostly fungivorous, but may consume a wide variety of foods, including microalgae. The present study is focused on the integrative taxonomy of *H. nitidus* from the caves of Slovakia, several Balkan countries, Georgia, and Iran. Based on cryptic diversity known in this species, geographic distance and potential isolation of its populations, we hypothesize a distinct genetic diversity within *H. nitidus* populations. Similar to other widely distributed species, we expect that this taxon represents in fact a species complex. For the collection of *H. nitidus*, four common methods are used, which include: (1) pitfall trapping, (2) baiting, (3) extraction of organic material (wood remains, bat guano, baits) in a high-gradient apparatus, and (4) visual searching and hand collecting of individuals by brush and tweezers. The specimens of *H. nitidus* are morphologically studied in order to identify the characters that are specific for particular geographic and molecular lineages. The molecular analyses will be performed based on different mitochondrial and nuclear markers followed by sequencing of DNA fragments, for this purpose we will use different statistical and cluster analyses in program MEGA to construct concatenated phylogenetic trees. This study will be helpful in understanding the morphological and genetic variability and divergence time of *H. nitidus* lineages in relation to the paleoecological history of a given area.

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Revealing the genetic structure of *Allorhiscosoma sphinx* (Verhoeff, 1907) – a millipede inhabitant of the Western Carpathian caves, Slovakia

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The millipedes of the European family Attemsidae preferably inhabit the interface between the surface and subterranean environment and include a few basically troglomorphic species. Cryptic speciation may be an expected phenomenon attemsidae millipedes, due to their isolated and frequently stenoendemic occurrence along the microclimatic surface-subterranean gradient. Three endemic taxa belonging to the family Attemsidae are found in Slovakia, especially *Allorhiscosoma sphinx* (Verhoeff, 1907), *Mecogonopodium carpathicum* Mock and Tajovský, 2008, and one undescribed species. *A. sphinx* probably represents a complex of isolated populations (cryptic species) due to its relatively extensive distribution over central Slovakia in comparison with other taxa that have local endemics distribution range. Using mitochondrial molecular marker COI we analysed genetic diversity of five *A. sphinx* populations inhabiting caves in five geomorphological units, namely the Low Tatras, Veľká Fatra Mts., Slovak Paradise, Revúcka vrchovina Highlands, and Strážovské vrchy Mts. Preliminary results based on 17 individuals showed intrapopulation genetic distances ranging from 0.00 % to 0.43 %, while genetic distances between populations varied from 0.16 % to 1.95 %. Totally, two clusters were distinguished in the neighbour-joining tree, one of which was divided into several subclusters. A population from the Revúcka vrchovina Highlands created separate cluster, population from Veľká Fatra Mts. was separated in two subclusters, while other populations were grouped into a single subcluster. The highest genetic diversity was found in a population from the Veľká Fatra Mts., in which each individual showed its own unique haplotype. Since the remaining populations had uniform haplotypes, we can assume that there is no gene flow due to effective geographical barriers associated with the fragmentation of karst units.

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Low diversity of Crustacea and Annelida in municipal wells in Krakow, Poland

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The studies were performed in 2019-2020 in 91 city wells. Material was collected twice a year, by filtering 100 L of water through a plankton net (50µm mesh size). Beside Annelida and Crustacea invertebrates belonging to four groups were found: Microturbellaria, Nematoda, Collembola and Diptera larvae. Copepoda were determined from the second year of the study only. In the majority of studied wells crustaceans and annelids were not found, and if they were present, one or two taxa only. Sum of individuals from particular taxa found during the studies of well fauna was low (1-14) beside *Acanthocyclops vernalis* (108 ind.) *Bathynella natans* (38) and *Pseudocandona* sp. (61). However, the diversity of fauna was low in the studied wells but stygobionts were represented by five species found in a few wells only: *Trichodrilus cernosvitovi*, *Niphargus* cf. *tatrensis*, *Bathynella natans*, *Diacyclops languidoides* and *Typhlocypris* cf. *eremita*. Most of those species are common in subterranean waters of Southern Poland but *Bathynella natans* is very rarely stated in our country. Surprisingly, among annelids a few *Aeolosoma* sp. and *A. hyalinum* were found, moreover one, very young individual of genus *Rhynchelmis* (Lumbriculidae) was present. Enchytraeidae were most common; they were represented by three semi-aquatic taxa and young, not determined individuals. Among the copepods, beside one stygobiont, five species typical of astatic and small water bodies were found. Moreover small numbers of immature stages of nauplius and copepodits were recorded. Unpredictably ostracods were recorded only in three wells and they were represented by *Typhlocypris* cf. *eremita*, *Pseudocandona* sp. and unidentified juvenile individuals of the family Candonidae. Studied wells are completely closed from above and many of them are isolated from surface water that is why settlement routes by surface water fauna are hard to trace.



Modelling the distribution of European Leptodirini (Leiodidae) with a special focus on diversity hotspots and their threats

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The tribe Leptodirini (Leiodidae) is one of the most species-rich subterranean beetle taxa worldwide—the second largest group after the Carabidae Trechini. More than 900 species in ca. 240 genera are known so far, mostly in the Palearctic Region, with the largest radiations in the Mediterranean basin. This area includes the north and east of the Iberian Peninsula; some Mediterranean islands such as Corsica, Sardinia and Sicily; the Southern Alps; Italian and Balkan peninsulas and Carpathian Mountains. All Leptodirini inhabit caves, litter or deep soil layers. Often restricted to very small areas, or even known from one single cave, Leptodirini species have many narrow endemic species, and for almost all species the limit of their distribution is unknown. This lack of basic knowledge about their distribution makes it difficult to evaluate their vulnerability to direct and indirect human impacts like habitat fragmentation, soil degradation, intensive forest management practices, pollutions and karst destruction due to quarrying and mining activities. In our project we have created a georeferenced database of all published Leptodirini sample localities of European species. In a next step, we will combine these data with the geological and hydrological available information to model the potential distribution area of the group. From the results, the Hotspot regions of Leptodirini diversity will be underlined in order to address the potential conservation issues.



The first genome of *Garra longipinnis* – more to come!

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Garra longipinnis is a cyprinid fish endemic to Northern Oman. This species has both troglomorphic cave populations found in two nearby underground locations in the Hajar mountains and surface populations that inhabit several wadis in Northern Oman. Our previous research compared the genetic diversity of these cave populations with their surface counterparts, revealing that the cave population of *G. longipinnis* is genetically isolated from surface populations. This isolation is relatively recent, dating back between 0.8 to 0.1 million years ago, and the cave population has experienced genetic bottlenecks in the past. These findings establish *G. longipinnis* as an excellent model for studying cave fish evolution. In our current project, we aim to address the following questions:

- 1) Is genetic differentiation among cave and surface populations the result of adaptation to the cave environment or due to random fixation from genetic drift?
- 2) Are adaptive genetic changes the result of de-novo mutations or due to shifts in standing genetic variation?
- 3) Are troglomorphic characters the result of changes in protein structure of genes and/or due to regulatory changes modifying expression patterns?

To uncover the genomic basis of the troglomorphic phenotype, we will perform population genomic analyses using comprehensive genome-wide data generated through Whole Genome Resequencing (WGR). We will use large sample sizes from both cave and surface populations of *G. longipinnis*, as well as from a population of a closely related surface species. Initially, we constructed a draft genome of a surface specimen of *G. longipinnis* using sequence data from the MinION Nanopore sequencing platform (Oxford Nanopore Technologies, ONT). This yielded a draft genome approximately 1.2 GB in length, consisting of 8,888 scaffolds with an N50 of 650 kbp. We further assessed the completeness of the draft genome using the BUSCO pipeline v. 5.3.2, which indicated a high level of completeness with over 95% of vertebrate-specific benchmarking universal single-copy orthologous (BUSCO) genes detected as complete copies.



Exploring Cultivable Stress-Tolerant Bacteria in the Skin of *Speleomantes italicus*

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The skin microbiota protects organisms from external agents and environmental stress rising due to anthropogenic activities and climate change. As part of project FFORIC24.35, we aim to characterize the skin microbiota of European plethodontid salamanders (genus *Speleomantes*), a group of eight strictly protected species that are either endemic or sub-endemic in Italy. The present study focused on the cultivable bacterial communities associated with individuals from a cave population of *S. italicus* from Abruzzo region. The cultivable microbiota of twelve individuals was sampled and isolated using the Nutrient Agar medium by swabbing the collected samples on plates and then incubating them at 29°C for 48 hours. After purification, we characterized the bacterial isolated strains based on morphological traits and bacteriological classifications. Each strain was also tested for heavy metal stress, drought tolerance, and exopolysaccharides (EPS) production. The isolates with interesting characteristics were identified using DNA barcoding and phylogenetic analysis. The isolation yielded seventeen strains, the majority of which being classified under the Actinobacteria and Firmicutes. Interesting outcomes were observed from the heavy metal stress test, emphasizing the capacity of seven strains to withstand moderate to high levels of copper, zinc, nickel, cadmium, aluminium, and lead. Additionally, some selected bacterial strains exhibited positive results in drought tolerance and EPS production, indicating their potential involvement in maintaining the hydration of salamander skin. Molecular identification and phylogenetic analysis allowed us to identify most isolates as *Bacillus* sp. and *Streptomyces* sp. Understanding the cutaneous microbiota of *Speleomantes* increases our knowledge about its implications for amphibian ecology and health, leading to improved conservation strategies. Our preliminary results lay the groundwork for describing the cutaneous microbiota of the genus *Speleomantes*, exploring its potential role in contributing to the production of cutaneous secretions and mitigating environmental stresses.

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Zonation of springs: eucrenal - a door to the subterranean; hypocrenal - a window to the true epigeal from the perspective of macroinvertebrates

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Springs are fascinating ecosystems because they can serve as ecotones between subterranean and epigeal systems, as well as between aquatic and terrestrial environments. In these spatially limited environments, invertebrates from various origins (surface-dwelling, spring-obligate, and subterranean species) coexist and may compete for resources. However, the mechanisms that enable these populations to coexist in what are likely resource-scarce conditions remain poorly understood. The focus of our research is on benthic macroinvertebrate communities in three karst springs along the eucrenal-hypocrenal gradient. We were interested in determining whether the same drivers operate in the eucrenal (which is closer to the subterranean environment) and the hypocrenal (which is closer to the epigeal environment). The aim of this research was to identify the primary factors influencing the structuring of macroinvertebrate communities, specifically whether these drivers are physical and chemical water parameters, hydrological parameters, number of microhabitat types, or biotic interactions. Additionally, the aim was to provide a more comprehensive understanding of benthic life in karst springs and to extend knowledge about benthic macroinvertebrates across the eucrenal–hypocrenal gradient of karst springs, both from a taxonomic perspective and in terms of functional feeding groups. We considered the following parameters: temperature, conductivity, oxygen concentration and saturation, and pH values from physicochemical parameters; discharge from hydrological parameters; number of microhabitats as a habitat parameter; and number of species and individuals as a measure of biotic interactions. A total of 74 taxa were recorded, with 59 taxa inhabiting the eucrenal and 61 taxa inhabiting the hypocrenal. Of the taxa found in the eucrenal, 50.8% were also recorded in subterranean ecosystems, whereas in the hypocrenal, this percentage is 37.7%. The only crenobiontic species in the examined springs were representatives of the water mites and the snail genus *Bythinella*, which do not tend to be found in subterranean environments. We used SIMPER analysis to calculate the faunal dissimilarity between the eucrenal and hypocrenal (61.47%) and to identify key species: *Gammarus fossarum* (46.19%), *Ibisia* sp. (13.31%), and *Niphargus* sp. (8.4%) for the eucrenal, and *Ibisia* sp. (26.43%), *Gammarus fossarum* (25.38%), and *Cordulegaster bidentatus* (9.6%) for the hypocrenal section. In both sections of the springs, the functional group of shredders predominates, as expected. Unexpectedly, there is a high prevalence of predators, especially in the hypocrenal section, indicating functional differences between the spring itself and headwater ecosystems. According to principal component analysis (PCA), the number of microhabitats and the number of specimens were the main explanatory factors for the eucrenal, whereas in the hypocrenal, the main explanatory factor was the number of species. The results of PCA analyses indicate a more pronounced role of biotic factors and the number of microhabitats, in contrast to previous findings that primarily emphasized the importance of physicochemical parameters.



Contribution to subterranean *Oncopodura* Carl & Lebedinsky, 1905 (Hexapoda, Collembola) from Iran and Balkan with Notes on the taxonomy of the genus

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Oncopodura Carl & Lebedinsky, 1905 is an eyeless genus of the family Oncopoduridae (Entomobryomorpha) involving both edaphic and subterranean species. Elongated mucro, the presence of scales on the body, thickened sensilla on Ant IV and the presence of hooks and spines on dens are considered characteristic features of this genus. The present study has two aims: (1) Investigation on cave dwelling *Oncopodura* in Iran and the Balkans; (2) Notes on the taxonomy of the genus. A new species named *Oncopodura moghanensis* sp. nov. was found from Moghan cave in Iran. *O. cavernarum* Stach, 1934 was collected from caves in Croatia and Slovenia. Also, *O. jugoslavica* Absolon & Kseneman, 1932 was found from cave in Bosnia and Herzegovina. *O. moghanensis* sp. nov. can be distinguished from other congeners by 6 long subequal lobes in PAO, each subdivided into 3–6 fingers; dens with 7 dorsal feather-like macrosetae; at basal part with 1 dorsoexternal and 2 dorsointernal hooks, at the distal part with 1 dorsoexternal and 3 dorsointernal hooks. *O. cavernarum* Stach, 1934 with PAO with 1 simple lobe; unguis elongated, untoothed; dorsal side of manubrium with 12+12 ciliated setae and 4+4 long smooth setae; dens at basal part with 1 dorsointernal hook, at the distal part with 1 dorsoexternal and 1 dorsointernal hook. Also, *O. jugoslavica* Absolon & Kseneman, 1932 has morphological characteristics such as PAO with 4 short, equal single lobes; unguis elongated, untoothed; dorsal side of manubrium with 17+17 ciliated setae and 5+5 long smooth setae; dens at basal part with 1 internal hook, at the distal part with 1 external big and strong dentated hook and 2 internal hooks. More recently, Deharveng (1988) divided *Oncopodura* representatives into two basic groups. (1) *O. tricuspidata*-group: Ant IV with 4 thick sensilla (S-setae) arranged in a line, and 1 thickened sensillum at the base of segment (sometimes absent); PAO small, with 3-4 rounded lobes, or absent; unguis with large basal lamella, dental hooks smooth, but with strong denticles on their outer surface. (2) *O. crassicornis* group: Ant IV with 4 short and thick sensilla arranged in a curved line, thickened sensillum at the base of segment absent; PAO large with 6 elongated lobes; unguis without large basal lamella (external tooth); pretarsal seta long; dental hooks finely scaly-ciliated, lacking strong denticles. However, since Deharveng's proposition, numerous species have been described with various combinations of characters and difficult to assign them to any of these species groups. Therefore, we refused to use this species sorting.



Relationships between hydraulic properties and invertebrate community structure in the hyporheic zones of rivers in Texas, USA

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The hyporheic zone (HZ) in the porous and permeable sediments beneath rivers and streams is where surface water and groundwater interact. As habitat and refuge for epigeal and groundwater-obligate species (both HZ specialists and more ubiquitous groundwater generalists), the HZ plays an important role in influencing overall community composition in river or stream ecosystems. Permeability allows fluxes of water into and out of the HZ and is directly linked to the presence, size, and connectivity of pore spaces. Permeability, therefore, is a master variable defining habitat availability and influencing habitat quality in the HZ. However, relationships between water, DO, and nutrient fluxes, permeability, and HZ invertebrate community composition (e.g., invertebrates' richness and abundance) are poorly characterized and understudied. Using data collected in 2021-2022 from 60 sites across 9 Texas rivers, our objective was to determine how fluxes and permeability are related to HZ invertebrate community composition. At each site, we placed 3-5 spikes across riffles at a depth of 30-50 cm using the Bou-Rouch method. At each spike, we collected invertebrates, which were sorted and identified to the lowest practical level in the laboratory. Hydraulic conductivity and gradients were measured at each of the spikes. Hydraulic conductivity, specific discharge, and gradient were compared to invertebrate community metrics (abundance, richness, and diversity) using linear modeling and Akaike information criterion. Results from 2021 sites suggest a positive relationship between hydraulic conductivity and invertebrate abundance ($p < 0.001$, $R^2 = 0.41$) and invertebrate richness ($p < 0.001$, $R^2 = 0.5$). Although the invertebrate community is dominated by epigeal taxa (e.g., Ephemeroptera, Trichoptera, Diptera), we also found a diverse groundwater-obligate fauna including harpacticoids, cyclopoids, ostracods, mites, described and undescribed snails (Cochliopidae), bathynellids, Ingolfiellida, amphipods, and isopods. Preliminary data from 2021-2022 sites also suggest that higher abundance and richness of groundwater-obligate taxa in HZ communities is correlated with higher hydraulic conductivity values. This is probably because higher hydraulic conductivity is associated with shorter residence times, which is often correlated with important environmental parameters (e.g., dissolved oxygen, temperature, etc.) in groundwater systems, including the HZ.



Macroinvertebrate communities in caves and subterranean habitats with anthropic origin

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Habitats such as caves, mines and railway tunnels share similarities with regards to their environmental conditions, such as high relative humidity, thermal stability, and lack of light. However, the fauna residing in these subterranean ecosystems are facing increasing threats due to human activities, making conservation efforts challenging. This study aimed to investigate potential differences in the structure of invertebrate communities between natural caves and subterranean habitats of anthropic origin. A total of 21 subterranean habitats, including 9 caves, 7 mines, and 5 railway tunnels, were sampled to assess the structure of invertebrate communities across three distinct sectors within each habitat: entrance, transition, and deep zones. Invertebrates were collected from both the floor and the wall in each zone, and various environmental factors such as entrance height, air temperature, relative humidity, altitude, and the presence of aquatic habitats were recorded for each habitat. All taxa were identified to the lowest taxonomic rank possible, and four key assemblage traits of the invertebrate community were analyzed: abundance, taxon richness, diversity, and composition. The study found that the interaction between zone and substrate, surface habitat, and entrance exposure were the most significant environmental variables influencing the structure of invertebrate communities. Taxon richness was observed to be lower in the deep zone compared to the entrance and transition zones in all habitats, underscoring the importance of trophic resources. There was a substantial overlap in invertebrate communities composition between caves, mines and tunnels. However, all analyzed assemblage traits were lower in tunnels compared to mines and caves. There is no differences in the taxon abundance, richness and diversity of mines located in karst areas compared to caves. Overall, the results suggest that subterranean habitats of anthropic origin play a crucial role in supporting biodiversity, as some of them harbor invertebrate communities similar to those found in natural caves (e.g. mines from karst areas). These man-made voids could serve as important refuge and substitute habitats for local fauna, highlighting the need for their preservation in landscape management strategies, especially in the face of increasing disturbances such as climate change affecting subterranean ecosystems.



Unearthing subterranean biodiversity: insights into genus *Niphargus* in the Western Carpathians

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The Palaearctic genus *Niphargus* (Amphipoda: Niphargidae) represents one of the most species-rich groups of freshwater amphipods globally, with species predominantly inhabiting groundwater environments. The Western Carpathians—spanning the Czech Republic, Slovakia, Poland, and Hungary—provide a unique setting for studying *Niphargus* diversification due to their geographical position, isolation, landscape heterogeneity, well-preserved environment, and minimal impact from Quaternary glaciations. These factors make the Western Carpathians particularly valuable for biodiversity studies. Existing knowledge about *Niphargus* in the Western Carpathians, derived mainly from pre-molecular morphological studies, identifies only a handful of nominal species in the region. Among these, *N. tatrensis* is widespread in the northern parts, while *N. aggtelekiensis* is confined to the central and southern karst areas of the Western Carpathians. However, these historical data likely underestimate the true diversity of the genus. In this study, we investigated the taxonomic structure and phylogenetic relationships among *Niphargus* individuals collected intensively over several years from various subterranean habitats, including both karst and non-karst areas of the Western Carpathians. We extracted genomic DNA from more than 540 individuals and amplified two fragments of the nuclear 28S rRNA gene, a fragment of the nuclear gene histone 3 subunit A (H3), and a fragment of the mitochondrial cytochrome oxidase I (COI) gene. Unilocus species delimitations suggest that the dataset comprises at least thirty molecular operational taxonomic units (MOTUs). Phylogenetic relationships reconstructed using Bayesian inference and maximum likelihood methods revealed that these MOTUs belong to several phylogenetically distinct lineages. Many of these lineages likely represent new species, thus significantly expanding the known diversity of the genus in this region. Our genetic analyses provide the first comprehensive insights into the *Niphargus* species of the Western Carpathians, uncovering a remarkable phylogenetic diversity within this mountain range. Future studies should focus on integrating morphological, ecological, and genetic data to further delineate species boundaries and elucidate the biogeographic patterns underlying the rich *Niphargus* diversity in the Western Carpathians.



Life-history traits in *Niphargus balcanicus*: new observational data from hatching to two years of age

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Acquisition of life-history data requires long-term observations of individuals and/or populations either in their natural habitats or in the laboratory, both of which are demanding or hardly feasible in subterranean species. Most of the available life-history data for subterranean species, especially for invertebrates, derives from non-systematic and anecdotic observations, leaving plenty of space for systematic research. Subterranean amphipods from the genus *Niphargus* are no exception in this respect. In this contribution, we present preliminary results obtained from the raising of the offspring of two *N. balcanicus* females from hatching until approximately two years of age. We collected two ovigerous females in a cave in Southeastern Bosnia and Herzegovina in March 2022, labelled them as “A” and “B”, and kept them alive in the laboratory until the eggs hatched in May and July 2022, respectively. We placed 58 “A” and 51 “B” juveniles each in their own petri dish and kept them in constant darkness at 11-13 °C. On a weekly basis, we checked their condition, fed them with enchytraeids and noted how much they ate since the last feeding. We removed the remains of food and other debris and put a fresh alive enchytraeid in the petri dish upon each check. To quantify growth rates, we recorded the presence of exuviae, and measured body length on a subset of juveniles from both groups every five weeks using a stereomicroscope. We analyzed the survival and growth rates, molting patterns, feeding habits, and assessed the relations between growth, molting frequency, and feeding habits. We inferred the approximate size of the species at sexual maturity from the development of secondary sexual characters. These observations represent rare data available for an early growth of *Niphargus*, and the first of this kind for *N. balcanicus*. The latter is a relatively large and functionally distinct species with a narrow distribution range. As it is recognized as endangered, getting insights into its life-history traits is even more valuable. By observing the offspring in regular intervals for such a long time, we gained the much-needed experience in setting the grounds for similar observations or experiments in the future.



Sub-BioMon - Developing and testing approaches to monitor subterranean biodiversity in karst

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Subterranean habitats and their biota are among the most challenging ecosystems to monitor, for several reasons. Generally, subterranean species are rare, difficult to find and narrowly endemic, many of them are morphologically almost identical, and can often be distinguished only by molecular analyses. The diversity of subterranean species varies greatly in space and can reach very high numbers in very restricted regions. In addition, there are difficulties in accessing subterranean habitats. Caves present rare, direct natural access points to subterranean domain. Although traditionally regarded as the most important subterranean habitat, we are increasingly aware that most subterranean species live in the fissure systems behind these large voids. However, caves are protected as a habitat type under the EU Habitats Directive, and need to be monitored. There is no standardized sampling method to monitor the subterranean biota, especially the exclusively subterranean species (troglobionts) in caves. The development of precise and harmonized monitoring is the central challenge of the three-year international project Sub-BioMon (<https://www.sub-biomon.net/>), which is funded by the EU Biodiversa+ mechanism. The project began in April 2024 and involved partners from six European countries. These countries differ in terms of the proportion of karst, number of troglobionts, and scope of activities already dedicated to performing national monitoring of subterranean habitats. The aim of the Sub-BioMon project is to establish standardized methods and protocols that will allow a comparison of the state of the subterranean biota in different geographical regions and over time. This project considers the dual role of caves as habitats. Caves are habitats or parts of habitats for several species. In contrast, many species live in extensive fissure systems that are inaccessible to humans, and their detection via caves may be inconsistent. We will first identify the subterranean bioregions at different spatial scales and examine which could be used as monitoring units. We will then standardize the sampling procedures. We will evaluate the experiences and practices of the participating countries and identify the simplest subterranean fauna monitoring protocol that provides reliable results. Third, to ensure the accurate and rapid identification of samples, we will explore a range of DNA-based molecular approaches (DNA barcoding, eDNA) and test their efficiency in routine species identification and detection using the selected taxa. An important part of this project is the involvement of the relevant stakeholders. These include government and conservation organizations, authorities managing nature parks and show caves,



as well as cavers and the general public. We will use citizen science to develop an early warning system for the status and imminent threats to underground karst areas, as well as explore whether it is possible to include a wider public in any monitoring activities. The project results will directly benefit policymakers and the authorities who are obliged to implement the monitoring and report the results to the international community.



***Niphargus* diversity from South-West France through the lens of the barcoding fragment of cytochrome-oxidase I (Crustacea, Amphipoda)**

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Niphargus is the most speciose amphipod genus inhabiting groundwater of the Western Palearctic, counting over 450 described species. The genus is known for its problematic taxonomy, including large within-, yet sometimes negligible between-species variation, often associated with cryptic diversity. With the onset of the molecular methods, it became clear that morphological variation captures between one half to one third of genus's genetic species diversity, raising the need to revise past taxonomic and faunistic efforts using molecular markers. Research efforts of several laboratories in Europe have been dedicated to this task during the past two decades. Hence, *Niphargus* was a subject of intense exploration in parts of Great Britain and of Pyrenees, Alpine and Carpathian arch, Dinaric region and Caucasus. Surprisingly, France remained relatively understudied, despite the long tradition in *Niphargus* research and the fact that the very first molecular studies on *Niphargus* originated from this country. Here, we fill this gap and revise diversity of *Niphargus* in France using molecular markers. We accumulated a large number of samples originating from dedicated sampling campaigns of stygofauna in the south-western parts of the country, samples collected during exploration of isopods and other occasional sampling events. The samples covered altogether over 260 locations. Of these, we selected 434 individuals, whose morphology corresponded to 26 nominal species. We isolated DNA and amplified the Folmer's fragment of the mitochondrial COI marker. To test for species diversity, we employed unilocus species delimitation Assemble Species by Automated Partitioning (ASAP) and identified 136 Molecular Operational Taxonomic Units (MOTUs). Notwithstanding the limits of ASAP and similar statistical species delimitation techniques, more than 80 MOTUs need to be investigated as putative new species, indicating that *Niphargus* diversity in France is probably as underestimated as in the rest of this genus range. We detected up to 6 MOTUs within the morphological species identified as *N. ciliatus*. Moreover, we assessed phylogenetic origin of *Niphargus* collected in France. We selected one individual per MOTU and amplified three additional nuclear markers (two 28S fragments and Histone 3, subunit 2). The phylogenetic hypothesis unveiled that *Niphargus* from France comprise lineages derived from deep splits within the genus and recent speciation events. The study closed an important knowledge gap in molecular-spatial coverage of *Niphargus* and the available barcodes will contribute to future integration of *Niphargus* into biodiversity studies.



Insights into population structure and relatedness of olms (*Proteus anguinus*) in Postojna-Planina cave system Slovenia

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The olm (*Proteus anguinus*), the first described subterranean animal in the world, is an ambassador of conservation of subterranean animals. However, very little is known about the size of populations and their genetic structure, gene flow within and among olm populations, and even less about relatedness of individuals. These are important from the conservation perspective. The Postojna-Planina Cave System is a home to a large and relatively easily accessible population of olms on several sites within the system, and therefore one of the best places to study population biology of the olm. A set of specific and polymorphic microsatellite markers, together with a large sample of skin swabs, enabled us to gain insight into the fine-scale structure and other parameters of the population. An extensive mark-recapture study in 2015 and 2016, followed by further sampling until 2023, resulted in 1803 successfully genotyped samples using 22 microsatellite loci. From the total number of olms captured in one year, approximately 20% were recaptured. Most of the recaptured individuals showed high site-fidelity as they were caught on the same or the nearest section of the subterranean Pivka River during following years. A single individual was recaptured the next year far away and upstream from the first capture site. Despite the high philopatry of the majority of individuals, the entire population seems to be in a nearly panmictic state. Hence, movements reaching several kilometers at some point in the life cycle of reproducing individuals have to occur. Weak genetic structure was detected between the two extreme points – the upstream Postojna Cave and the downstream Planina Cave. Additionally, we tested the possibility of determining genealogical lineages and relatedness of individuals in the population through parentage analysis. Being the first comprehensive population genetics analysis for a subterranean tetrapod, these results pave the way for a new conservation approach for subterranean animals, tailored to the level of individual sites and sub-populations.



Including data on subterranean biodiversity into NarcIS - a national nature conservation information system in Slovenia

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Accessible, standardised and up-to-date data on the distribution of species, habitats and conservation documents are an important source for the efficient planning of conservation strategies and implementation of conservation measures at national and international level. The integration of all available biological data and relevant documents and measures for nature conservation are important part in spatial planning. To provide an effective service to all stakeholders in Slovenia, a common nature conservation information system started to get established in 2021 as part of the four-year project LIFE NarcIS – NATuRe Conservation Information System (LIFE19 GIE/SI/000161, co-funded by the EU Life Programme). The project brings together 8 partner institutions with at least 11 data sources, to form a national »one-stop-shop« for all publicly funded biological and nature conservation data and documents. NarcIS will become a central national service and a useful tool for professional conservationists, experts, decision-makers, as well as the general public. One of the databases that contributed to NarcIS at the start of the project and at the early stage of development of the system, is the SubBioDB - Subterranean Biodiversity Database, managed by the Subterranean Biology Lab (SubBioLab). We have a long tradition of collecting and managing data on subterranean species from various sources. The collection of such data started decades ago and evolved into a strong relational database with well defined and set structure, that has become a central tool for our research work. It contains distributional and molecular data on the subterranean species of the Western Balkans and for some taxonomic groups also wider geographical regions. The inclusion of our data in the NarcIS brought along several challenges and new hope for the conservation of the subterranean biota. The first challenge was to update and harmonise the taxa names in the various contributing databases. While some taxonomic groups are well resolved, and species checklists have been published for the country (e.g. some crustacean groups, leeches), many groups lack specialists and need further revision. During the LIFE NarcIS project, we cleaned and updated the data from various sources, including digitising some old datasets from the collection curated by SubBioLab. We also updated the SubBioDB and added new features to facilitate the transfer of the data into NarcIS. Finally, we have initiated Citizen Science activity to facilitate data collection and raise public awareness in relation to subterranean habitats and their biota. We launched Life Underground website (<https://zivo.podzemlje.si>), where the public is invited to report observations of subterranean species from caves or other habitats. The NarcIS is a unique opportunity for the vertical integration of surface and subterranean biota into comprehensive conservation efforts in Slovenia. To empower the next generation of conservation experts, we are engaging and educating students about the opportunities offered by NarcIS and other tools useful for the data-driven era of integrative conservation in future.



Intraspecific genetic variation in surface and cave mites of the family Damaeidae (Acari, Oribatida) in Slovakia

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Oribatid mites (Acari) form one of the most abundant and diverse groups of soil mesofauna. Members of the family Damaeidae are adapted to a wide range of habitats, from surface to subterranean environments. Surface-dwelling species can show high genetic variability, which is related to the heterogeneity of the given environment, and the absence of dispersal barriers. On the contrary, cave-dwelling species are more isolated and their genetic variability is generally lower compared to their surface relatives. The present study is aimed at evaluating the genetic variability of oribatid mites in the surface-cave ecological gradient that have not been studied in detail so far. Altogether, 19 localities in eight karst geomorphological units were selected. At each locality, cave entrances together with adjacent surface habitat were sampled. In the cave entrance, we obtained oribatid mites by individual collecting from cave walls and leaf litter that was sieved from the entrance and surrounding habitats. Damaeidae species, which were frequent in surface habitats throughout Slovakia, were selected for molecular analyses (*Damaeus auritus*, *D. gracilipes*, *D. riparius*, *Epidamaeus berlesei* and *Kunstitamaeus tecticola*). Only three cave-associated species were collected in sufficient numbers, specifically *K. lengersdorfi*, *Belba clavigera* and *E. berlesei*. *B. clavigera* and *E. berlesei* are restricted to ecotone, while *K. lengersdorfi* occurs also in aphotic parts of caves. *K. tecticola* and *K. lengersdorfi* probably represent ecologically vicariant species. A total DNA was non-destructively extracted from the whole specimens. A fragment of mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified. PCR products were successfully obtained in 48 % of surface specimens, while 32.5 % of cave-associated specimens (90 sequences altogether). Preliminary results show more pronounced isolation and limited gene flow in cave-associated species compared to surface species. For example, genetic distances between the populations of surface *K. tecticola* from selected localities ranged between 0.3 and 35 % for the COI gene. The results of this research contribute to a better understanding of gene flow rate between and among populations of ecologically diverse forms of oribatid mite communities.

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Biodiversity patterns in the urban groundwater of Halle (Saale), Germany

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In urban areas, the shallow groundwater is severely impaired by various anthropogenic influences, which is reflected e.g. in increased temperatures and local hydraulic changes. The groundwater ecosystems, with its invertebrate fauna as well as microorganisms, are therefore under growing pressure. Although the communities contribute to the purification of groundwater, the functional role of biodiversity in urban aquifers has so far been insufficiently explored. Our project aims to study the spatial and temporal dynamics of key abiotic factors and their influence on biodiversity patterns in the city of Halle (Saale), Germany. A thermal as well as physicochemical gradient along increasing urban influences and its impact on the abundance and composition of groundwater microbial and faunal communities will be derived. Assuming that the geological background superimposes anthropogenic impacts, field measurements were carried out throughout different hydrogeological settings in the study area. Due to the complex hydrogeology in the city centre and the outskirts, the shallow subsurface of Halle offers a broad spectrum of hydraulic, thermal and physicochemical conditions, ideal for the unravelling of abiotic drivers of the composition of microbial and faunal communities in groundwater. As part of a full-year monitoring divided into 5 field campaigns, repeated sampling was carried out at 91 groundwater wells. Using a multiparameter probe, key physicochemical parameters (T, EC, pH and dissolved oxygen) were measured on-site. Furthermore, groundwater fauna was sampled from each monitoring well by means of a net sampler. Subsequent to sorting the samples to major taxonomic groups, specimens were passed to experts for morphological and molecular analysis. Major ions, nutrients and dissolved organic carbon (DOC) were analysed from both from well water as well as pumped groundwater. Microbiological analyses comprise the characterisation of the prokaryotic communities including 16S rRNA gene targeted amplicon sequencing, total counts of prokaryotic cells and microbial activity measurements (concentration of intracellular ATP). The field data reveals large seasonal and spatial differences in abiotic factors. Groundwater temperature, which is closely related to the building density, covers a range of up to 12 K. Dissolved oxygen concentrations vary between 0.01 and 10.14 mg/L. Preliminary analyses of the microbiological communities reveal a wide range in bacterial abundance in the shallow urban groundwater, ranging from $1 \cdot 10^5$ to $9 \cdot 10^{10}$ bacterial 16S rRNA genes per litre. The presentation will provide early insight into the composition and abundance of groundwater fauna including patterns of its spatial and temporal distribution in relation to selected abiotic variables.



Diversity and Conservation of Arthropods and Bryophytes in the Volcanic Caves of the Azores

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Caves are among the richest and most interesting extreme environments for arthropods and bryophytes in the Azores. The Azores caves are home to a remarkable number of endemic troglobite arthropods most of them with very restricted distributions and classified as CR and EN by IUCN red list. Moreover, a large number of bryophyte species occur in cave entrances, and species of conservation concern are more likely to occur in cave entrances, whereas more common species may also be found in the surrounding areas. Some caves within the Azores have been identified as biodiversity hotspots, harboring unique assemblages of arthropods. These caves provide important refuges for these species, particularly given the isolated nature of the islands and the presence of different microhabitats within each cave system. In this study we sampled with standardized sampling the arthropod (pitfall trapping e direct search) and bryophyte flora of 11 caves in Terceira Island (Azores). In addition, we also sampled bryophyte species growing on rocks outside their entrances. We characterized the bryophyte communities considering the diversity and number of species, and investigated the life strategies, environmental preferences of light, water, and soil reaction. Our findings also suggest that comparing the bryophyte species found inside and outside caves is useful to study their taxonomic and functional diversity, as well as their ability to adapt. This approach may also reinforce the evidence for the importance of protecting caves as a refuge for bryophyte threatened species. The unique and fragile cave ecosystems of the Azores face several threats, including human disturbance (tourism), habitat pollution, and climate change. Conservation efforts are crucial to protect the endemic arthropod troglobite species and rare bryophytes, many of which have very limited distributions and are vulnerable to environmental changes. Effectively protecting the biodiversity and functioning of subterranean ecosystems while accommodating human development is challenging but achievable, as demonstrated by the recent success of subterranean protected areas in the Azores' oceanic islands. These findings show the importance of ongoing monitoring and research efforts to safeguard these unique habitats and their biota.



The northernmost and isolated among blind julid millipedes in Europe: the genus *Typhloiulus* in the Western Carpathians

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Twenty-eight species of the genus *Typhloiulus* (Julidae, Typhloiulini) are recognized as valid, with distribution in the Balkan Peninsula and southwestern Carpathians in Europe. Due to a long morphotaxonomical effort, the species diversity and distribution are well described. All species in the genus *Typhloiulus* are blind and usually depigmented. They are dwellers of subterranean habitats and rarely of soil. An isolated occurrence of *Typhloiulus polypodus* (Loksa, 1960) was documented from the type locality, the Anna Cave in the Bükk Mts., Hungary. Some other unidentified specimens were noted from the caves situated at the Slovak-Hungarian border. We made a taxonomical revision of all material, sampled in two caves in the Bükk Mts. (Hungary) and three caves in the Slovak-Aggtelek Karst (Slovakia, Hungary). We have confirmed the morphological identity of all materials with *T. polypodus*. Redescription and genetic identification of the material are under study. We correlate distributional data with the geology of the territory. The species belong to the morphotype „long, terrestrial“, while there are several species known as „short, hydrophilous or semiaquatic“. The longest specimens reach the limits of the number of body rings within the family Julidae. Interesting intraspecific body dimension differences are discussed. We also contribute a phylogeny and potential crypticism in the genus based on molecular data (COI) and complete data on bionomy and microdistribution, which are unknown in the species as well as in the genus. The rarity of the occurrence of cave millipedes in the region, with low numbers of relic troglobionts, motivates us to protect their habitats. One cave open to the public, where this millipede was probably extinct, is a negative example where practical care has been in contradiction to nature conservation.

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Development of eDNA monitoring of *Congeria jalzici* in Slovenia

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The cave clam (*Congeria jalzici*) is only known from four locations in Croatia and two locations on a very small area in south-eastern Slovenia. In 1971, only shells and no live animals were found in the spring of the Krupa River. Since the spring is the site of an environmental disaster in which dozens of tons of pure polychlorinated biphenyls (PCBs) were dumped in nearby waste disposal sites, the presence of *C. jalzici* there was questionable. In 2010, the first live specimens were found in Slovenia in a cave, two kilometers downstream from Krupa spring. As the importance of *Congeria* conservation has been recognized, it is listed on the European Union Habitats Directive, and according to the Directive we are obliged to monitor the species and report on its conservation status. As part of the LIFE project, LIFE-IP NATURA.SI (LIFE17 IPE/SI/000011), we have started to develop a methodology for monitoring of *C. jalzici*. As both known and potential locations are difficult to assess, eDNA method was developed and used. We detected eDNA of *C. jalzici* on both locations, including Krupa spring and thereby confirmed the presence of a living population there. We surveyed other potential sites in the vicinity (springs and caves), but no new population was discovered. On one location we regularly take water samples to test for possible variability in *Congeria* eDNA detection with an aim to determine the best sampling method for further regular monitoring of *Congeria*.



***Schmidtea polychroa* as a proof-of-concept model for investigating cave-related pigment loss in planarians**

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All troglobitic animals, regardless of their phyla, share many different traits, with the loss of body pigment being one of them. Why this is so universally the case is a fascinating question, especially when considering the differences in body pigment types across the tree of life. There are some species that have both surface and cave morphs, like *Astyanax mexicanus*, but usually the research of cave-related adaptations has to be conducted on different, although closely related species, which *a priori* have many interspecific differences. Freshwater planarians (Tricladida) are a particularly interesting case when investigating cave-related pigment loss, since there are species that utilize porphyrins and ommochromes as body pigments. Although cave planarians are distributed almost world-wide, and we have succeeded in establishing laboratory cultures of several troglobitic species, very little is known about the molecular mechanisms underlying pigment loss in these taxa. To investigate cave-related pigment loss while avoiding the mentioned interspecific differences, we decided to focus on different strains of the epigeal species *Schmidtea polychroa* (Schmidt, 1861). Seven naturally occurring surface strains differing in pigmentation are kept in the flatworm collection at the Max Planck Institute for Multidisciplinary Sciences in Göttingen. One of these, originating from a spring in Campania, Italy (Spol55), is unpigmented, with the exception of the eye cups. In contrast, the coloration of the Dalmatian strain from Croatia (Spol189), is dark brown. *S. polychroa* is a sister species to a well-established model organism *Schmidtea mediterranea*, whose body and eye pigments are characterized. Thus, *S. polychroa* an ideal *proof-of-concept* model to investigate the underlying cause of body pigment loss. Combining different methods, we confirmed that the pigment types responsible for *S. mediterranea* body pigmentation, ommochromes and porphyrins, are the same in Spol189. By using whole-mount in situ hybridization (WISH), we confirmed that the RNA expression patterns of the known pigment cell markers *PBGD-1*, *thrD-1* and *KMO-1*, are similar in *S. mediterranea* and Spol189. *PBGD-1* and *KMO-1* are known to be a part of the pigment synthesis pathway and are showing dendritic morphology, while *thrD-1* shows a more punctate signal and its role in the pigmentation process is unknown. An additional pigment cell marker, *tolloid-like 2*, has been identified based on the cell transcriptome atlas, and its expression pattern characteristic for pigment cells has been observed. We hypothesized that pigment cells are still present in the body of Spol55 *but* have lost their function. Our preliminary data shows that the expression of *PBGD-1*, *thrD-1* and *KMO-1* are extremely reduced in Spol55, while expression of *tolloid-like 2* is only moderately decreased. All these markers show higher expression in the regenerating parts of the white strain. Taken together, these results suggest that Spol55 has retained pigment cells, but that they have lost



the ability to synthesize pigment. Determining the exact mechanism of pigment loss in this model will establish markers and assays we will use to characterize pigment cells in the troglobitic planarian species.

Physiological impacts of salinity and pesticides on the Yucatan isopod *Creaseriella anops*

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Groundwater supplies half of the world's population with drinking water and supports human activities in regions where surface water is scarce. Consequently, this critical resource faces significant pressure from both climate change and anthropogenic stressors. In the Yucatan Peninsula, over-extraction of freshwater, poor wastewater management, and unregulated pesticide use pose major threats to groundwater quality. Despite these threats, the impacts on groundwater-restricted species (stygobionts) are often overlooked in ecological risk and vulnerability assessments. The Yucatan Peninsula is home to endemic stygobiont species that thrive in anchialine environments. Observations and physiological studies have shown that *Creaseriella anops* inhabits the fresh and brackish groundwater of this region. Being widely distributed in both anchialine and fully freshwater systems, its populations are exposed to various anthropogenic pollutants and frequently encounter acute salinity changes due to vertical halocline stratification. Therefore, we used *C. anops* as a model species to study the effects of acute salinity changes and simultaneous exposure to the agricultural pesticide Chlorpyrifos on its physiological response. Individuals were exposed for 96 hours to two salinities (fresh and brackish) and two concentrations of Chlorpyrifos. To assess their physiological state, we measured metabolic rates, indicators of oxidative stress, shifts in antioxidant enzyme activity, inhibition of acetylcholinesterase (AChE), and lactate levels as an indicator of anaerobic metabolism. Our preliminary findings provide insights into the combined effects of salinity fluctuations and pesticide exposure on the physiological health of this species, emphasizing the need to include these species in ecological risk assessments to ensure the conservation of subterranean biodiversity in the face of increasing environmental pressures.



Cenote biodiversity through citizen science

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The cenotes of the Yucatán Peninsula (Mexico) are not only emblematic natural features but also priority ecosystems for conservation. These freshwater sinkholes serve as crucial water sources and economic assets for local communities while acting as biodiversity sanctuaries for numerous species. Despite their ecological importance, significant gaps in biological knowledge persist due to the extensive number of cenotes—approximately 7,000 across over 40,000 km². This knowledge gap hampers conservation efforts, necessitating external support to document and monitor biodiversity. We present a new initiative that leverages citizen science, using the iNaturalist platform, to document cenote biodiversity through public participation. Engaging citizen scientists increases biological data collection and enhances public awareness and environmental sensitivity. The first project reared 97 iNaturalist projects and documented over 1,463 species, highlighting the cenotes' ecological value and emphasizing their conservation necessity. The initiative has successfully engaged 651 observers who have shared more than 9,098 photographs, mostly of epigeal species. The stygobiont community is particularly hard to photograph, as it mostly requires advanced photographic experience and equipment additional to cavediving training or an interest in dry caving. This project aimed to increase efforts of citizen observation of stygobionts through the iNaturalist portal. There are currently 408 observations of 17 stygobiont species (out of a possible 66 species) that are present in the Yucatan Peninsula. The most observed species are the palaemonid *Creaseria morleyi* and the mexican blind brotula *Typhlias pearsei*. The collected biological data will be integrated into the Cenoteando-UNAM Big DataBase, facilitating open access (excluding sensitive data) and ongoing updates to support comprehensive cenote conservation strategies.



The harpacticoid fauna (Crustacea, Copepoda) from interstitial environments of the Vjosa River and some tributaries in Albania, including the description of a new species of *Nitocrella* Chappuis, 1923.

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During the study of hyporheic samples from the rivers Vjosa (4 sites), Bença (3 sites), Drinos (2 sites), Dishnica (1 site), and Drinos (1 site), we found three species of harpacticoid copepods as new records for Albania. Additionally, an unidentified parastenocaridid copepod was collected in interstitial sediments of three sites of the Vjosa River. The most common species was the canthocamptid *Elaphoidella karamani* Chappuis, 1936, recorded in 9 of the sampled sites. The morphological variability of that species based mainly on shape and ornamentation of the caudal rami in several specimens, was examined. A second canthocamptid species, *Attheyella wulmeri* (Kerhervé, 1914), was found in the rivers Drinos and Dishmica. The third species is an ameirid harpacticoid of the genus *Nitocrella* that does not fit to any known species of the genus and will be described as a new species. It was collected in the hyporheic zone of the rivers Benço and Drinos. It belongs to the hirta species group of the genus *Nitocrella*, characterized by 3 to 4 appendages on the exopod of the fourth pair of legs. The new species carries 4 appendages on this leg. Other differential traits of the new species are leg 3 endopod 2 with 2 setae and leg 2 exopod 2 without inner seta. We discuss its relationship with central and southern European species of the hirta-group, namely *N. juturna* Cottarelli, 1975, *N. fedelitae* Pescei, 1985 and *N. pescei* Galassi & De Laurentiis, 1997 from Italy, *N. calcaripes* Damian & Botosaneanu, 1955 from Hungary and *N. maggii* Pesce, 1983 from Greece. Its nearest congener is *N. maggii*, sharing the same chaetotaxy of second endopodite of legs 2, 3 and 4 with setae formula 1:2:1. Other differential characters are leg 5 basoendopod extended until $\frac{3}{4}$ of exopod, armed with 3 spines, and exopod bearing 3 spines. The new species has caudal rami of rectangular shape, in contrast to *N. pescei*, *N. juturna*, *N. calcaripes*, *N. fedelitae*, and *N. maggii* (conical shape). Male differential diagnosis of the new species are: legs 1, 2 3 and exopod of leg 4 armed like in female, leg 4 exopod 3 with 5 setae (4 in female). Leg 5 basoendopod with 2 spines on each side, exopod with 4 spines. A determination key of females of the 18 species of the hirta-group of *Nitocrella* is provided.



Fig. *Nitocrella albanica* n. sp. female. Dishmica River, 21.09.2022. Photo: S. Gaviria

Life's Toxic Sanctuary in Movile Cave (Dobrogea, Romania): insights into ostracod shell chemical composition, ultrastructure and epibionts

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Ostracods are small bivalve crustaceans that encapsulate in their shell information on the properties of water in which they live. The network of pores and canals ultrastructure regulate the osmotic exchanges between the haemolymph of the organism and the ionic concentration of the water. As such, their carapaces have distinct geochemical signals (e.g. Mg, Ca, and Sr) determined by those of the water. Additionally, the sensorial sensilla hairs or bristles traversing the pores and canals projected on the external side of the valves connect the organism with the environment by detecting the chemical elements in the water. Their presence has a special meaning in challenging environments where toxic elements (H₂S, CH₄ and/or low oxygenation) for other life-forms are present. Third, epibiosis is a facultative association among two organisms, frequently found in marine environments with stressful conditions such are thermal vents. When dealing with demanding conditions both the host and epibiont may develop adaptive mechanisms in tandem, increasing the fitness and the capacity of the first to occupy specific ecological niches. If epibionts of epigeic ostracods have received extensive attentions especially in marine habitats, the association and implications for cave species is less understood. We aim here to provide preliminary data on chemical composition and ultrastructure and epibionts in an ostracod species (*Pseudocandona movilaensis*) from Movile Cave (southeast Romania), by means of electron microscopy techniques. Movile Cave is known to show challenging features for life forms, i.e. high concentrations of H₂S (up to 30 mg/L) and CH₄ (1-2%); low oxygen concentration up to hypoxia and anoxia and elevated water temperature (between 19-21°C). The cave represents a remarkable evolutionary hotspot for several species and their symbionts, both showing specific physiological and ecological traits. The results of energy-dispersive X-ray spectroscopy (EDX) analyses applied to the dorsal part of the ostracod shells (20 individuals) revealed the presence of elemental carbon, oxygen, and calcium. Environmental scanning electron microscopy (ESEM) further evidenced a particular structure of the pores on dorsal face adorned with a rim and the presence of relative dense chemosensory sensilla for a Candoninae ostracod. The carapace also shows the presence of 5-7 loricas of *Lagenophrys* sp., a peritrichous ciliate, having a specific design on dorsal face hitchhiked by sulphur-oxidizing *Thiothrix* bacterial ectosymbionts. We view this nested biotic interaction as adaptive strategies, highlighting the role of the interplay between symbiotic host and particular water chemistry conditions. We finally outline the significance of shell geochemistry and ultrastructure in ostracods thriving in extreme continental cave habitats and discuss the implications of symbiosis in the expansion of their abilities to exploit challenging habitats.



Conservation, monitoring and restoration assessment of the world-class cave biodiversity hotspot in Portugal

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Below the ground live some of the most rare, endangered and unprotected species worldwide. Neglected in major conservation policies, cave animals show high endemism patterns and constitute a unique natural heritage, and simultaneously provide important services for humanity linked to carbon and nutrient cycling. Despite being isolated in the underground; they are directly impacted by pollution and environmental changes aboveground. Recently, a single cave in the karst area of the Algarve (Barrocal, Portugal) has been identified as a world hotspot for subterranean biodiversity, due to its incomparable richness in endemic species. Many of those are only known from this unique site that faces several acute threats, such as urbanisation and degradation of the corresponding surface influence areas. These threats call for its study in order to generate useful information for its protection and a framework for its future ecological assessment, and for restoration of degraded areas. Here we show the framework of the Barrocal-Cave project, which will fill this gap, laying the foundations for the conservation of this world-class cave biodiversity hotspot. We will assess its biodiversity and conservation status, implement a long-term cave monitoring program, and assess its ecological restoration needs in degraded areas of the cave and its surface influence area. Finally, aligned with the stakeholders, this project will prepare a formal proposal for a legal framework for the protection of this unique environment, ensuring its sustainability. This also includes the implementation of a specific outreach program for raising awareness on the value and uniqueness of cave ecosystems as a legacy for future generations.

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STYGOTOX: A quality assessed database of (eco)toxicological data on stygofauna and other aquatic subterranean organisms

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Despite the global importance of groundwater and the increasing pressure on this vital resource, risk assessments that consider groundwater ecosystems are rare and knowledge of stressor effects on groundwater fauna lags far behind that known for surface water fauna. In many jurisdictions, water quality guidelines for surface waters may be applied to groundwaters and/or used in risk assessments for the protection of groundwater ecosystems. This practice assumes that surface dwelling species are a suitable proxy for groundwater species, but this assumption is questionable because the sensitivity of specialist groundwater fauna to stressors may differ to that of their surface. A recent review compiled ecotoxicological data for stygofauna prior to 2019 (Castaño-Sánchez et al., 2020), however there is no accessible database that includes those, and more recent ecotoxicological data in a readily downloadable and searchable format. This paper compiles the toxicity data on stygofauna and other aquatic subterranean organisms in one (eco)toxicological database. The database currently contains records from A total of 50 studies were found, containing 520 toxic endpoints covering 43 different stressors. These compounds were tested on subterranean organisms from four phyla, 12 orders, 24 genera, and 55 species. The studies included were published between 1976 and December 2023 using fauna collected in thirteen different countries. The suitability of the studies was assessed in order to indicate the completeness of reporting and their suitability for use in hazard and risk assessment. The database will be updated with the publication of new results. Authors will routinely search for relevant publications and welcome submissions from other authors, with the goal of maintaining a database that contains all current ecotoxicological data pertaining to aquatic subterranean organisms. This compilation provides a valuable source of data for future development of toxicity testing protocols for groundwater organisms, and to support decision-making, ecological risk assessments and the derivation of water quality criteria for the protection of groundwater ecosystems.

The database is freely available at DOI: [10.25949/24030285](https://doi.org/10.25949/24030285)



Six years of cave biodiversity conservation campaign: Cave Animal of the Year in Italy

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The "Cave Animal of the Year" initiative, inaugurated in Germany in 2008, seeks to enhance knowledge and awareness of subterranean fauna and promote cave protection. Each year, a specific cave animal is spotlighted through a range of activities and awareness campaigns, aimed at promoting the conservation of cave ecosystems and their unique inhabitants. This German Speleological Society (Verband der Deutschen Höhlen VdHK) initiative, which was honored with the France HABE prize in 2014 by the International Union of Speleology (UIS), since 2018 has expanded internationally with the support of the Italian Speleological Society ETS. In 2019, *Limonia nubeculosa* (Common Cave Limonid fly) was chosen for its ecological significance. The following year, *Plectogona sanfilippoii* (millipede) was highlighted for its role in decomposition processes within caves. The campaign took a significant leap in 2021, marking the start of the common UIS initiative "International Cave Animal of the Year" with *Italodytes stammeri* (cave beetle) being the focus. In 2022 within Italy, finally bats the *Miniopterus schreibersii* was selected to emphasize the importance of preserving cave ecosystems, while in 2023, the genus *Niphargus* (small crustaceans) was chosen to highlight the conservation of life in underground waters. For 2024, the genus *Speleomantes* (European cave salamanders) has been designated as the Cave Animal of the Year. These amphibians, known for their protrusible tongue and cutaneous respiration, inhabit areas near cave entrances and are particularly cherished by Italian speleologists. Despite their ecological significance, they face multiple threats, among which habitat destruction, climate change and infectious diseases. Nevertheless, poaching may be an additional threat. In 2024, 26th the International Conference on Subterranean Biology will take place in Sardinia from September 9 to 14. This international event, organized jointly with the 6th International Symposium on Anchialine Ecosystems, will be an important platform for promoting the protection of caves and subterranean biodiversity. A comprehensive awareness campaign will support these efforts. This campaign includes since the first year, the distribution of brochures, flyers, and posters, the creation of interactive games and workshops for tourist caves guides, engagement through social media, school lessons, collaboration with tourist caves and sector associations, and regular observations and monitoring of cave biodiversity. The "Cave Animal of the Year" initiative underscores the importance of conservation and public awareness to ensure a safe future for the fascinating and diverse life forms inhabiting our caves. For more information and to get involved, visit <https://animalidigrotta.speleo.it/>



The influence of scale and environment on the beta diversity of troglobitic organisms in subterranean ecosystems of Carajás National Forest – Brazil

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A modern model for the distribution of subterranean biodiversity predicts that the isolation of cave communities depends on the scale from which it is observed. Isolation manifests at larger scales and involves significant geological discontinuities that impact subterranean connectivity. In contrast, at local scales, subterranean connectivity between caves would be maintained through spaces and the rock's porosity, allowing for the exchange of individuals and homogenization of species composition. An excellent approach to identifying these patterns in cave communities is the analysis of β diversity and its components turnover and nestedness. To test the above-mentioned assumptions in ferruginous subterranean ecosystems, the present study analyzed the variability in the composition of troglobitic organisms (β diversity) between caves located in the Carajás National Forest (FLONA), a protected area in southeastern Amazonia, evaluating the effect of environment and space on this variability. We compiled occurrences of 133 troglobitic species distributed across 471 cataloged caves in the FLONA. We assessed the partitioning of total β diversity in caves of different sizes (Resolution – size calculated through vertical/horizontal developments) and at three different spatial extents (Local - caves on individual plateaus; Intermediate - caves in different mountain ranges; Regional - entire dataset). We used distance-based redundancy analysis (db-RDA) and multiple regression on distance matrices (MRM) to evaluate the effect of environmental variables and spatial structure on species composition. We found that species turnover between caves peaked at the intermediate spatial extent and that the scale resolution and spatial extent were determinants in defining the patterns of influence of environmental and spatial factors on the β diversity of cave communities. Larger extents (intermediate and regional) depicted the dominant influence of space and geological characteristics, while cave heterogeneity was significant at the local scale. These results support the proposed theoretical model for cave biodiversity in ferruginous landscapes, indicating a higher redundancy in species composition at the local scale. Testing such models advances the knowledge of Neotropical subterranean biodiversity, improving the recognition of the limits of differentiation in the species composition of cave assemblages and elucidating the correct scales at which conservation actions towards them should be focused.



Cavernicolous terrestrial isopods (Oniscidea) from Georgia, Caucasus

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Up to date, 39 species in 22 genera and 9 families of terrestrial isopods were known from Georgia, of which 21 species and subspecies were recorded from 31 caves. In recent years, during investigations in many Georgian caves, a large number of cavernicolous Oniscidea has been collected, including several new species and one new genus. With the new species identified, the total number of terrestrial isopods known from Georgian caves is now 28 species and subspecies. Ten species and subspecies in the genera *Ligidium* and *Caucasoligidium* (Ligidiidae), *Buddelundiella* (Buddelundiellidae) and *Cylisticus* (Cylisticidae) do not show troglomorphic traits and are considered to be troglaphiles. All the other species and subspecies show absence of body pigmentation, reduction or absence of eyes and, in a few cases, elongation of the appendages, and are considered to be troglobionts. These troglobiotic taxa comprise the following species: *Ligidium* n. sp. (Ligidiidae); *Caucasonethes borutzkyi*, *Psachonethes* n. sp., *Trichoniscus aphoniscus* with three subspecies, *T. gudauticus* with two subspecies, *Trichoniscus* n. sp. 1, *Trichoniscus* n. sp. 2, *Trichoniscus* n. sp. 3 (Trichoniscidae Trichoniscinae); *Colchidoniscus kutaissianus* with two subspecies, *Colchidoniscus* n. sp., *Mingrelloniscus inchkuricus*, n. gen. et n. sp., *Haplophthalmus* n. sp. (Trichoniscidae Haplophthalminae); and *Borutzkyella revasi* (Buddelundiellidae). All these taxa belong to the Oniscidean sections Diplocheta (Ligidiidae) and Synocheta (Trichoniscidae and Buddelundiellidae), while no troglobiotic Crinocheta have been discovered so far in Georgian caves. Troglobiotic isopods are characterized by a high level of endemism at species and often at genus level. All the troglobiotic species of Oniscidea from Georgia are endemic to the country. Six species are single cave endemics, i.e., *Ligidium* n. sp. from Jortsku Cave, Zemo Svaneti Region, *Trichoniscus* n. sp. 2 and *Haplophthalmus* n. sp. from Sataplia I Cave, Imereti Region, *Trichoniscus* n. sp. 3 from Daghetis Damaluli Eklesia Cave, Qvemo Kartli Region, *Mingrelloniscus inchkuricus* from Inchkhuri Cave, Zemo Svaneti Region, and *Borutzkyella revasi* from Akui Cave, Abkhazia Region. All the other Georgian troglobiotic species occur in more than one cave, often in the same karstic area or region. Three genera of Trichoniscidae Haplophthalminae (*Colchidoniscus*, *Mingrelloniscus* and a new genus) and a genus of Buddelundiellidae (*Borutzkyella*) are endemic to Georgian caves.



Fig. *Colchidoniscus kutaissianus kutaissianus* from Melouri Cave

TrAQ: a free behavioural tracking software for subterranean fauna

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We introduce TrAQ, an innovative MATLAB-based software designed for two-dimensional tracking of unmarked single animals in Open Field video analysis, requiring minimal user intervention. TrAQ efficiently identifies the animal within a user-defined arena, offering precise quantitative measurements of the body centroid and the positions of two extremities. Users can select any arbitrary arena shape, and the software automatically calculates a comprehensive range of kinematic behavioral parameters. An optional shape erosion feature enhances its flexibility in removing animal's appendices or small debris. Developed by the University of L'Aquila, TrAQ is a free, versatile application that is not species-specific and can be adapted to various 2D behavioral assessments with no need for dedicated video hardware. Initially applied to groundwater copepods and later tested on mammals, it is suitable for both aquatic and terrestrial animals of any size and colour. The quantitative results have been successfully validated against commercial software. TrAQ is particularly useful for describing the locomotion traits of subterranean fauna, providing valuable insights into their movement patterns. To ensure user-friendliness, we have included intuitive GUIs and a detailed instruction manual outlining the initial steps. During our presentation, we will demonstrate the software's basic functionality and provide a download link. The TrAQ script can be customized to meet specific users' requirements by contacting the developers at davide.dicenso89@gmail.com and angelo.galante@univaq.it.



Preliminary data on the presence of trace elements in cave water and soil

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Cave soil is a complex matrix composed by materials produced *in situ* and by inputs from surface environments. External inputs are mediated by different atmospheric agents, such as wind, gravity and water fluxes, but also by species able to exploit both surface and subterranean environments (troglophiles and troglonexes). Independently by the way used to get into the caves, external inputs may be contaminated by pollutants of diverse typology and origin. Once pollutants get into the cave, they are unlikely recycled and can produce substantial harmful effects to the local biodiversity, compromising the entire ecosystem. Nonetheless, besides being bioaccumulated through the local trophic pyramid, pollutants from cave soil can penetrate groundwater, polluting our most important reserve of unfrozen freshwater and therefore representing an important hazard for human health. Developing a monitoring protocol is of high importance to preserve the ecosystem functionality and to avoid negative impacts on human population. We here present a preliminary data produced from the first study that aimed to assess pollutants in cave soil and water from multiple Italian caves. We specifically focused on the concentration of the following biologically available and potentially toxic metals: As, Ba, Cd, Co, Cr, Cs, Cu, Ga, Li, Mn, Mo, Ni, Zn, Rb, Se, Sr, Y, Zr, Ag, Ba, Tl, V, Hg, Pb, Bi and rare-earth elements from La to Lu. Trace elements analysis was conducted using the inductively coupled plasma mass spectrometer (ICP-MS, PerkinElmer, Elan-DRCe model). Samples were prepared according to the following phases: drying; weighing; acid digestion; evaporation; bring to volume; storage. The water analyzes have been completed, while the soil analyzes are currently underway. From a first, summary evaluation, the content of trace elements in the cave waters did not demonstrate particularly high values, and when this occurred (as for example for iron and aluminum) the high concentrations would seem to be attributable to the chemical properties of the rocks crossed by the water during percolation. This allows to hypothesize that potential pollutants are not driven into the cave from water fluxes. The assessment of pollutants in cave soil (still under analysis at the moment) will clarify the abundance and diversity of trace elements and will allow to speculate on potential origin.

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Reviewing biodiversity and distribution of subterranean fauna in China

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China covers a territory of approximately 9,600,000 km² and extends about 5,500 km from north to south and over 5,200 km from west to east. Owing to this huge geographical extension spanning from 53° 33' 39.506" North to 17° 59' 58.56" North in latitude, climates are extremely diverse, ranging from subarctic in the far north and alpine in the higher elevations of the Tibetan Plateau to subtropical and tropical in the south. China has also many karst areas, covering about 2,000,000 km², of which 1,300,000 km² are bare karst and 700,000 km² are buried karst. Although knowledge on Chinese caves is extensive, knowledge on subterranean fauna is still rather poor and fragmentary. More than 700 species exclusive of subterranean environments are currently known. About 98% of the species found in Chinese caves are endemic to the country. Based on the present knowledge, the areas of China with the highest number of troglobiotic/stygobiotic species are Guizhou and Guangxi Provinces. The high concentration of troglobiotic and stygobiotic species in these areas is due to biogeographical (these provinces are located on the border between the Palaearctic and Oriental biogeographical regions) and geological (the huge extent of the karst and the high number of caves) factors. It also reflects the large number of studies carried out in comparison to other provinces of China. The rapid multiplication of field and laboratory researches during these last decades has led to the discovery of new vertebrate and invertebrate species, while taxonomy has increasingly integrated molecular approaches. Species conservation issues have also been recently assessed for several groups. In the last years at least, investigations on airborne fungi and parasitic Laboulbeniales, as well as Bacteria from cave soil, air, and dripping waters, have been carried out. Current knowledge on these different aspects of the biodiversity and distribution of subterranean fauna of China is analysed and summarized in this contribution.



First insight into the microbiota of gypsum subterranean environments to study their evolution and protection

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Bacteria inhabiting subterranean environments have evolved metabolic strategies to cope with the absence of sunlight and the scarcity of organic compounds. Despite caves can be considered, from a certain point of view, extreme environments for life, they have been found to be hotspots of microbial diversity attracting research interests focusing on the discovery of novel bacteria and metabolic pathways also in relation with the microbial interaction with the rock substrates and the speleogenesis. Microorganisms are also bioindicators of the cav health being some bacterial groups potential proxies of human impact and environmental contamination. The study of the diversity and metabolic potential of microorganisms colonizing caves can therefore provide information on the evolution and protection of subterranean environments and might result in the discovery of novel microbes and metabolites with biotechnological interest. Up to date, most of the studies conducted on subterranean environments have focused on limestone caves, whereas the microbiology of gypsum caves is still understudied. Within this project (in the framework of PNRR-DM118/2023), we provide a first description of the microorganisms colonizing the gypsum caves that are part of “Parco Regionale della Vena del Gesso Romagnola” recently included as UNESCO World Heritage Site. For this purpose, we have first studied two different gypsum caves, i.e. the touristic cave La Tanaccia and the cave Bules, characterized by a sulfidic spring in the deeper part. Microbial characterization and abundance analyses were conducted through targeted metagenomics and qPCR assays. Geochemical analyses were conducted to assess the nature and abundance of macro- and micro-elements as well as organic matter composition. Microbial community composition analyses indicated that La Tanaccia is dominated by bacterial members belonging to different groups involved in nitrogen cycle like *Nitrosococcaceae*, *Nitrospiraceae*, *Nitrosomonadaceae*, including nitrogen fixation like *Beijerinckiaceae*. Other bacteria highly abundant in coloured patinas from the deep part of the cave are associated methane and/or methanol oxidation mostly belonging to the families *Methyloligellaceae* and *Methylomirabilaceae*. These results indicate that inorganic nitrogen compounds and C₁ compounds are possible fuels for the microbial growth in La Tanaccia. Moreover, some biofilms from the wall of this cave showed the high abundance of *Pseudonocardiaceae* potentially involved in CO₂ fixation and carbonate mineralization processes. Conversely, Bules cave is dominated by sulphate reducing and sulphur oxidizing bacteria belonging to the families *Desulfocapsaceae*, *Geobacteraceae*, *Sulfurovaceae* and *Sulfurimonadaceae*. The microbial composition can be related with the high presence of inorganic sulphur compounds both in the gypsum substrate and in the rising sulfuric waters. In conclusion, this work provides a first insight into the biodiversity present in gypsum caves to



envison the role of bacteria in shaping the only UNESCO World Heritage Site with a subterranean localization and their use as bioindicators for the protection of gypsum caves.

Is tail fat accumulation higher in hypogean vs epigean populations of *Speleomantes*?

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European plethodontid salamanders (genus *Speleomantes*) are troglophile amphibians that are able to inhabit both epigean and hypogean environments. These two types of habitats are characterized by different ecological conditions: contrarily to the former, the latter is typically characterized by absence of light, high microclimatic stability and scarce trophic resources. Due to these characteristics, the fauna specialized in living in subterranean environments has developed multiple adaptations, among which there is the ability to resist starvation and to efficiently storage more fat tissue. With this study we want to verify whether the hypogean populations of *Speleomantes* present a higher accumulation of fat compared to the epigean ones. We used 1061 high-quality photos of individuals, balancing between age classes and sexes, whose weight and total length were known. The photos were taken during different periods (autumn and spring) and years. We used the ImageJ program to estimate the tail area and length, and then we calculated the ratio between them (fat tail index; hereafter, FTI). Considering that *Speleomantes* store fat in the tail we used the FTI as a proxy of fat accumulation. We used Generalized Linear Mixed Models to assess potential correlations between individuals' FTI and multiple variables. We considered as independent variables the population habitus (subterranean vs. surface), sex (m,f,j), species and month of sampling. The results reported no significant difference in fat accumulation between epigean and hypogean populations ($p = 0.27$), hence it seems that subterranean populations did not increase their ability to store fat. On the other hand, significant differences were highlighted for the variables of sex ($p < 0.01$), species ($p = 0.028$) and month of sampling ($p = 0.003$). Our results demonstrated that females accumulate more fat, probably because they invest more in reproduction. March was the month in which *Speleomantes* showed the wider FTI; these results confirm our knowledge on the higher foraging activity of these species at the end of Winter. Finally, we observed a significant difference in FTI between species; however, we do not have enough information to produce an hypothesis yet.



Fig. *Speleomantes italicus*



Exploring the methods to monitor subterranean biodiversity via caves in Slovenia

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Assessment of the conservation status of subterranean species and/or communities is severely hampered by the lack of standardized and validated methods for biological monitoring. Subterranean species are an indispensable part of biodiversity, which is also recognized at the national and international levels. Caves are listed as a habitat type with special protection status in the EU Habitats Directive. Consequently, EU countries have the obligation to monitor and report the status of this habitat every six years. Despite this, there has been no monitoring of the subterranean biodiversity of caves in Slovenia. The only exceptions were sporadic attempts to monitor the cave beetle *Leptodirus hochenwartii* and regular monitoring of caves as part of an established national monitoring of bats. However, neither approach addresses the troglotic communities that are important for assessing the status of caves as habitats. A first step in this direction was taken in 2021 as part of the national project LIFE-IP NATURA.SI (LIFE17 IPE/SI/000011). In this project, we started to develop biological monitoring of caves as a habitat type. This task was conducted in several steps. First, we systematically reviewed the scientific literature from around the world to identify existing and verified schemes to monitor subterranean biodiversity. We planned the monitoring programs including also our own experiences and available reports from unpublished (grey) literature. We analyzed the sampling methods in terrestrial and aquatic habitats separately. For terrestrial species, we selected three caves in a karst massif in central Slovenia. We conducted i) visual inspections in the caves, ii) set baited traps (containing no preservatives) at approximately equal distances, and iii) standardized spatial and temporal visual inspections around the traps. To test the efficiency of sampling aquatic fauna in subterranean rivers, we applied direct collection and quantitative sampling of benthos in the Postojna-Planina cave system. The approach was repeated in terrestrial habitats in four seasons, whereas the subterranean river system was sampled in summer and winter. We investigated the efficiency of sampling, minimum effort required to obtain representative samples, and optimal combination of methods and seasons to adequately assess the diversity of local communities. In addition, we pointed out that the identification of subterranean taxa is difficult due to cryptic diversity and the lack of taxonomists. Therefore, we suggest that more emphasis should be placed on DNA-based methods for species identification in routine monitoring, but in close collaboration with the morphological identification of species to assess molecular identification. Based on our results, we have provided initial recommendations for the future development of biological monitoring of caves in Slovenia, as well as other regions with rich troglotic communities.



Validating COI metabarcoding primers for detection of subterranean fauna

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Subterranean ecosystems host diverse and ancient fauna, yet their study is hindered by substantial sampling difficulties. Environmental DNA (eDNA) metabarcoding is a promising tool to help monitor these ecosystems, but it faces challenges such as primer bias and non-target amplification. Therefore, rigorous validation of metabarcoding primers are essential to ensure accurate and comprehensive assessment of subterranean fauna diversity. This study addressed a need for robust primer validation through *in silico* and *in vitro* analyses, providing insights into primer performance across a range of subterranean taxa. Our objectives were to evaluate the efficacy of COI metabarcoding primers for assessing subterranean fauna diversity, addressing the challenges posed by traditional sampling methods. *In silico* analyses involved curating COI sequences from the Barcode of Life Database (BOLD) and selecting 14 primer combinations for subsequent *in vitro* testing using mock communities. Results demonstrate varied primer performance in PCR efficiency and detection limits across subterranean taxa. One assay (BF1/jgHCO2198) was able to detect 82% of taxa in the mock community, but only in at high DNA concentrations of target taxa. The greatest proportion of subterranean fauna taxa detected in a diluted mock community was 68% with the fwHF2/fwHR2n primer combination. Our findings underscore the potential of COI metabarcoding for assessing subterranean fauna diversity while highlighting the importance of primer selection and optimization. Overall, this study contributes valuable methodological development for future studies targeting the conservation and management of subterranean fauna biodiversity.



Echoes of the underworld: eDNA pathways for cave monitoring

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Karst regions constitute 22% of Europe and host a unique and highly endemic part of its biodiversity. However, identification of subterranean species is particularly problematic because of the general lack of taxonomists. Most species are also rare, endemic, and cryptic, and species turnover is very high between different regions. Furthermore, an extensive part of subterranean habitats consists of cavities and crevices that are inaccessible to humans. All of these drawbacks make consistent monitoring of these habitats extremely resource-demanding or even nearly impossible. Environmental DNA (eDNA) has emerged as a noninvasive alternative for assessing information on both single species and entire community structures, making it a perfect tool for the detection and identification of subterranean species. Here, we propose the development of a DNA-based molecular procedure using eDNA to overcome this "taxonomic barrier." This project focuses on the karst system of Monte Albo (Sardinia), which hosts an aquifer accessible through a large cave with deep siphons. The outlet, a spring captured by an aqueduct, is an important water resource in the region; however, hydrogeological studies have revealed that cave waters contribute to a small part of the aquifer outlet, suggesting aquifer compartmentation and inaccessible groundwater areas. The entire Monte Albo is part of a UNESCO MAB reserve and Nature 2000 site following the European Habitats Directive. However, it is still in need of an efficient monitoring plan, required both by the Directive regulation and by a proposal to withdraw water from the aquifer for drinking purposes. To do so, we filtered the water using a fine mesh filter to recover biological debris contained within the cave water during different hydrological seasons and at different cave locations. eDNA was then extracted from the filter using a dedicated kit (Sylphium eDNA Isolation Kit). The extract was later sequenced using distinct approaches (PCR-free, DNA barcoding) and sequencing methods (Illumina and Nanopore) to compare outcomes depending on the chosen method. In parallel, more traditional methods, such as species morphotaxonomy, will be used to produce a reference list and barcode to validate novel methods. This project will provide insight into the usability of novel biomolecular methods, such as eDNA, as fast, efficient, and affordable monitoring tools when traditional monitoring becomes too complex and time consuming.



Blood samples as an invaluable source of information on the health and reproductive status of the olm *Proteus anguinus*

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The olm, *Proteus anguinus*, is classified as vulnerable by the IUCN and is protected by national laws. In captivity, where we only have a limited number of animals, our long-term goal is to establish breeding pairs of these remarkable animals. Successful captive breeding depends on the fulfillment of several conditions, including the appropriate stage of the reproductive cycle of both sexes and ensuring overall good health without chronic stress effects. A major challenge we face is the reliable identification of olm's sex, as the animals are not sexually dimorphic. To address this, we have used non-destructive methods such as visual inspection of the animals and blood sampling to gather essential information. The study has been running since 2015 and we have analysed samples from twenty olms. Most of them were collected in the Postojna-Planina Cave System (*P. a. anguinus*; n=19), and one was from Bela Krajina (*P. a. parkelj*). The olms were captured and kept in captivity in accordance with the permission of the Ministry of Environment and Spatial Planning (Document No. 35601-8/2016-4) and the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (Document No. U34401-25/2013/11). Blood sampling was performed under anesthesia to ensure the safety and well-being of the olms. Although the amount of blood collected is minimal (e. g. up to 200 µl for an adult olm with a body weight of 20 g), it provides invaluable insights into various aspects of the olm's health and reproduction. From a single blood sample, we can: (1) make blood smears to assess overall health based on the leukocyte profiles and evaluate chronic stress of the animals based on neutrophil to lymphocyte ratios - stressed animals often have a ratio that is three times higher than normal. (2) By using microhematocrit capillaries and centrifuging them, the same small amount of blood can also be used to: (i) determine the hematocrit value to detect potential health conditions affecting the olm's well-being, (ii) use the blood plasma to measure sex steroids (estradiol, testosterone) to identify the sex of the olm, and (iii) detect the presence of the protein vitellogenin to identify females with developing oocytes. A combination of these molecular markers also helps to detect oocyte degeneration, which is a fairly common phenomenon in captive olms. (iv) In addition, we can use blood cells to establish primary cell cultures - a crucial step to obtain metaphase chromosomes, which provide important insights into olm genetics and aid in cytogenetic studies in a non-destructive manner. With all these approaches, we strive to ensure the well-being of our olms in captivity while increasing our knowledge of these endangered species. This research not only supports their conservation, but also enhances our ability to establish successful breeding programs for the olms, contributing to their long-term survival.



A multi-faceted approach to the assessment of alpha and beta diversity patterns in a groundwater biodiversity hotspot: conservation implications and future challenges

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The global biodiversity decline prompted the development of in-depth assessments of biodiversity and alternative conservation strategies for areas and individual species. Quantification of biodiversity with the aim to identify conservation-priority areas shifted from traditional measurements of local (α) and regional (γ) taxonomic diversity (TD) to inclusion of between-site differences in community composition (β) and other diversity facets, i.e. phylogenetic (PD) and functional (FD) diversity. In parallel, species-level conservation is increasingly more inclusive: besides species conservation status and endemism, attributes such as phylogenetic and functional distinctiveness are already being considered. These contemporary approaches have been explored in a variety of habitats and taxonomic groups, yet not in groundwater ecosystems. Groundwaters as habitats are poorly represented in conservation agendas and are often protected only indirectly. The development of conservation plans is facing various challenges, among which data deficiency on multiple diversity facets and poor knowledge on environmental drivers of diversity patterns are the most evident. Here, we explored the niphargid amphipod diversity in the Western Balkans (SE Europe), a subterranean biodiversity hotspot, which now represent a model system for multi-faceted and multi-scale biodiversity assessments from the conservation perspective. We used distributional, taxonomic, phylogenetic, and functional trait data for Niphargidae MOTUs (molecular operational taxonomic units) present in the region and 1) recognized MOTUs of high conservation priority based on multiple criteria, 2) analyzed α - and β -diversity patterns for all three facets, 3) evaluated the overlap of the most diverse communities with protected areas and 4) analyzed correlations between β -diversity patterns and environmental drivers. We found that both high-priority MOTUs and most diverse communities only weakly overlapped with current protected areas. Further, we detected high MOTU endemism, highlighted also by high taxonomic β -diversity with prevailing replacement component and geographic distance being the most important predictor of the total β -diversity in all three facets. Based on the findings, we list recommendations for future research and conservation efforts. On a MOTU level, these include promotion of species descriptions and IUCN assessments. On a community level, we suggest carrying out targeted sampling campaigns at the under-sampled areas while also complementing phylogenetic and functional trait datasets. Furthermore, it is timely to shift from the model systems to holistic community assessments. Lastly, we recommend investing efforts into defining biogeographic units based on groundwater connectivity, which would enable more accurate spatial scaling, contribute to better understanding of environmental drivers shaping biodiversity patterns, and be useful for the development of monitoring schemes.



Learning from non-scientists: how can citizens assist to fill groundwater knowledge gaps?

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Since the mid-1800s Australia has been engaging citizens in scientific research, presumably as the vast and remote nature of the continent coupled with a relatively small population has meant the collection of meaningful data requires additional efforts. Using citizen scientists (CS) to aid data collection in surface ecosystem is logistically simple and engaging volunteers in well understood ecosystems with charismatic biota is relatively easy. However, groundwater ecosystems are neither well known, easy to sample or have any ‘trophy’ species, thus engaging CS in these unknown environments is difficult. Using two case studies, we discuss how non-scientists can help fill knowledge gaps in groundwaters and their connected ecosystems. Firstly, CS farmers were engaged to collect water quality and biological data for the assessment of groundwater health within the shallow alluvial aquifers of the semi-arid and sparsely populated Namoi catchment in north-west New South Wales NSW. In this project, sampling was undertaken on 4 sampling occasions on 6 large properties between 2016-2018, coinciding with agricultural activities. Results were compared to samples taken in the same catchment by scientist between 2013-2018. Results indicated a ‘moderate’ groundwater health score (using the GHI) on many farms, highlighting several stygofauna species and potential water quality issues. The second case study involves integrating cultural knowledge with western science by engaging with remote communities in Wilcannia and Menindee in far western NSW to help identify areas of groundwater discharge into river systems- locally known as Ngardji holes. These two examples of engaging with communities outline how scientists can both learn from, and work with, non-scientists to further our knowledge of groundwater ecosystems. Both projects have aided researchers by helping to fill data-gaps and have highlighted the importance of raising the profile of these poorly understood but important ecosystems. The projects have successfully utilized landscape knowledge and on-ground logistical capabilities to enable the study of groundwater ecosystem dynamics. As the need and urgency for knowledge on groundwater quality and health is much greater than the limited number of professionals in this field can supply, we should be actively seeking to fill this data gap using both citizen scientist and local community knowledge.



CENOTEANDO - a group effort to contribute to the census, multidisciplinary description and biological monitoring of the Yucatán Peninsula sinkholes (cenotes), Mexico

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Cenoteando (www.cenoteando.mx) is a non-profit organization and working group that focuses on the shared development of a repository and database of all available data and information on the flooded caves or sinkholes of Yucatán, also known as “cenotes”. The multidisciplinary team’s objectives for the project cover both biophysical and socioeconomic variables with the hope to create and deliver impact assessment metrics to better manage tourist activities and other uses in the Yucatán cenotes. The initiative has three branches:

- 1) A community focused working group that deals with environmental education, citizen science, communication, and promotion of tourism management best-practices in cenotes
- 2) An academic focused working group, that centers on the taxonomy and ecological function of the unique underwater trophic web processes in the Yucatán Cenotes.
- 3) A data repository focused working group that aims at storing all obtainable information for each cenote known in a findable, accesible, interoperable, and reusable (F.A.I.R.), publicly available data repository. Access the GitHub project at: <https://github.com/cenoteandoDB/cenoteando>.

We are committed to the biological and geological conservation and responsible use of the cenotes, as well as to the investigation of their resources, to create products for scientific, academic, social and tourist purposes that positively optimize the use of these natural assets that are so important for the local ecosystem. This presentation will share the history of the working group and provide details and insights into our current activities, projects, initiatives and potential for collaborations.



A collaborative database to fasten sustainable biodiversity data acquisition: the World Asellidae Database

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Collaborative databasing has become crucial to biodiversity sciences because the amount of data needed to address broad-in-scope questions typically exceeds the production capabilities of even the most performing laboratories. Yet, collaborative databases that encourage joint-production and sharing of data before publishing are still few, even though they are key to minimize work duplication among data producers, thereby promoting sustainable data production. Here, we present the World Asellidae Database (WAD), a tool specifically designed to provide scientists with an efficient and sustainable way to jointly produce multiple-species ecological and molecular resources to study life evolution in groundwater. The Asellidae (Pancrustacea, Isopoda) is one of few families of aquatic metazoans containing both surface and subterranean species, thereby providing an excellent model for understanding eco-evolutionary processes taking place during the transition from surface to groundwater habitats. First, we describe the guiding principles and content of WAD. The tool functions as an open laboratory in which species occurrence data and DNA sequence metadata are made available to all as data production flows, hence well before data are published. WAD contains about 10,000 distributional records for approximately 500 species, 2,000 specimen lots and metadata - from sampling of specimens to PCR and chromatogram settings - for more than 9,000 DNA sequences belonging to two mitochondrial and three nuclear genes. Second, we use molecular data available in WAD for two mitochondrial genes and two nuclear genes to produce a worldwide dated phylogenetic reconstruction of the Asellidae family to be used in future comparative studies of species traits in surface and groundwater asellids. Third, we use that phylogenetic reconstruction and body size data from published articles and morphological measurements made on specimen lots referenced in WAD to test for changes in male and female body size during transition from surface to groundwater habitats. We provide the first, phylogenetically controlled evidence that the difference in body size between surface- and groundwater species is sex dependent. Body size of males is significantly larger in surface water than in groundwater species whereas female body size does not differ between surface water and groundwater asellids. We propose that male conflicts for mating with large receptive females selects for large males in surface water whereas male competition for finding rare receptive females selects for smaller, more agile males in groundwater. Fecundity selection potentially favors large female body size in both surface and groundwater habitats.



Far below elephants and rhinos: the role of regional and local predictors on invertebrate richness in African caves

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To develop effective conservation strategies that protect cave ecosystems and mitigate the impacts of climate change on their communities, it is crucial to understand the factors influencing their diversity patterns. Despite the African continent's size and ecological significance, studies on cave biodiversity remain scarce. This study aims to understand how local and continental environmental factors affect the diversity of subterranean invertebrates in Africa. Samplings took place in Morocco, Kenya, Namibia and South Africa. The invertebrate survey involved active visual searches and manual collections within defined sectors (3x10m) and quadrants (1x1m), with three collectors ensuring comprehensive coverage until all invertebrates were sampled. Substrate and resource types were visually quantified in each sampling unit and used as proxies for cave resource availability. We categorized environmental variables into physical and trophic categories. To assess habitat heterogeneity, we used the Shannon index for substrate diversity, creating variables for Diversity of All Substrates (DIV), Diversity of Shelters (DIVA), and Diversity of Organic Resources (DIVR). We calculated zeta diversity, a measure of species turnover between sites, with the order indicating the number of sites considered. For example, zeta diversity order 4 represents the average number of species shared by four sites. Multi-Site Generalized Dissimilarity Modelling (MS-GDM), which combines zeta diversity with GDMs to analyze multi-site species turnover, was used to identify environmental variables affecting widespread species distributions. For the total African subterranean fauna sampled, our models at zeta order 2 indicated that annual precipitation had the most significant effect on species turnover, followed by mean annual temperature and distance. At zeta order 3, distance showed the highest effect, followed by mean annual temperature and annual precipitation. In the Mangroves biome, models could not be produced due to zero turnover values up to order 2. In contrast, in the Tropical and Subtropical Grasslands, Savannas, and Shrublands biome, human alterations, distance, and temperature seasonality were the most important factors at zeta order 2 to 4, highlighting the variability in responses to different variables across biomes. This study provides valuable insights into the relationships between environmental factors and biodiversity in African caves, offering crucial information for conserving subterranean habitats in the context of climate change. Our findings highlight that caves possess a high degree of rare species, evidenced by zeta diversity reaching a maximum at order 4.



Extraordinary diversity and patterns of distribution in tiny subterranean crustaceans: Bathynellacea of Western Australian arid zone

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Northern Western Australia is a recognised hotspot for subterranean fauna. This rich biodiversity has largely been revealed during environmental surveys related to mining developments. Bathynellacea are small interstitial/subterranean crustaceans. Their Pangean distribution suggests that their ancestors were already present in the Carboniferous-Permian period, possibly living in the warm seas and spread before Pangea breakup. Bathynellacea have been restricted to groundwater habitats for a long time, as suggested by the absence of extant surface species, potentially millions of years longer than contemporaneous taxa within the same assemblages. Their present-day ranges are influenced by a combination of restricted habitats, complex lifestyles, and biogeographical patterns reflecting also ancient hydrology. Providing ecosystem services such as water purification and nutrient cycling, species in this important stygobitic group are still poorly described. To date, only 60 species, representing two families (Bathynellidae and Parabathynellidae), are formally recognised in Australia. Parabathynellidae include ten mostly endemic genera, and two genera that have ‘cosmopolitan’ and ‘Gondwanan’ distributions: respectively, *Hexabathynella* (25 described species), and *Atopobathynella* (19 described species). Bathynellidae are more neglected with only 5 species described in the country. However, recent morphological and molecular studies conducted have revealed an abundance of species within these two families, with intriguing distribution patterns. Understanding Bathynellacea diversity and species boundaries is crucial for the conservation of groundwater habitats in a landscape increasingly impacted by human activities.



Stygofauna Mundi: a comprehensive global biodiversity database of groundwater-related habitats across marine and freshwater realms.

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Groundwater ecosystems are inhabited by unique assemblages of animals, often with restricted distributions and highly specialized traits. Those assemblages sustain ecosystem functioning and contribute to important ecosystem services. Knowledge of the species occurring in those habitats and the main ecological and historical drivers for their distribution is important not only from a theoretical point of view, but also to ensure their optimal conservation and groundwater resources management in the perspective of global change. Unfortunately, despite more than a hundred years of research, most information on the species occurring in groundwaters is either not directly accessible, or scattered across a myriad of publications, personal collections, and databases with often a narrow scope and interoperability. To enhance the accessibility of this information, we have built Stygofauna Mundi. Stygofauna Mundi, named after the compendium published by Lazare Botosaneanu in 1986, is a relational, interactive, interoperable, and open-access database aimed at gathering all available information related to animals inhabiting groundwater environments and connected ecotones. We mined all records available in each data source independently, allowing us to trace the origin of each entry. We included all records for aquatic species found in groundwaters, whether they are permanent residents or present only as aquatic or semi-aquatic life stages. We linked and validated all taxonomic names after the Catalogue of Life, Worms-APHIA platform, GBIF, and GenBank. Each name is connected to a set of metadata including the level of subterranean specialization, lifestyle, and available DNA sequences. Functional traits will be incorporated in a subsequent version. For each locality, Stygofauna Mundi includes coordinates (available for 87% of the localities so far), as well as geographical and geological data interoperable with most regional and national cave cadasters and open access databases, such as Global Lithological Map, HydroAtlas, GMBM Mountain Inventory, Global Hydrogeological Maps 2.0, World Administrative Regions, Marine Ecoregions, and OpenStreetMap. The database contains 388,872 records, extracted from 11,661 data sources written in 28 languages. These records account for 52,120 localities and 31,338 taxonomic entities. The localities include springs (N=18,958); marine, coastal, and inland caves and mines (N=12,361); interstitial environments in lakes, seashores, and rivers (N=10,453); wells and boreholes (N=9,326); as well as groundwater-fed and anchialine pools (N=1,022). While they cover all continents, records originate mostly from Europe (N=24,511) and North America (N= 13,216). The most taxon rich are Hexapoda (N=5,407), followed by Copepoda (N=3,892), Nematoda (N=3,802), Mollusca (N=3878), Platyhelminthes (N=2,200), Annelida (N=2,038), Amphipoda (N=2,014), and Acari (N=1,765). By means of Stygofauna Mundi, we will describe firstly the taxonomic and geographic biases that affect global knowledge on groundwaters. We will then expand on the habitat classification by Botosaneanu in the original Stygofauna Mundi combining data on the hydrology, geology, chemical profiles, and community composition within localities. Next, we will study the main drivers for changes in taxonomic alpha and beta diversity at a global scale, both across different animal lineages and groundwater ecosystems. Finally, we will explore how to use these data for boosting global conservation plans for the groundwater ecosystems, e.g., by proposing a spatially-explicit strategy to better incorporate groundwater biodiversity into the expanding national and international networks of protected areas.



The Dinaric Cave Tube-Worm *Marifugia cavatica* Absolon and Hrabě 1930 (Annelida: Serpulidae): New Insights into Distribution and Biology

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The Dinaric cave tube-worm (*Marifugia cavatica* Absolon and Hrabě 1930) is the only known subterranean representative of the serpulid family (Serpulidae, Polychaeta) in the world. It inhabits the underground waters of the Dinaric region, which spans several countries in Southeast Europe, including Italy, Slovenia, Croatia, Bosnia and Herzegovina, Montenegro, Serbia, and Albania. The Dinaric Karst is renowned for its extensive limestone formations, distinctive karst topography, and unique subterranean biodiversity, including one of the most iconic subterranean species, the olm (*Proteus anguinus* Laurenti, 1768), the largest obligate cave tetrapod. For *M. cavatica*, we still lack comprehensive data on its distribution, biology, and genetics. The species is categorized as Data Deficient according to the Red Book of Cave Fauna of Croatia, highlighting the need for further research. Therefore, the goals of our research are: (1) to collect and compile all available published and unpublished data on the distribution of the species, and (2) to track the rate of settlement and growth of *M. cavatica* as a basis for future monitoring of the species' population. Through this research, we compiled a distribution list and documented the species in 159 localities across the Dinaric region. Of these, 68 are confirmed living populations, 4 show fossil tubes, 16 contain tube fragments, and 71 localities require further investigation to determine whether they support living populations. The distribution of *M. cavatica* coincides with that of other stygobiont inhabitants of the Dinaric Karst, such as bivalves of the genus *Congeria* and the olm (*P. anguinus*). Recent research on *P. anguinus* indicates the existence of nine major lineages along the Dinaric Karst that could be considered distinct species based on molecular divergence. This could similarly apply to *M. cavatica*, necessitating molecular research on hydrologically separated populations of the Dinaric cave tube-worm. For our second goal, we established permanent plots in two caves with a scale to monitor the settlement and growth trends of the population and individual *M. cavatica* over time. In Tounjčica Cave, we installed one stone surface, and in Jama nasuprot Torka Pit, we installed surfaces of different materials (terracotta, stone, and PVC) to investigate the species' substrate preferences. Additionally, we examined multi-year photographs of Dinaric cave tube-worm individuals from Jama nasuprot Torka Pit. Our results revealed valuable insights into their annual growth patterns. The analysis of this selected population showed an annual growth of 0.7 ± 0.4 rings (growth segments), equivalent to 0.2 ± 0.1 mm annually. The results of this research provide a foundation for the development and future application of a monitoring method, as well as insight into the species' distribution. Sites listed as unknown in this study, literature-referenced sites, and sites where only tubes are noted without further indication of whether they belong to living populations are the first ones that require more detailed investigations.



Life below the City of Vienna - Drivers of groundwater fauna distribution in an urban ecosystem

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Over thousands of years, groundwater fauna has adapted to life in a challenging, yet relatively stable subsurface environment. However, groundwater habitats are neither homogeneous environments, nor isolated from (surface) impacts, but are tightly interconnected with other ecosystems and influenced by different environmental dynamics. Since groundwater ecosystems are not easily accessible and observable environments, little is known about key factors driving groundwater fauna distribution and habitat suitability. Previous studies have shown that groundwater fauna is rather unevenly distributed, and that high biodiversity is found in patchy hotspots rather than in clear spatial patterns. Although predictors for the distribution of individual species have been identified in some cases, overall habitat suitability criteria in relation to richness and biodiversity are vague, as communities are shaped by a combination of diverse species requirements. Urban areas pose various anthropogenic pressures, such as surface sealing, lack of dissolved oxygen, and heat accumulation, which may affect groundwater fauna habitat suitability. In the project 'Heat below the City', 150 groundwater wells in the city of Vienna, Austria, have been sampled and a large number of abiotic, as well as biotic variables, including abundance, richness and diversity of groundwater fauna, have been analysed to identify the main drivers of groundwater ecosystem biodiversity and functioning. Samples were collected in Autumn 2021 and Spring 2022 to account for seasonal dynamics. Our results show that even below highly urbanised areas such as the city centre of Vienna, groundwater fauna biodiversity can be impressively high. Within the most common group of groundwater invertebrates, the crustaceans, 47 stygobiont and stygophile species could be found, including several species endemic to the region as well as species previously undescribed in Austria and worldwide. A habitat suitability analysis combining 6 major environmental factors (geology/permeability, oxygen availability, temperature, depth of the groundwater table, degree of surface sealing and distance to surface water) presumed to govern the presence and distribution of groundwater fauna were linked to crustacean species richness and biodiversity. Together with the application and comparison of several groundwater ecosystem health indices - still in progress - we will develop an integrative and sustainable groundwater management strategy that combines groundwater use and quality aspects with conservation and protection strategies for groundwater biodiversity.



Mercury content in aquatic organisms from anchialine caves, Croatia

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Anchialine ecosystems are fascinating and unique environments characterized by their distinct physical and biological features. It is known that in some anchialine caves at the Croatian Adriatic Sea coast mercury amounts in the water column and sediment are significantly elevated. As mercury (Hg) contamination is a significant environmental issue, its content in anchialine cave organisms can provide important insights into the ecosystem health of these unique habitats. We analyzed Hg amounts in the water column and in animals from seven anchialine caves from the National Park “Kornati Islands” and one from a coastal anchialine cave Mandalina near the city of Šibenik, all located on the coast of the central Adriatic Sea. In total, Hg was measured in 97 organisms (*Troglocaris*, *Niphargus* sp. and some unknown amphipods), caught manually by a cave scuba diver. Most of the organisms were of insufficient mass to be analyzed separately, and composite samples containing 5 to 10 organisms were analyzed. Hg, in all matrices, was measured by cold vapor atomic absorption spectrometry (CVAAS) with Elemental Mercury Detector 3200 (Thermo Separation Products, USA), using the 253.7 nm absorbance peak. In comparison to marine organisms from the Adriatic Sea, Hg concentrations were elevated in most of the analyzed organisms, with some organisms having very high Hg concentrations. Hg amounts ranged from 9 to 552 ng/g. In Mandalina Cave, *Niphargus* sp. showed the highest Hg content of all organisms (552 ng/g), while the highest content of all *Troglocaris* was also determined. It is interesting that Mandalina Cave had quite low concentration of Hg in the water column. However, the cave is home to a large colony of bats, which are a known source of mercury. Knowing the mercury content in anchialine cave organisms is crucial for assessing the health of these unique habitats. Ongoing research and monitoring are essential to estimate the impacts of mercury contamination and preserve the biodiversity of anchialine cave ecosystems.



Impact of Tetrachloroethylene on Groundwater Ecosystems: Sub-Chronic Effects on the Locomotion Behavior of Two Harpacticoid Copepod Species

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Aliphatic chlorinated hydrocarbons, particularly tetrachloroethylene (also known as perchloroethylene, PCE), persist as significant groundwater contaminants, often exceeding safe drinking water thresholds. This study fine-tunes the groundwater risk assessment of PCE on the sensitivity of groundwater-obligate (stygobitic) species. Through sub-chronic exposure trials on two copepod species, the stygobitic *Moraria* sp. and the non-stygobitic *Bryocamptus zschokkei*, we challenged the current European Predicted No-Effect Concentration (PNEC) of PCE in groundwater. Specifically, we applied a correction factor of 1000 to the current PNEC of PCE in water (50 ng/L PCE) and investigated the sub-chronic effects on the locomotion traits of the adult females of the two species. We used TrAQ, a MATLAB-based software developed by the University of L'Aquila. The software, designed for two-dimensional tracking of unmarked single animals video recorded in Open Field tests, is free and can be adapted to various 2D behavioral assessments with no need for dedicated hardware. Our findings revealed significant locomotion impairments in both species at a concentration of 50 ng/L PCE, suggesting the inadequacy of the existing European groundwater risk assessment procedures. *Bryocamptus zschokkei* exhibited particularly high mortality rates and persistent post-exposure impairments. The absence of recovery after PCE clearance in *B. zschokkei* underscores its higher susceptibility to PCE compared to *Moraria* sp., potentially due to differences in metabolic rates. This finding highlights the potential of *B. zschokkei* as a surrogate species for groundwater risk assessment. We advocate for refining groundwater risk assessment methodologies to better account for the vulnerabilities of stygobitic species.



Groundwater fauna below the city of Munich and relationships to urbanization effects

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Our project focuses on the groundwater ecosystem below the city of Munich, Germany, as a role model for understanding the spatial and temporal dynamics of groundwater biodiversity within a quite homogeneous, shallow urban gravel aquifer. In general, the city's groundwater is subject to various stressors, including increased temperature, chemical pollution, and an altered hydraulic regime. Facing these challenges, the shallow aquifer serves as habitat for a diverse invertebrate fauna. To date, ecological studies on shallow aquifers mainly focused on natural and arable environments, with little attention to biodiversity and of its relationships to anthropogenic factors in urban groundwater habitats. Almost 100 groundwater wells were repeatedly sampled in the course of two field campaigns within the city limits of Munich and a few sites located in the more natural vicinity. Physical and chemical parameters, along with hydrochemical analyses—including major anions, nutrients, and dissolved organic carbon (DOC)—were performed on water samples collected from the wells in comparison to freshly pumped groundwater. Groundwater fauna were collected from the well bottoms using a net sampler and, where possible, determined to species level. Overall, dissolved oxygen concentrations were found to be high throughout the city's groundwater, while DOC concentrations were low, indicative of oligotrophic aquifer conditions. On the other side, groundwater temperatures exhibited a clear elevation within the city centre, correlating with the degree of urbanization and well depth. Among the 201 sampled wells, 81.6% were found colonized by fauna during the two sampling campaigns. The most abundant taxonomic groups identified were cyclopoids, ostracods, and amphipods. Crustaceans collectively comprised 56–63% of the collected specimen, consistent with the typical composition of porous aquifer groundwater fauna communities. Out of the 56 species identified, 21 were classified stygobiont and 18 stygophilic, with some noteworthy discoveries such as the first record of *Nitocrella psammophyla* from Germany. The presentation will highlight the spatial and temporal trends of faunal abundance and diversity, examining their relationships with different aspects of urbanization. We will discuss the relationship between faunal biodiversity and various abiotic factors and categories, including depth of groundwater table and land use.



Nation-wide standardization of groundwater ecosystem health assessment - a microbiological-ecological approach

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Microorganisms can be viewed as engineers that drive the Earth's biogeochemical cycles. This is also true in aquifers where microbes mineralize organic compounds, recycle nutrients, and (im)mobilize metals, including priority pollutants. In fact, microbes provide essential ecosystem services including the purification of water. In comparison to fauna, the microbiome is characterized by a faster response to dynamics in environmental conditions in terms of activity and biomass. In addition, microbes are ubiquitously present in aquifers. In summary, microorganisms may serve as ideal bioindicators for the assessment of the ecological status of groundwater and aquifers. The recently developed D-A-C index approach integrates some basic and easy to measure features of microbial communities. In detail, the density of prokaryotic cells (D), the microbial activity (A), and information on the available energy, i.e. the quantity and/or quality of dissolved organic carbon (C), promise useful information on the microbiological-ecological water quality. This approach has been applied successfully in case studies on the local to regional scale (Fillinger et al. 2019; Retter et al. 2021). For its use in a national monitoring program, however, an important next step is the delineation of reliable reference conditions. This requires the identification of key factors influencing microbial community biomass and activity, as well as the composition of dissolved organic matter (DOM). In our study, the D-A-C components were recorded for more than 1,961 groundwater wells distributed throughout Austria. The prokaryotic cell density was recorded via flow cytometry. Here, in addition to pure cell numbers, the proportion of cells with low and high nucleic acid content (LNA/HNA) was taken into account as an additional activity measure. Intracellular ATP, a proxy of microbial activity, was quantified by means of a bioluminescence assay. The concentrations of dissolved organic carbon (DOC) and the quality of DOM were determined in a TOC analyzer and by fluorescence spectrometry, respectively. DOM quality was assessed by means of different indices, i.e. the humification index (HIX), the biological index (BIX), and the fluorescence index (FI). In a first step, reference sites were identified based on hydro-chemical thresholds derived from the Austrian GeoHint report, which provides natural background values for 27 physical-chemical parameters (Briellmann et al. 2018). About 10% out of 3,700 of groundwater samples, could be classified 'hydro-chemically undisturbed'. Still, the abundance and activity of microbes revealed a high variability in this reference dataset. In a second step, the type of aquifers, the lithology, the season, the redox state, the land cover, and the type of well were tested for its influence. Pre-existing assumptions, like the occurrence of higher cell numbers in anoxic groundwater could be confirmed. Moreover, new insights regarding the effect of lithology, the quality of DOM, as well as the effect of land cover were obtained. A first classification scheme for Austrian groundwaters and related reference conditions for the microbial communities will be introduced.



Spatial and temporal analysis of groundwater fauna

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Groundwater is an important global resource, providing water for industry, irrigation, geothermal uses and potable water. Moreover, groundwater harbours the world's largest terrestrial freshwater biome with ecosystems, inhabited mainly by invertebrates (stygo fauna) and microbes that undertake important services including water purification, as well as carbon and nutrient cycling. Despite some local studies on the spatial and temporal variation of groundwater fauna and the general influence of environmental processes on this ecosystem, even the most basic knowledge about these ecosystems is lacking in most parts of the world. This study aims to provide a spatial and temporal overview on groundwater fauna (stygo fauna), including the global distribution of groundwater fauna research. Moreover, shifts in groundwater fauna due to natural or anthropogenic impacts are examined on a regional scale. To achieve this, an extensive review of accessible groundwater fauna data was conducted by analysing 859 studies. This analysis revealed an exponential increase in the number of groundwater fauna studies since the 1920s together with changing paradigms in the research focus and sampling methods. Moreover, studies on groundwater fauna are spatially uneven, and large gaps in the spatial and temporal distribution of groundwater fauna research, particularly in Africa, Asia and the Americas were identified. With a sampling density of 0.011 samplings/km² and a high frequency of sampling (28.8 samplings/year since 1876), stygo fauna in Germany is one of the best-studied worldwide. Studies in Germany focus on biodiversity and ecological aspects while applying ecological research to address groundwater management and monitoring requirements. We here use long-term groundwater data from Southwest Germany to identify shifts in groundwater fauna due to natural or anthropogenic impacts over the past 20 years. Comprehensive analysis of metazoan groundwater fauna and abiotic parameters from 16 monitoring wells revealed no overall temporal trends for fauna abundance, biodiversity and no significant large-scale trends in abiotic parameters. However, seven wells exhibit shifting or fluctuating faunal parameters, linked to natural causes, such as decreasing dissolved oxygen contents and fluctuating groundwater temperatures, or linked to anthropogenic impacts, such as construction activities and changes in land use. In the future, a more evenly distributed stygo fauna sampling effort, especially in currently underrepresented areas of the world, and more long-term studies of groundwater ecology with higher temporal resolution are necessary to provide a more comprehensive perspective on stygo fauna biodiversity, services and functional significances. This is important to increase our knowledge about the sensitivities of these ecosystems to anthropogenic activities as well as climate change and is fundamental to the effective management of these ecosystems. To ensure that groundwater ecosystems are maintained and preserved in the future, stronger policies and monitoring requirements need to be implemented.



An improved Feulgen method reveals genome size variations within and between surface and subterranean populations of *Asellus aquaticus* (Crustacea, Isopoda)

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Genome size is a fundamental biological trait that varies significantly among eukaryotic species. However, research on its intraspecific diversity is limited. Previous studies comparing surface and subterranean isopod species found that cave species have generally larger genomes than surface ones; hence, we wondered whether the same pattern would be observed within species. To fill this gap, we examined intraspecific variations in genome size in *Asellus aquaticus*, a freshwater isopod crustacean with surface and cave-dwelling populations. A total of 39 specimens were collected from surface and subsurface populations in Slovenia and Romania, as well as seven individuals from a hybrid F1 population obtained by crossing hypogean males with epigeal females (both from Romania). We used our refined Feulgen Image Analysis Densitometry protocol, which is based on three standards instead of one, includes various checks of the theoretical expectations of the methods and returns a precise genome size estimate as well an estimate of its coefficient of variation. We found C-values ranging from 0.979 to 2.45 pg within surface populations, from 1.21 to 4.11 pg for subsurface populations and from 1.75 to 2.91 pg for the F1 population. Intrapopulation genome size variations ranging from 1.25 to 2-fold were detected. For surface populations, variation was similar among males and females, but for cave populations, the variation was 1.7-2 times larger in males than in females. Cave populations also had larger average genome sizes than surface populations, with the hybrid F1 population having a genome size intermediate between those of its parent populations. The observed variations have various possible explanations, including whole-genome duplication, copy-number variations, supernumerary chromosomes and/or variations in the relative amount of non-coding sequences versus gene-coding ones. Whole-genome sequencing will be required to find out which one(s) of these hypotheses is correct.



Genome skimming and amplicon sequencing: novel DNA-based techniques shed light on the taxonomy and phylogeny of the *N. thuringius* - *N. dolenianesis* species complex (Crustacea, Amphipoda, Niphargidae)

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Subterranean fauna is an important contributor to global biodiversity. A case example is the genus *Niphargus*, the most common and species-rich subterranean amphipod genus of the West Palearctic. Although niphargids inhabit almost every kind of groundwater in Europe and the Middle East, the taxonomy of most clades in the genus remains unresolved. To improve our understanding of Alpine endemic clades of the genus *Niphargus* and to investigate the impact of glaciation on their phylogeography, specimens were collected at many unsampled sites, both in formerly glaciated areas and in areas very close to the Quaternary glacial maximum. All type localities of species present in the Alps were sampled and complemented with previously published sequences of Alpine species available in GenBank. In this presentation we focus on results concerning the *Niphargus thuringius* - *Niphargus dolenianesis* species complex, which inhabits springs and alluvial waters in the pre-Alpine area. It is distributed along the southern margins of Quaternary glaciations in the whole pre-Alpine area and in some nearby alluvial sites. In total over 350 Alpine sites were visited and sampled, however this species complex was found in 25 sites. To overcome the limitations of Sanger sequencing, we sequenced our amplicons using Nanopore, a faster and cheaper technique allowing easier reconstruction of the two haplotypes of heterozygous individuals. For resolving deeper nodes, we also used genome skimming, i.e. low-coverage sequencing allowing to reconstruct high-copy number markers such as mitogenomes (mtDNA), nuclear ribosomal DNA (rDNA), and nuclear histone 3 (H3), using Nanopore and/or Illumina. These novel approaches, applied here for the first time to *Niphargus*, allowed us to clarify the phylogeny and biogeography of this species complex. We notably confirmed that it is a monophyletic group distributed from the western pre-Alps in Piedmont to western Slovenia throughout the entire pre-Alpine area and identified its sister clade, which includes species from the Balkan area (Slovakia and Bosnia-Herzegovina).



Delimiting species in the dark: a benchmark of DNA taxonomy workflows for subterranean organisms

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Morphological taxonomy of subterranean species is very much obscured by strong evolutionary pressures, which result in morphological stasis after speciation as well as morphological convergence between long diverged lineages. A glimmer of hope in this forlorn darkness comes from DNA taxonomy, which holds the potential to unravel true species boundaries unabated by selective pressures acting on their morphology. However, DNA-based approaches are not trivial to apply to subterranean organisms, as many of them assume implicitly that species are panmictic, without strong population differentiation. This is at odds with the biology of many cave species, which exhibit limited dispersal and large genetic differences between populations. Simulation studies have repeatedly suggested that tree-based approaches rooted in coalescent theory, such as GMYC (Generalized Mixed Yule-Coalescent), PTP (Poisson Tree Processes) and BPP (Bayesian Phylogenetics and Phylogeography), are prone to mistaking differentiated populations for distinct species, whereas distance-based, such as ABGD (Automatic Barcode Gap Discovery) and ASAP (Assemble Species by Automatic Partitioning) and allele sharing-based (haploweb) approaches appear less sensitive to such issues. To test this theoretical expectation, I present here the results of a benchmark of species delimitation approaches on several representative empirical DNA sequence datasets obtained from cave-dwelling animals and draw from these results general recommendations on the most effective way to delineate species of subterranean organisms.



Insights from genome skimming of Remipedia (*Xibalbanus* spp.)

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Remipedia is a class of elusive insect-like blind crustaceans adapted to the marine water layers of anchialine caves around the world. Together, the Yucatan Peninsula and Cozumel Island host four species including *Xibalbanus tulumensis* (the first documented venomous species) and *Xibalbanus fuchscockburni* from coastal caves of Quintana Roo, *Xibalbanus cozumelensis* of Cozumel and *Xibalbanus cokei* from marine caves in Belize. Species delimitation based on morphology and the inclusion of molecular tools has supported distinct species for the three Mexican *Xibalbanus* species, while DNA sequence data from specimens collected from the type localities have only been available for *X. fuchscockburni* and *X. cozumelensis*. *Xibalbanus cokei* has only been evaluated based on morphology alone. The goal of this study was to expand the molecular data available for all *Xibalbanus* species and from their respective type localities to serve as species references. Initial screening via DNA Barcoding (i.e., COX1) supports that *X. cozumelensis* is distributed across multiple caves in Cozumel Island, previously thought to be endemic to a single cave, but highlighted the need to further evaluate the other three Yucatan Peninsula species using genomic approaches. Low pass whole genome sequencing (i.e., genome skimming) offers a low-cost alternative to reconstruct whole mitochondrial (mt) genomes, circumvents PCR amplification steps, and allows for screening of phylogenetically informative ribosomal and protein coding genes. Gene data generated and evaluated from genome skimming confirms that the Yucatan Peninsula hosts a single broadly distributed *Xibalbanus* species and implies that single gene analyses may have led to incorrect species designations due to PCR biases caused by the presence of competing nuclear mitochondrial pseudogenes.



Unveiling the unknown: first insight into molecular diversity and DNA barcode library of epikarst-dwelling invertebrates in the Western Carpathians

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Epikarst, the uppermost zone of the karst landscape, represents a critical interface between soil and carbonate rocks. It forms a perched aquifer that stores, gradually releases infiltrated precipitation and serves as a subterranean aquatic habitat. Between 2019 and 2020, we conducted extensive sampling of epikarst invertebrates using 27 filtering devices in four caves of Demänová valley, northern Slovakia, Central Europe. Our integrative approach combined conventional morphological techniques with molecular tools, specifically DNA barcoding, to evaluate the invertebrate diversity of this understudied subterranean habitat. Utilizing the 3' fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene, we successfully generated 784 DNA barcodes representing 36 morphospecies from 17 orders and 6 animal classes, with a predominant representation of Arthropoda (98.3%). Morphological identification revealed 10 obligatory aquatic species, including four stygobionts restricted to groundwater habitats, nine amphibiotic species, and 17 terrestrial species, including five troglobionts associated with the cave environment. Our study provides unique documentation of the genetic and species diversity of Metazoa within the epikarst, with more than 70% of Barcode Index Numbers (BINs), treated as rough equivalents of species, being new to the Barcode of Life Data Systems (BOLD) database. Importantly, 24 out of 28 morphologically identified subterranean species lacked sequence coverage in BOLD, emphasizing the novelty of our findings. Notable cases of intraspecific divergence were observed, particularly in subterranean taxa, highlighting the existence of morphologically indistinguishable cryptic species within the fragmented karst system. Our data provide evidence for undescribed species of the harpacticoid copepod of *Elaphoidella* genus and suggest the potential presence of a new-to-science family within the order Amphipoda. In the case study of the Demänová caves, we demonstrate that the genetic and species diversity of invertebrates associated with seeping epikarst waters is vastly understudied. Our findings shed light on the overlooked realm of subterranean biodiversity, emphasizing the need for future extensive investigations into groundwater life diversity in the Western Carpathians and adjacent regions. Furthermore, it provides a novel DNA-based perspective for future comprehensive investigations of subterranean biodiversity and marks the initial phase of a broader research endeavour focused on the biodiversity of the epikarst.



Using genomic tools to enhance biodiversity assessments in anchialine caves

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Molecular tools allow non-invasive biodiversity assessments and metapopulations analyses. Low pass whole genome sequencing is a cost-effective method that circumvents PCR bias, one of the biggest caveats in metagenetic studies. Our goal is to characterize benthic metazoan biodiversity via metagenomic analysis of environmental samples, determine the best genomic tools and workflows, and evaluate the utility and shortfalls of current public molecular databases for documenting stygobionts and poorly known taxa. In this study we analyzed DNA derived from sediment samples from two anchialine caves with direct connection to the ocean: El Aerolito in Cozumel Island, a cave with outstanding biodiversity and density of stygophiles and stygobionts; and Aayin Aak in the Caribbean Coast of the Yucatan Peninsula, a cave with low richness but high density of stygobionts. We show that a minimum of 12 Gb (giga bases) of sequence data is needed to have sufficient sequencing coverage to capture low abundance taxa, and that the poor representation of most invertebrate taxa in molecular databases remains as the major challenge towards metazoan characterization at the community level. We identified representatives of 11 phyla of aquatic invertebrates, including taxa not previously reported at the study sites. However, we also detected exogenic DNA from terrestrial and marine taxa. Exogenic DNA represents a challenge for future studies utilizing metagenomic tools, especially in caves with direct connection to the ocean, but also an opportunity to better understand connectivity with adjacent ecosystems. Although our analyses focused on metazoans, our data also allows for the evaluation of prokaryotes and single celled eukaryotes, thus, providing with the ability to conduct holistic biodiversity assessments within aquatic subterranean ecosystems.



Developing environmental DNA (eDNA) methods for detecting troglobitic fauna in a diversity hotspot

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In the non-karstic geologies of the Pilbara, Western Australia, troglofauna occur in the complex networks of solution tubes and vuggs throughout isolated geological units. As a result of this isolation, highly diverse troglobitic communities have evolved to inhabit niche conditions, such as individual mesas of channel-iron deposits, which are then vulnerable to proposed mining activities targeting the deposits. Currently, the monitoring of these communities occurs via drillholes that have been installed for resource exploration, using methods such as borehole scrapes and baited colonisation traps. These monitoring methods are time consuming, with colonisation traps required to be in place for a minimum of six weeks, logistically expensive, and requiring a diverse species-level taxonomic knowledge. In recent years, environmental DNA (eDNA) methods are being increasingly deployed across global habitats as a method of community detection. eDNA methods can isolate DNA that has been shed into the environment from local organisms, which can then be used to identify species and the communities present. A popular eDNA collection method is the isolation of DNA from water, which in subterranean environments has proven effective at detecting groundwater-inhabiting fauna. However, there are currently barriers to applying eDNA collection methods to drillholes that do not intersect the water table for the detection of troglofauna. A new eDNA substrate and collection method is required to target cave fauna. In this study we develop and explore three methods of eDNA sample collection at eight drillholes in a known troglofauna hotspot in the Pilbara, and compare these methods to those accepted in local governmental guidance statements utilising morphological identification. We target novel subterranean substrates by developing a unit to isolate DNA from air, collecting drillhole scrapes, and isolating DNA from sediment at the base of drillholes. While results for this study are still pending, we expect all the target methods to be successful in detecting troglobitic fauna. Results from other eDNA studies (non-subterranean focus) have demonstrated that the diversity and concentration of DNA can vary greatly between sampling methods. Given the enclosed subterranean system, we anticipate that airDNA sampling methods have the potential to detect broadly across the subterranean environment. If successful, these methods can provide a noninvasive tool for monitoring troglobitic fauna in compliance and impact assessment, as well as the detection of target and conservation significant species.



A comparison of eDNA metabarcoding and metagenomics for assessing groundwater communities

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The analysis of DNA in environmental samples provides an unprecedented ability to explore the diversity and function of subterranean ecosystems. This is particularly the case for microbial assemblages, which, prior to this revolution, have been difficult to characterise. Analysis of environmental DNA (eDNA) has been applied across all ecosystem types but has seen relatively little application in groundwaters although this situation is rapidly changing.

eDNA can be analysed using metabarcoding and/or metagenomics approaches. Metabarcoding is focused on taxonomic identification using specific marker genes, while metagenomics provides additional genomic-scale information, enabling both taxonomic and functional characterization of the environment. Both methods are effective in characterising microbial communities from across a variety of environments, but they differ in the analytical cost and complexity. In this study, we compared metabarcoding and metagenomic approaches to approaches to characterise the microbes in shallow alluvial and fractured sandstone aquifers and considered the suitability of these methods for use in routine environmental monitoring and assessment. Analysis of metagenomes compiled from several different gene databases showed differences in the functional assemblages of samples from different aquifer types and from pre- and post-purge bore samples. Overall, metagenomes derived from different databases all responded to a similar suite of environmental variables. Taxonomic assemblage information derived from the metagenomes also showed separation of samples by aquifer types and pre- and post-purge bore samples, consistent with the patterns derived from metabarcoding of the same samples. Metagenome analysis provides a very large amount of information on the functional genes present within a sample, which is more detailed than can be inferred from metabarcoding analyses. However, although metagenome analysis provides a greater depth of information on microbial functions within aquifers, this comes at considerably higher cost and greater complexity of analysis. Metagenome and metabarcoding data were similar in their ability to discriminate samples based on taxonomic composition. We recommend that metabarcoding is sufficient for the analysis of microbes for the purpose of routine monitoring, and that the benefits of metagenome analysis for monitoring are not yet outweighed by the additional cost and analytical effort. However, it is likely that as the cost and complexity of metagenome analyses decrease, such analysis may be more accessible for routine monitoring, and will be a very valuable tool for monitoring and assessment of groundwater ecosystems in the future.



Tracking the elusive blind cave eel (*Ophisternon candidum*) - results of an opportune eDNA degradation study

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The blind cave eel (*Ophisternon candidum*) is classified as endangered by the IUCN Red List of Threatened Species and is one of three Australian stygobitic fish species. This species is rarely collected by net hauling (the most common stygofauna sampling method used in Australia), and the use of Environmental DNA (eDNA) has been invaluable in determining species' distribution and persistence. The use of eDNA however, is not without its limitations. In a natural system, DNA commonly degrades quickly, due to numerous factors including microbial processes, water chemistry, ultraviolet light and heat. These factors vary across environments, and so the rate of DNA decay will be different in different systems. Time until eDNA is undetectable ranges from hours to weeks, and little is known about how long eDNA persists in subterranean environments. In September 2023, during a stygofauna monitoring survey (not targeting blind cave eel) conducted in the Robe Valley (Western Australia) on behalf of Rio Tinto, a live specimen was collected from a groundwater bore using a 70mm haul net. The specimen was held in groundwater from the collection site for approximately 15 minutes, after which it was released back into the bore. This groundwater was retained to conduct an ad hoc eDNA degradation trial. Using passive eDNA sampling, samples were taken at different time intervals, over a 72-hour period. Eel eDNA was targeted using two approaches, both focusing on the 16S gene region: a metabarcoding approach, and a species-specific probe, amplified using qPCR. Eel eDNA was detected at all time intervals, in all replicates, using both approaches. Noting limitations to the experimental design, considering how infrequently blind cave eel specimens have been collected (eight specimens to date from the Robe Valley catchment) the results of this study provide the first insight into longevity of blind cave eel eDNA and can help interpret, movement of DNA in groundwater habitats and the distribution of this elusive species.



Pairing phylogeny and historical geology to understand the evolutionary radiation of leptodirine cave beetles (Coleoptera, Leiodidae, Cholevinae)

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Despite a long history of research coupled with elaborated alpha taxonomy and the fact that they represent one of the hallmarks of speleobiology, we are still missing a satisfying phylogenetic framework for the predominantly subterranean coleopteran tribe Leptodirini. While previous, morphology-based attempts became entrapped in convergences, resulting with erroneously interpreted relationships, molecular attempts addressed only regional pools of taxa, thus providing only limited insights into one of the largest subterranean radiations in the world. In an attempt to resolve the long unfinished riddle, we assembled a combined dataset, including both morphological (16 traits) and molecular (nine molecular markers) information. Questions on the tribe's spatial and temporal origin, dispersal pathways and main biogeographical patterns, as well as tempo and mode of morphological evolution, were tested by gathering a fourth of all known Leptodirini representatives from all over the group's distribution range, i.e. Spain to Iran. Our results point towards a single 'surface-to-subterranean' transitional event, followed by a cascade of dispersal events combined with smaller, localized, and repetitive diversifications. Overall, diversification events seem to be paired with the geological formation of the Mediterranean region and adjacent areas during the Oligocene and the Miocene, and well reflecting the ephemeral connections between the existing landmasses. The archipelago-like setup, with mountainous ranges across Europe and Asia Minor acting as islands, enabled establishing of regional monophyletic lineages of subterranean beetles and their in-situ evolutionary radiation. While most of the regional species' pools comprise several convergent body plans (edaphic to pholeounoid), both species richness and morphological diversity peaks in the Western Balkan's Dinaric Karst, where a plethora of morphologically 'ultra-evolved' species can be found. Our molecular results may also shed a new light on the extant taxonomy of Leptodirini, as they undermine the morphological foundation of some of the existing subtribal taxa. By connecting biological processes and mechanisms with the extant biodiversity patterns, our analysis presents one of the first comprehensive overviews of Leptodirini within the evolutionary ecology framework, and presents a stepping stone towards improved understanding of how communities within the subterranean realm are assembled at local and continental scales.



Leptodirini beetle males with five versus four protarsomere segments; it doesn't really matter

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Among the beetle families most taxa have pentamerous tarsal formula i.e., tarsi compound of five articles or tarsomeres. However, since extreme variability among beetles, some families have reduced number of tarsal articles, varying from five to three, and even reduced at a single tarsomere. Quite often the tarsal number is different among males and females of the same species, and males often have enlarged and dilated protarsomeres. This character is stable and enables us to easily separate beetle families at a glance. Pentamerous protarsi at both males and females are a plesiomorphic feature common to the Leiodidae beetles and to tribes of the Cholevinae subfamily. The only exception here is the mostly subterranean Leptodirini tribe, where the protarsal tetramery is synapomorphic in females of all species, but also present in well-characterized Teleomorphes phyletic lineages of Supraflagellates in systematics proposed by Jeannel. Within the majority of other Leptodirini taxa the males have pentamerous protarsi. In all the later Leptodirini systematic supra-generic division attempts, the male 5-segmented versus the 4-segmented protarsi were so far one of the first considered as well as the main morphological character considered. After twenty years of collecting specimens of the most of described Leptodirini genera from the most of the group distribution from the Pyrenees to Asia Minor, and after using standard methods PCR-amplified six gene sequences of studied taxa at the Ljubljana University, we reconstructed phylogenetic relationship of the tribe. Our results significantly shake the fundamentals of the present systematics of the tribe Leptodirini proposed by Gueorguiev and adopted by Newton and Perreau. The presently recognized subtribes Pholeuina, Leptodirina, Bathysciina, Bathysciotina, Antroherponina and Spelaeobatina, appears to be significantly polyphyletic. Our recent molecular phylogeny results are not yet published and comprehensive comparative morphological character study is on the slow progress. In this presentation we are focused on the one morphological character the protarsal formula. As one of our main observations is that the tetrameric male protarsi appeared in the Leptodirini evolution independently several times in different phylogenetic and geographically well-defined clades. Sometimes different protarsal numbers appear even among closely related genera. Considering our recent molecular results, a protarsal formula is useful morphological character for Leptodirini genera separation, but phylogenetically applying it as an informative morphological character for supra-generic systematics seems to be a less important and protentional misleading.



Fig. Male *Bathysciotes khevenhuelleri* with 4-segmented protarsi and male of *Adelopidius neumanni* with 5-segmented protarsi. Both taxa are closely related and phylogenetically belongs to the same North Dinaric clade of Leptodirini beetles.



From the dark side of paradise: Three new cavernicolous species discovered in lava tubes on Hawai'i Islands

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The Hawaiian Islands are recognized as a hotspot of unique biodiversity yet it is rare for new native Hawaiian arthropod species to be described, particularly from subterranean habitats. Three new species of cave dwelling invertebrates including a terrestrial amphipod, a planthopper, and a beetle have recently been described from lava tubes on the island of Hawai'i. The Hawaiian Islands are known to harbor a rich and diverse fauna of troglobionts. More than 70 troglobiont species have been documented from the main islands of the Hawaiian archipelago, the majority of which are from lava flows formed by Kilauea Volcano on the youngest island, Hawai'i. Since 2016, bioinventories of lava tube systems on Hawai'i continue to document the existence of previously unknown, cave-adapted species from flows on three of the five volcanoes on the island (e.g. Hualalai, Mauna Loa, and Kilauea). During surveys, individuals were collected in lava tubes by hand and aspiration and sent to taxonomic specialists for description. 'Cave-hopper' amphipods are rare, with only four cave obligate species known globally from distant geographic locations, each in its own genus. The new species, *Spelaeorchestia pahoehoe*, was compared with the only other known cave amphipod from the Hawaiian Islands, an endangered species from Kaua'i island, and with a closely related cave species from Japan. Given the rarity of amphipod troglobionts, a full conservation assessment is necessary to document the range and potential threats to *S. pahoehoe*. The first cave-adapted species of the endemic Hawaiian planthopper genus *Iolania*, *Iolania frankanstonei* is shown to be related to the single species found on the surface. This discovery indicates that adaptive shifts, where a species exploits an open habitat or food resource, are a major pattern in cave species evolution on young islands. The beetle, *Paratachys aaa*, is the only troglobiont in a genus with four Hawaiian surface species, and occupies the dark zone of numerous, recently developed lava tube caves within the Mauna Loa and Kilauea regions. Given its geographic range across the island there is evidence of dispersal through underground voids to exploit new habitat. While the fauna of Hawaiian lava tubes has been known for 60 years, the discovery of new species on Hawai'i Island indicate that additional bioinventories are necessary to fully assess species richness and to support conservation planning efforts for these species. The description of these new species also provides opportunities to explore the underlying evolutionary models and processes of speciation on this youngest Hawaiian Island.



The individual fossil of the poorly known subterranean beetle subfamily Catopocerinae (Coleoptera: Leiodidae)

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The subfamily Catopocerinae is a poorly known group of beetles within the family Leiodidae, with all extant species lacking eyes (anophthalmic). They inhabit North America (three genera, 48 species), Siberia (one genus, three species), and Japan (one genus, one species). Most species are endogean, with only one cave-dwelling (troglobiotic) species, having a slender, elongated body form similar to the highly evolved European Leptodirini tribe within the subfamily Cholevinae (leptodiroid morphology). Recently, a Cretaceous fossil have been discovered, with all distinctive characters of the extant endogean species of the subfamily: abdomen with five visible coxosternites instead of six in most of Leiodidae and prosternum longer than the width of the procoxae. However, unlike its extant relatives, this fossil possesses well-developed eyes and wings. In this study we describe in detail the morphological characteristics of the Catopocerinae subfamily, with a particular focus on the fossil specimen. The fossil was scanned using high-resolution propagation phase contrast synchrotron X-ray microtomography - (340 nm resolution) but also nanotomography (60 nm resolution). The nanotomography is applied for the first time to a Leiodidae fossil, providing unprecedented details of the aedeagus and endophallus morphology. Tomographic scans were performed at ESRF (European Synchrotron Radiation Facilities) in Grenoble (France) on beamlines BM05 and ID16B.



An unexpected diversity of Palpigradi at the northernmost distributional limit in Europe

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The micro-whip scorpions or palpigrades (Palpigradi) are among the least known and mostly overlooked arachnid orders due to their tiny size. In southern and central Europe, altogether 38 species inhabit exclusively the caves. *Eukoenenia spelaea* represents a complex of species differing in only a few morphological features or whose taxonomic status is completely unclear. In recent years, due to intensive collecting activities, numerous populations have been sampled in Italy, Slovenia, Croatia, Slovakia and Hungary, allowing more thorough morphological and molecular analysis of these taxa. In this study, we focused on *Eukoenenia* populations from caves in the Western Carpathians (Slovakia and Hungary) and Slovenia. This is the first study of the Palpigradi molecular phylogeny carried out at the infrageneric level. Individuals were collected from 10 sites in seven orographic units in the Western Carpathians, while a population from Slovenia served as a reference site for the taxonomic and phylogenetic comparison. Genomic DNA was non-destructively extracted from whole specimens and the mitochondrial fragment of the COI gene was amplified. Species delimitation was performed using the ASAP online platform, and Mega XI was used for standard distance analyses with the Tamura-3 parameter algorithm. A total of 48 successfully obtained sequences were clustered by a neighbour-joining tree into five groups corresponding to the species (MOTUs) delineated by ASAP. The genetic distance within the karst units reached only up to 0.7 %, while it was up to 39 % between the individual units. The high genetic diversity indicated three separate lineages in the Western Carpathians with independent phylogenetic histories. The Mantel test revealed a weak correlation between genetic and geographic distance. Two of 15 haplotypes were dominant and involved four neighbouring orographic units, while the haplotypes of the other four orographic units were unique (Fig. 1). Despite the relatively high cryptic diversity, several morphological traits, which allow distinguishing among different molecular species (MOTUs), were revealed, and can be successfully used as diagnostic traits. The phylogeny tree with divergence age estimates is placed in the context of the geological history of the Western Carpathians. The study was supported from the Slovak Research and Development Agency, project APVV-21-0379, from the Slovak Scientific Grant Agency, project VEGA 1/0438/22.



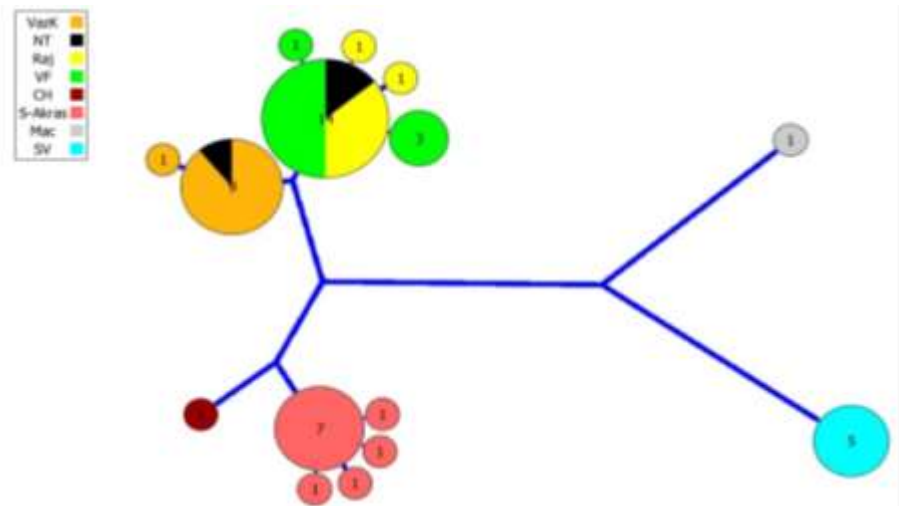


Fig. 1: Haplotype network of cave populations of the *Eukoenenia spelaea* complex in the Western Carpathians Mts and Mačkovića jama in Slovenia as a reference site. Abbreviations of karst areas/caves: VazK – Važecký kras, NT – Nízke Tatry, Raj – Slovenský raj, VF – Veľká Fatra, CH – Čierna hora, S-Akras – Slovensko-Aggtelcký kras, Mac – Mačkovića jama (Slovenia), SV – Súľovské vrchy.



Disentangling the diversity of the cave centipede *Lithobius stygius* species complex (Chilopoda: Lithobiomorpha: Lithobiidae)

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The centipede *Lithobius stygius* Latzel, 1880, described from Postojna-Planina cave system (Slovenia), is among the first scientifically described cave centipedes in the world. Since its discovery, many similar specimens were reported from caves throughout the Balkan Peninsula under several taxonomic names that are currently recognized as its synonyms. The unusually wide distribution for a subterranean species, as well as unresolved taxonomy of many populations, raised the need for the study of morphological and genetic diversity of this taxon. In the presented study, we first redescribed *L. stygius* and specified the variability of morphological characters based on the type series and the newly collected genetically evaluated specimens from Postojna-Planina cave system. The morphological examination showed concurrence between new material, type specimens, and Latzel's original species description. Some variation was detected in characters such as the number of ocelli, antennal articles and forcipular teeth, as well as in plectrotaxy. By providing good redescription and information on the morphological variation within the type population, we set the basis for future morphological comparisons with other populations currently assigned to *L. stygius* and for their taxonomic clarification. Secondly, we used phylogenetic approaches to disentangle the relationships within the *L. stygius* complex, which includes morphologically similar populations occurring in Balkan caves. The genetic analysis included samples of *L. stygius* complex and few samples of presumably closely related surface species (altogether 285 specimens from 142 caves and 9 surface localities), and additional published sequences of Lithobiidae representatives. We inferred the multilocus phylogeny of the group based on three genetic markers (COI, 16S rRNA, 28S rRNA) and performed unilocus species delimitations using COI. The results show that the specimens belong to several lineages, but together with nominal species *L. stygius*, *L. agilis* C. L. Koch, 1847, *L. erythrocephalus* C. L. Koch, 1847, *L. schuleri* Verhoeff, 1925, *L. atkinsoni* Bollman, 1887 and *L. peregrinus* Latzel, 1880 they form a monophyletic group within the genus *Lithobius* Leach, 1814. Specimens first attributed to the *L. stygius* complex were split into more than 40 MOTUs with predominately limited distribution. Higher level clustering reveals complex phylogeographic patterns, including wide and overlapping distribution of species groups. Newly acquired genetic data demonstrate that the present understanding of the *L. stygius* species complex taxonomy largely underestimates the degree of its diversity. In addition to the nominal species, it likely encompasses taxa that are currently synonyms of *L. stygius*, have uncertain taxonomic status (*L. illyricus* Latzel, 1880), or are new species, each with its own distribution, separate evolutionary history, and potential ecological differences in relation to surface/caves.



New stygobiont Neoniphargidae (Amphipoda) and groundwater biodiversity in small non-karst aquifers along the Mount Lofty Ranges and Fleurieu Peninsula of South Australia

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Globally, stygofauna research is focused on karstic groundwater systems, where most subterranean biodiversity hotspots are found. Stygobionts also inhabit interstitial spaces in other geologic settings, but taxonomic richness is often comparatively low. In recent decades, Australia has emerged as a continent with rich stygofauna, with regions of exceptional biodiversity in unusual geologic settings such as the isolated calcrete aquifers, alluvial aquifers, and fractured rock systems. About a decade ago, sampling in small, isolated groundwater systems in fractured and porous-media non-carbonate aquifers in the Mount Lofty Ranges and Fleurieu Peninsula of South Australia discovered undescribed stygofauna. Based on preliminary molecular (*COI* barcoding region) and morphological evidence, amphipods were assigned to the family Neoniphargidae. Deep divergence between populations at sites in nearby watersheds suggested that there may be additional undiscovered stygobiont diversity in the region. In 2023, we resampled known sites and sampled more than 50 new sites to better characterize stygofauna diversity in the region. Sites include low-discharge, hillslope springs/seeps, and mountaintop springs in non-karstic fractured bedrocks, and a well in clastic alluvium in southern Adelaide. Most samples were collected just beneath organic material at the springhead, and multiple samples at some sites show that stygobiont occurrence is heterogeneous and strongly associated with the point of discharge. A few samples were collected from shallow bores which intersect the sediment-bedrock interface beneath a seep or spring. Bou-Rouch pumping was used at a few sites where upwelling points could be located. Some springs with stygofauna records desiccate during seasonal drought, indicating that stygobionts also inhabit permeable fractures beneath and adjacent to the point of discharge. Stygobionts were found at 16 sites, multiple new species of Neoniphargids have been found at 9 of these sites, and two sites have two sympatric species of amphipod: a larger and a smaller species. Aside from amphipods, stygofauna in this region rivals the richness found in many karst regions, with several sites having one or more species each of undescribed Syncarida (Parabathynellidae, Psammaspididae), Isopoda, Harpacticoida, Cyclopoida, Ostracoda, and *Hydracarina*. Currently, described Neoniphargidae are a mix of stygobiont and epigean species from caves, high altitude lakes, and springs across eastern Australia, and two stygobiont species in Western Australia. Our molecular evidence indicates a monophyletic group (= genus) in the Mt Lofty region with several divergent lineages (= species), sister to but clearly divergent from Tasmanian and NSW neoniphargids included for comparison. Morphological characters align with current diagnoses of Neoniphargidae and indicate a new genus is necessary. Thus, we provide evidence for a newly expanded distribution of stygobiont taxa within this family. Similarly, the Psammaspididae (Syncarida: Anaspidae) is an enigmatic stygobiont group currently only known from two monotypic genera and species from Victoria and Tasmania. Our results expand the distribution of this family with a possible new genus and species. Our findings suggest that alpha- and beta-diversity of stygobionts may be high in non-karst settings, and that these settings deserve more attention and investigation. Agricultural water demands and climate change likely pose the most serious threats to these restricted ecosystems.





Fig. An undescribed genus and species of stygobiontic Psammaspididae. Representatives of this family were found in spring, interstitial, and borehole samples across the Mount Lofty Ranges and Fleurieu Peninsula of South Australia, often occurring together with Bathynellacea, Amphipoda (Neoniphargidae), Isopoda (Heterias), Ostracoda, and other stygobiontic taxa.

Niphargus (Amphipoda, Niphargidae) research in Romania. Diversity, pitfalls and shortcomings

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Amphipods of the genus *Niphargus* have been encountered and described from caves and groundwater in Romania for more than 100 years. Until recently, the identification of *Niphargus* species relied solely on description of morphological characteristics. Currently, we know that *Niphargus* species description is practically impossible without the analysis of phylogenetic markers (at least the mitochondrial COI and 28S rDNA). Cryptic species are encountered in distant places, while the distribution of most *Niphargus* species is generally rather narrow. Romania hosts 55 *Niphargus* species; however, the actual diversity of these amphipods is expected to be far higher. Several pitfalls hamper the accuracy in constructing of a species list and the *Niphargus* species distribution maps in Romania. Many *Niphargus* species descriptions that relied on morphology alone are insufficient in terms of the described characters and geographic position of the type localities. In many cases, type localities are vague or they may have been lost or destroyed. The largest impediment is neglecting this research on *Niphargus* species diversity and distribution. The data we have so far originated especially in the older literature and our limited contribution. Based on our research, many new *Niphargus* species await description. Sometimes in the past, when specimens were collected and identified, they were assigned to species with doubtful presence in Romania. Populations found in close proximity that have been classified into different species are likely to actually belong to one single species, whereas morphologically identical specimens found in distant places may have been incorrectly assessed as the same species. Most species are represented by a single point on a distribution map, so, the degree of endemism can be very high. *Niphargus* species in Romania had been collected mainly from cave environments, but also from interstitial habitats along the rivers. We mainly have knowledge on the presence of *Niphargus* species from places that have been visited by the scientists interested in the study of these amphipods. As a result, most of the diversity had been reported from the main karst areas in Romania, such as the Apuseni Mountains in north-west, the Plateau of Mehedinți in south-west, or the Sarmatian Plateau of Dobrogea in south-est. *Niphargus* species are missing, although hypothetically largely present, in the historical province of Moldavia (Eastern Romania), a territory as large as one third of the present day Romania. We aim to continue our work on the taxonomy and systematics of *Niphargus* species in Romania with i) extensive sampling, ii) massive sequencing of phylogenetic markers, iii) redescription of species with incomplete consideration in the older literature, iv) description of the new species, v) establishment of possible phylogenetic relationships between the different species alongside their geographic occurrence, and vi) construction of distribution databases.



A hidden diversity in cave-associated oribatid mite from Central Europe

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Kunstitidamaeus lengersdorfi is one of the most widespread oribatid mites found in karst and non-karst caves in Central Europe. In contrast to other Oribatida, which are restricted to entrance zones, *K. lengersdorfi* regularly occurs in aphotic parts. Twelve localities from six karst units of central Europe located in Slovakia, Czech Republic, Germany, and Hungary (Slovak Karst, Slovak Paradise, Little Carpathians, Moravian Karst, Harz Mountains, Bükk Mountains) were included in morphological and molecular analyses based on fragment of the mitochondrial COI gene. Standard distance analysis was performed in Mega X. The online platform ASAP with K2P distance and neighbor-joining algorithm was used for species delimitation. Out of 140 specimens, 15 potential species were identified, corresponding to cave populations. The genetic distance between the karst units ranges from 4.7% to 28%, while between *K. lengersdorfi* and the congeneric outgroup it is 18% to 23%. The distance within the karst units was small, reaching up to 4.9%. A significant but weak correlation between genetic and geographical distance was confirmed by the Mantel test. Moreover, the lack of shared haplotypes indicates long-term isolation and independent evolution of the studied cave populations. Morphologically, the population from the Moravian Karst shows more distinct differences in diagnostic characters (sensillae and apophyses), while other populations, even genetically distant ones, seem to be more uniform in these characters. Precise morphological and morphometric study could explain such unexpected diversity either by the existence of cryptic taxa or by distinct “*Kunstitidamaeus lengersdorfi*” species complex. The study was supported from the Slovak Research and Development Agency, project APVV-21-0379, from the Slovak Scientific Grant Agency, project VEGA 1/0438/22.



Subterranean sulfidic biodiversity in the Sarandaporo canyon (Albania-Greece)

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Life without light was not considered possible prior to the discovery of chemoautotrophy-based deep-sea ecosystems in 1977. The subsequent discovery of a sulfidic subterranean ecosystems in Movile Cave (Romania) and later in a few other sulfidic caves showed that large amounts of nutrients produced *in-situ* by sulfur- and methane-oxidizing microorganisms can support unusually abundant and diverse subterranean biological communities including numerous endemic invertebrate species. Some of these display morphological, physiological, and behavioural adaptations that enable them to thrive in extreme environments (low pH, hypoxia, hydrogen sulfide toxicity). All the sulfidic subterranean ecosystems explored to date emerged as hotspots of subterranean biodiversity. Here we report on three exceptional caves (Sulfur Cave, Turtle Cave and Pixaria Cave) that contain sulfidic streams and pools, located in the Vromoner and Pixaria canyons, on the Sarandaporo River along the Greek-Albanian border. These caves were formed by sulfuric acid speleogenesis (SAS), and they consist of large rooms with walls covered by gypsum and yellow sulfur crusts. Surveys and observations conducted during recent field trips (2022 – 2024) unveiled the presence of abundant microbial biofilms and of rich communities of invertebrates thriving in the deep sulfidic sections of the caves. Stable isotope analyses show that both the aquatic and the terrestrial subterranean food-webs are chemoautotrophy-based, relying of carbon fixed *in situ* by sulfur-oxidizing microorganisms. Eight endemic arthropod species have been identified to date: one scorpion, two pseudoscorpions, one spider, two Collembola, two centipedes, and one beetle. An enormous colony of agelenid spiders covers several hundred square meters of cave wall in Sulfur Cave, where they feed on a dense population of chironomid flies. Food produced in the sulfidic stream and consumed by larvae of Coleoptera and chironomids is transferred into the terrestrial food web by the adults of these insects. Current studies are focusing on subterranean biodiversity, ecology, and geomicrobiology. Efforts are being made to engage the local stakeholders to foster conservation actions for the preservation of these unique cave ecosystems and the invaluable biological communities they host. In collaboration with the European initiatives “Save the Balkan Rivers” and “The Blue heart of Europe”, we are hoping to include these canyons and their caves in the regional national parks of Albania and Greece.



Filling in the Gaps for the Cavernicolous Species Records for Hawai‘i

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Although diverse arthropod assemblages have been found from lava tubes and caves throughout the Hawaiian Islands over the past 50 years, determining the number of cavernicolous species within the State has been a challenge. Early workers suggested from 70 to 75 troglobionts, but counting discrepancies were apparent and many species have been undescribed. To fill the gaps, we searched the scientific literature for Hawaiian species records, including peer-reviewed publications, books, conference proceedings, dissertations, unpublished government and technical reports, databases, and museum records. We also reviewed caving group newsletters to confirm cave names, spellings, and locations because some caves have been referred to by multiple names, such as a Hawaiian name, a general name or number given by cave mappers, and then another name used by biologists or archeologists. Overall, we uncovered more than 1400 records from ~250 different caves or cave sections. Of 344 different species, 82 were considered obligate cave-adapted species. Hawai‘i Island had the most records of any island and 54 distinct troglobionts. Regionally, the ‘Ailā‘au flows formed on Kīlauea Volcano have the richest cavernicolous fauna and 23 troglobiont species. Kazumura Cave (‘Ailā‘au flow) had more species than any Hawaiian cave, at 50, from which there were 17 different troglobiont species. Another regional hotspot on Hawai‘i Island was Pu‘uwa‘wa‘a (Delissea System) on Hualālai Volcano, with 20 troglobiont species. In total, 5% of biosurveyed caves on Hawai‘i Island had 10 or more troglobiont species. These results provide a benchmark for conservation and management decisions to protect the cave fauna of the Hawaiian Islands.



Fig. Skylight entrance of a Hawaiian lava tube cave.

Cold, dark and unknown: the natural history of ice caves

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The ice stored in caves is a widespread yet neglected cryospheric component. The cold-adapted biodiversity of ice caves has received very little attention from research, despite the potential abundance of endemic troglotrophic and cryophilic species and their consequent sensitivity to changing climate. Still very little is known about the physiological and morphological adaptations of ice cave species and, given the rate at which they disappear, research about these unique environments needs to be intensified. For this reason, the Museo di Storia Naturale and speleological groups in Verona (Veneto, Italy) began to study the ecology and fauna of ice caves in the Alps and Pre-Alps, establishing the first comprehensive biological research on this kind of environment in Italy. Research was conducted in four ice caves in the Alps and pre-Alps of Veneto and Trentino. In addition to faunal research, temperature monitoring and in some caves ice dating by the C14 method and stable water isotope analysis were carried out. In addition to the presence of endemic troglotrophic species, at least 5 of which are new to science, it is noticeable the constant presence in all the studied caves of some cryophilic elements, being well adapted to low temperatures (mainly Collembola and Diptera belonging to the genera *Trichocera* and *Chionea*). These cryophilic organisms, together with some troglotrophs, seem to constitute a kind of “ipogean glacial faunistic association”. The distribution and diversity of the collected species, with special emphasis on cryophilic and troglotrophic ones, the ecological effects of ice reduction in cave, and the analysis on different ice types and compositions are presented in this communication.



Towards a barcode reference library for subterranean fauna

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There are high stakes in biodiversity assessment especially for those associated with mineral exploration and resource developments. There is a clear need for accurate, fast and consistent species identification with new molecular methods potentially offering a significant alternative to traditional morphological approaches. Use of environmental DNA (eDNA) metabarcoding as a tool for detecting subterranean species inhabiting groundwater associated with mineral deposits is quickly gaining momentum in the area of environmental impact assessment. However, in subterranean ecosystems unknown invertebrate dark taxa with ancient and genetically diverse evolutionary lineages dominate, making meaningful taxonomic assignment of eDNA metabarcoding Operational Taxonomic Units (OTU) extremely patchy. Therefore, a custom library of reference sequences is essential. Using one of the oldest hydrogeological environments on earth, Western Australia's Pilbara region, we have collaborated with taxonomic specialists and industry partners to curate extensive, verified taxonomic data to establish a custom reference library of mitochondrial *COI* and *16S*, *18S*, *12S* rRNA gene sequences, which will be expanded over time to meet future needs. Here we present a major step forward in establishing a subterranean fauna barcode reference library that will revolutionise subterranean faunal community assessment, monitoring and species identification.



Time does tell! Interdisciplinary approach for the evaluation of the cave fauna of the Mecsek Mountains, Hungary

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The Mecsek Mountains, which are situated in the southwest of Hungary, are a fairly small karst region of *ca.* 545 km². The karst features, including caves, are constrained to the Western Mecsek where open karst can be found. The karstification possibly started as early as 30 mya and currently there are 229 registered caves known from the area. Surprisingly all the caves are relatively small, only 34 exceed 50 m in length. In contrast, the cave fauna of the region is rich and contains representatives of ancient lineages and many troglobionts show extremely high levels of endemism. Given the lack of huge systems with various microhabitats the unique and diverse cave fauna with plenty of relict elements can only be the result of long persisting adequate habitats. In stark contrast with this assumption, recent geological research using various methods showed that the minimum age of the recently active caves in the area is only approximately 2.5 million years. However, data also evidenced the existence of preceding phases of karstification, interrupted several times by sediment coverage of the surface and fill-up of the caves, and high marine or brackish water levels e.g. of the Middle Miocene seas or the Late Miocene Lake Pannonian, when only the highest peaks of the range remained dry as islands. After the recession of the Lake Pannon the cavities could be filled up by allochthonous sediments several times, which hindered karstification. This result is in accordance with the morphology of the caves but raises questions about our knowledge on the history of the local cave fauna. Over the last decades plenty of research provided additional data on the species. The results sometimes contradicted, sometimes reassured the ancient origin of the cave fauna of the Mecsek Mountains. We evaluated all the available literature and came to the conclusion that the contradiction only seemingly exists, and in fact the biological patterns and the geological results are in accordance. Most of the aquatic troglobionts are representatives of ancient lineages and show high levels of endemism, while the terrestrial elements have considerably wider distribution and often cannot even be considered as true troglobionts. Moreover, most of the aquatic species are from taxa with members known to live in interstitial habitats, therefore it is safe to assume that they could survive in the ancient, water and sediment filled caves during the intermittent periods of high water levels and sediment coverage when classic cave habitats were temporally absent in the area.



Forty-plus years of anchialine cave research

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My anchialine cave research began in 1977 when I obtained a job at the Bermuda Biological Station. Bermuda is a mid-Atlantic volcanic seamount, capped with eolian limestone, and possessing a well-developed karst topography. Numerous caves on this small island reach tidal, saltwater pools, with submerged passageways accessible only to cave divers. Brackish water at the surface of these pools increases in salinity to fully marine waters at depth. After obtaining training in cave diving, I systematically explored the caves, discovering a wealth of rare and endemic stygobitic fauna consisting primarily of crustaceans. One of the most unusual was *Mictocaris halope* representing the sole species in the new order Mictacea. At about the same time, Jill Yager discovered an exceptionally strange crustacean from anchialine caves in the Bahamas, which she described as the new crustacean class, Remipedia. Considering our discoveries in Bermuda caves, I was curious about what might be found in caves from volcanic islands elsewhere in the Atlantic. Learning about the presence of saltwater caves on Lanzarote in the Canary Islands, I organized a cave diving expedition there in 1983. On our first dive, we discovered a new species of Remipedia inhabiting a volcanic cave on the opposite side of the Atlantic Ocean. As with Bermuda and the Bahamas, this volcanic cave on Lanzarote contains a unique stygobitic fauna consisting primarily of crustaceans. My first expedition into the South Pacific took place in 1985 to the islands of Palau. Although numerous caves were found there, all were relatively small, at least in comparison to the caves we explored on Atlantic islands and the discovery of new species was limited. In 1987, I obtained support for a year-long expedition to explore and investigate anchialine caves on islands extending across the South Pacific Ocean. Starting in Tahiti, our expedition continued to the Cook Islands, Fiji, Tonga, Western Samoa, Niue, New Zealand, Tasmania, New Caledonia, Vanuatu, and the Solomon Islands, spending about a month in each location. As with Palau, the caves we found were relatively small (~100 m maximum in length) and few stygobitic species were observed. In 1989, I obtained a faculty position at Texas A&M University at Galveston where I worked for the remainder of my academic career. My anchialine cave research continued primarily in the Bahamas, Yucatan Peninsula, and Belize. I trained 15 graduate students, discovered more than 250 new anchialine species, published 276 journal articles and book chapters, and was featured in 20 TV documentaries on Netflix, BBC, National Geographic, History Channel, Discovery Channel, and public broadcasting channels in the US, Germany, France, and others.



Ten years of hyporheic zone investigation in Texas, USA: large-scale patterns and the importance of karst

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Texas, USA has a long history of groundwater ecology research, and the Edwards Aquifer is a recognized hotspot of stygobiont diversity. The state spans a dozen ecoregions and large precipitation and temperature gradients across an area over 695,000 km², making it an attractive study area for large-scale biogeographic investigations. Despite this, the hyporheic zone of Texas streams was essentially unstudied prior to 2015. Investigation of Texas hyporheic sites was initiated because of the need for more complete occurrence data for at-risk stygobiont species, and more recently, interest in how groundwater-surface water interaction may influence stream responses to climate change. Since 2015, nearly 200 hyporheic sites across the state have been evaluated, allowing for several novel insights. The effort has spurred renewed taxonomic discovery, resulting in the description of 8 stygobiont species. Multiple taxa previously known only from karst aquifers are now known to also occur in the hyporheic zone. A smaller subset of taxa (e.g., species of Microcerberidae, Asellidae, Parastenocarididae) are apparent hyporheic specialists and reach higher abundances than observed for stygobionts in other Texas groundwater habitats. Proximity to karst and karst features has repeatedly emerged as an important influence on stygobiont diversity in the hyporheic zone at multiple scales. At the reach scale, stygobiont richness is positively correlated with proximity to groundwater upwelling in karstic regions (GLM, $R^2 = 0.5$, $p < 0.01$) and positively correlated with proximity to karst in non-karstic regions (GLM, $R^2 = 0.4$, $p < 0.01$). At the landscape scale, stygobiont richness is also higher in karst regions than in all other lithologies (GLMM, $p < 0.01$), and community structure in the hyporheic zone varies in response to basin geology. The reasons for these relationships are uncertain, but we suggest two potential mechanisms related to environmental filtering and dispersal. First, we illustrate that hyporheic zone hydraulic conductivity is higher in karst areas than in non-karst areas ($\bar{x} = 0.09$ cm/s vs. 0.05 cm/s, AOV: $F = 5.08$, $p < 0.01$). We suggest that hydraulic conductivity, which influences dissolved oxygen, organic matter availability, and solute transport, is an important measure of habitat availability and habitat quality (environmental filtering). Second, we show that hyporheic zone stygobionts are largely a subset of upstream karst aquifer taxa: the nestedness component of β -diversity is marginally higher than turnover ($\beta_{\text{nestedness}} = 0.44$, $\beta_{\text{turnover}} = 0.36$, $t = 1.79$, $p = 0.08$), and HZ communities are always less diverse and nested within aquifer communities. We suggest that the biologically diverse karst aquifers of Central Texas serve as a major colonizer pool for overlying and downstream hyporheic communities (dispersal).



Tell me where you live and I will tell you what you eat – trophic position of funnel-web spiders from three ecological zones of caves with different nutrient source

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The funnel-web spiders of the genus *Tegenaria* have an outstanding diversity in caves in Israel, with a gradient of eye-bearing, eye-reduced and blind species. In many caves two *Tegenaria* species can be found together. While the eye-bearing species is present mostly at the cave entrance and twilight ecological zones, the eye-reduced and blind species can be found in the twilight and dark zones. We were asking whether the ecological zone, the ecological affinity (troglomite or troglophile) or the species identity determine if the troglomite and the troglophile species found together in the same cave share the same food-web, and what ecological factors, including different nutrient sources, affect *Tegenaria*'s trophic position in cave food-webs. To test these questions, we used $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ stable-isotope analyses of spiders from three ecological zones (entrance, twilight and dark) of nine caves in Israel. Three caves were inhabited by the frugivorous Egyptian Fruit Bat, three caves were inhabited by insectivorous bats, and three caves had no bat colonies. We found that *Tegenaria*'s of different species and ecological affinities, collected from caves inhabited by frugivorous bats have higher $\delta^{15}\text{N}$, and have a different isotopic signal of food source ($\delta^{13}\text{C}$) than *Tegenaria*'s collected from caves without bats. The trophic position, as suggested by the $\delta^{15}\text{N}$, and the isotopic signal of food source ($\delta^{13}\text{C}$) of *Tegenaria*'s collected from caves inhabited by insectivorous bats were found to be affected by their position along the cave: deep zone *Tegenaria*'s had higher $\delta^{15}\text{N}$ and a different isotopic signal of food source than *Tegenaria*'s from the entrance and twilight zones. Our findings suggest that trophic position of *Tegenaria* funnel-web spiders in caves are affected by the organic nutrient source (i.e., type of bat guano) and the zone within the cave. These findings have implications for the conservation of cave fauna in the Levant.

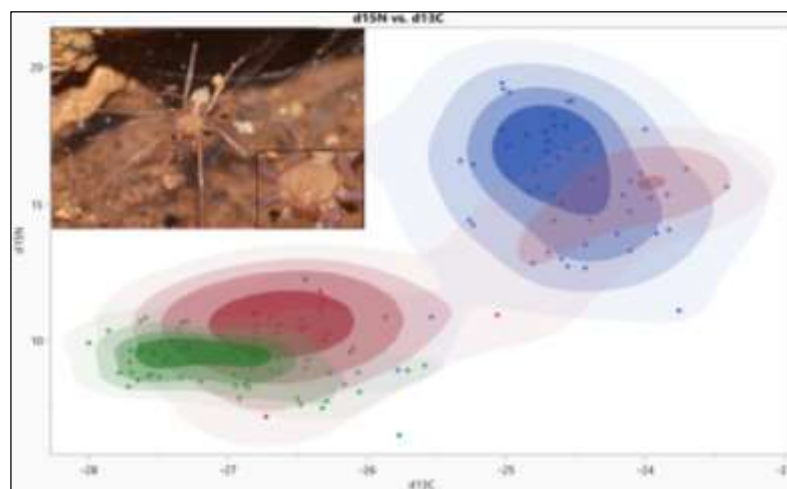


Fig. $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of spiders collected from caves in Israel. Blue dots are the values for spiders collected from caves with frugivorous bats (distributed in one cluster), red dots are the values for spiders collected from caves with insectivorous bats (distributed in two cluster, representing different ecological zones in the caves) and green dots are the values for spiders collected from caves without bat colonies (distributed in one cluster). Picture (upper-left) of the troglomite funnel-web spider species *Tegenaria trogalil* Aharon & Gavish-Regev, 2023 by Shlomi. Aharon



A stable isotope study of organic matter cycling and food web dynamics in Cenote Crustacea, an anchialine cave in the Yucatán Peninsula, Mexico

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The world's most extensive anchialine ecosystem is found in the karst aquifers of the Yucatán Peninsula, Mexico. Presently, our understanding of biogeochemical cycling and ecosystem functioning in anchialine caves is limited to a restricted number of studies in localities without a direct connection to the sea and focused primarily on the water column and excluded the benthos from their analyses. In this study, we present a dataset assembled during sampling campaigns between 2013 and 2014 from Cenote Crustacea, an anchialine cave with a seaside connection along the Caribbean coast of the Yucatán Peninsula. Our primary goal was to examine links between carbon and energy sources that sustain the food web in the water column and the benthos. The stable carbon isotopic signatures and high concentrations of dissolved organic carbon (DOC) and methane (CH₄) in the shallow fresh groundwater indicate that organic matter degradation in the overlying terrestrial soil is the primary source of these dissolved constituents within the cave. The rapid decrease of DOC and CH₄ is associated with isotopic shifts in the intermediate brackish water, indicating that microbial consumption of these carbon sources conveys carbon and energy into the food web. In the deepest saline groundwater, $\delta^{13}\text{C}$ -DOC values were consistent with the mixing of organic matter from terrestrial and marine sources. Bulk stable carbon and nitrogen isotopes measured from the cave fauna indicated different dietary preferences between the water column and the benthos. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from the free-swimming shrimp *Typhlatya dzilamensis* were more negative than the benthic fauna or the terrestrial and marine organic matter, suggesting that carbon from methane and/or other chemoautotrophically produced sources contribute, at least partially, to their diet and biomass. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ composition of deposit feeders is consistent with a primary dietary source obtained from dense and degrading terrestrial vegetation in the sediments. By contrast, the diet of suspension feeders and grazers living on the sediment surface was predominantly based on marine-derived organic carbon in the saline groundwater. Given the presence of heterogeneous carbon sources and the observed functional diversity of the benthic fauna, we propose that benthic species within the saline groundwater mitigate competition by feeding on different sources of organic matter.



Assessing Energy Source Effect on Food-Webs and Arthropod Assemblages in Levantine Caves

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Caves are unique habitats, characterized by the absence of sunlight and primary producers. Caves are often nutrient limited, and their food-webs rely heavily on external resources such as bat guano. We studied how guano affect arthropod food-web structure, trophic position of spiders and their assemblage in twilight and dark zones of nine caves in Israel; three caves were inhabited by the frugivorous Egyptian Fruit Bat, three caves were inhabited by insectivorous bats, and three caves had no bat colonies. We identified 36 spider species and morphospecies belonging to 15 families. The most abundant spider families were Agelenidae, Sicariidae, Pholcidae, Linyphiidae, and Filistatidae (that are absence from the three caves inhabited by insectivorous bats). Using multivariate analysis, we found that spider assemblage composition is significantly affected by guano and cave ecological zone to some extent. Using stable isotope analysis, we found that arthropods, including spiders, collected from the three caves inhabited by frugivorous bats had higher $\delta^{15}\text{N}$ and have different isotopic signal of food source (high $\delta^{13}\text{C}$) compared to those collected from the three caves inhabited by insectivorous bats and the three caves without bats. We also found that the trophic position, as suggested by the $\delta^{15}\text{N}$ and the isotopic signal of food source ($\delta^{13}\text{C}$) of spiders collected from the three caves inhabited by insectivorous bats were affected by their position along the cave: deep zone spiders were in a higher trophic level and have a different isotopic signal of food source than spiders from the twilight zone. These results were similar for the spider families Agelenidae, Sicariidae, and Pholcidae, that were collected from all the nine caves and two zones studied. Many cave-dwelling bats in Israel are negatively affected by anthropogenic activities and climatic changes. Moreover, most insectivorous bats in Israel are rare and threatened. As our results suggests that guano is crucial for cave-adapted arthropod food-webs in Israel, and any decline in bats, which will affect the level of guano in the caves, will also have a negative effect on the spiders and other arthropods, that their food-web is highly based on the guano as an energy source.



The origins and fate of dissolved organic carbon in the Yucatan Peninsula's karst subterranean estuary

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Anchialine caves within karst subterranean estuaries are important conduits for carbon transport and transformation prior to groundwater expulsion into the sea. To investigate the sources, magnitude, and biogeochemical reactions that regulate dissolved carbon species within these ecosystems, we analyzed the concentrations and $\delta^{13}\text{C}$ values of dissolved organic carbon (DOC), methane, and dissolved inorganic carbon (DIC) along a 6-km shoreline-perpendicular transect of flooded coastal caves, and an additional site, in the northeastern Yucatan Peninsula, Mexico. Significantly higher DOC concentrations following rain events compared to a mid-summer drought suggests precipitation is an important driver of the downward DOC flux into the aquifer. The primary source of DOC (on average, 678 μM ; $\delta^{13}\text{C}\text{-DOC} = -27.2\text{‰}$) in the shallow fresh (0.35 ± 0.05 psu) groundwater is decomposed mangrove and/or tropical forest vegetation. Evidence for net DOC consumption, along with positive and negative changes in $\delta^{13}\text{C}\text{-DOC}$ values, is consistent with microbe-mediated heterotrophic and cryptic chemolithoautotrophic processes that facilitate organic matter recycling, with the primary zone of carbon transformation being the upper aquifer's low salinity waters (meteoric lens). Downward diminishing DOC coinciding with a 4- to 5-fold increase in DIC concentrations while $\delta^{13}\text{C}\text{-DIC}$ becomes more positive implies that organic matter diagenesis also enhances carbonate dissolution. The tendency of $\delta^{13}\text{C}\text{-DOC}$ to become more positive along the seaward transport regime is consistent with increased mixing with DOC from the saline groundwater and/or the coastal ocean. In comparison to the coastal ocean DOC (158 μM , -19‰), saline groundwater DOC is less abundant (70 μM , on average) with a substantially more negative $\delta^{13}\text{C}$ value (-26‰). This pattern suggests most marine DOC entrained into the saline groundwater was removed, presumably by diagenesis, and replaced with a more negative $\delta^{13}\text{C}$ source, which is most likely terrestrial in its origin. These landscape-level observations reveal hydrologic and biogeochemical factors that regulate the internal functioning of an anchialine ecosystem and have the potential to influence the quality and quantity of carbon exported to the coastal sea.



Role of landscape of Fear (LOF) and Hunters' Horizon (HuHo) in affecting foraging activity in subterranean and ecotonal freshwater habitats

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Predator-prey interactions are among the strongest factors shaping activity and adaptations/plastic responses in surface environments. How those interactions affect stygobionts ecology and biology is an aspect that still understudied. With this study we assessed how Landscape of Fear (LOF) and the Hunters' Horizon (HuHo, i.e., higher prey availability) affect both groundwater and springs exploitation by some taxa of predators and prey, including stygobionts and surface animals that can be encountered in both groundwater and springs. These taxa include the olm *Proteus anguinus* the fire salamander (*Salamandra salamandra*), planarians of the genus *Dendrocoelum* and shrimps of the genera *Typhlocaris*, *Troglocaris* and *Spaeleomysis*. Within the context of the PRIN 2022 project “STygofauna at the Interface between surficial and Groundwater Ecosystem: CLIMate change effects on AQUatic Invertebrates Fitness, Energy and Resource use in Italian aquifers (STIGE - CLIMAQUIFERI)”, we surveyed multiple caves and springs in Italy across the regions of Lombardia, Friuli - Venezia Giulia and Puglia. We assessed if predator-prey occurrence and abundance varied depending on the LOF and HuHo conditions and how were them related to distance to the boundaries and period of the day (daytime/nighttime). We observed that *P. anguinus* occurrence was negatively related to increased LOF levels and positively to springs subject to flooding. LOF affected also the other stygobiont taxa, especially in spring habitats. The abundance of fire salamander larvae was positively related to HuHo both in groundwater and in spring environments. The period of the day affected most taxa occurrence. Our results represent a first step to assess how the interactive dynamics of HuHo and LOF shape temporal and spatial variation of groundwater and ecotone communities. Further investigations including metabolic requirements of both predator and prey will be able to use animals composing stygofauna as models to improve the understating of factors determining foraging activity in complex environments.



Activity and abundance on subterranean population of Monte Albo cave salamander (*Speleomantes flavus*)

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The Monte Albo cave salamander (*Speleomantes flavus*) is a plethodontid species endemic to the namesake massif located in the north-east part of Sardinia Island (Italy). *Speleomantes* are troglophile species that usually inhabit the first meters of natural and artificial subterranean environments, where they can find suitable microclimatic conditions. In this study we aim to assess the potential effect of different abiotic and biotic features on the abundance of subterranean populations of *S. flavus*, assessing potential divergences between sexes and life stage. We used data collected throughout the year from seven populations of the Monte Albo cave salamanders. Abiotic factors included both microclimate and cave morphology, while biotic data was related to the presence of species that can be prey or predators. Using Generalized Linear Mixed Models (GLMMs) we tested the potential effects of such biotic and abiotic factors on the abundance of different groups of *S. flavus*. Results demonstrated that individuals are generally more abundant close to the entrance, while in winter and summer its abundance tends to increase in deeper cave sectors. The presence of *Oxychilus oppressus* was positively correlated with the abundance of *S. flavus*, allowing us to hypothesize that these two species probably select similar abiotic conditions. On the other hand, *Metellina merianae* was negatively correlated with the abundance of *S. flavus*, probably because the former inhabits the cave sectors nearby the entrance, namely those more affected by external climatic conditions. Regarding sexes, abundance of males is positively correlated with dipterans and *M. merianae*, while no significant difference was observed in females. Furthermore, juveniles are usually found close to the cave floor, while adults seem to prefer high sectors of the cave during spring and summer. This research underlines biotic and abiotic factors that influence the Monte Albo cave salamander seasonal activity, and the importance of improving the species' knowledge to enhance its conservation status by proper conservation and monitoring activities.



Fig. *Speleomantes flavus*

Metagenomics of Bundera Sinkhole: Archaea, Bacteria and Viruses

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Bundera Sinkhole, situated 1.5 km inland in the desert landscape of north-western Australia, is the only deep anchialine ecosystem known in the Southern Hemisphere; a fraction of the fauna also occurs in the shallow anchialine system on Christmas Island in the Indian Ocean 2600 km to the north west. Bundera Sinkhole is located in the Ningaloo Coast World Heritage Area and its fauna was a significant factor leading its listing as a World Heritage property. Bundera Sinkhole supports the blind gudgeon, *Milyeringa veritas*, and a community of eukaryotes, mainly Crustacea, typical of similar habitats with an ampho-Atlantic distribution. These include Remipedia, Thermosbaenacea, Thaumatoctyrididae, Copepoda (Epactericidae, Pseudocyclopiidae; Calanoida, Misophrioida) and Isopoda (*Haptolana*), and the spionid polychaete *Prionospio*. The water in the sinkhole has no surface connection with the ocean – definition of anchialine – and has a tidal range of about 10% of the ocean tide and a temporal lag of several hours. As typical of anchialine systems the sinkhole has a highly stratified physico-chemical structure exhibiting a marked thermohalocline between about 8 and 16 m depth below which the water is at ocean salinity. The upper and lower bounds of the thermohalocline also represent density boundaries at which organic matter can accumulate and they are marked by anaerobic strata typified by the presence of hydrogen sulphide. Recent metagenomic studies of the microbiology of the sinkhole water throughout its depth (32 m) have revealed the presence of a remarkable biota of Bacteria, Archaea and Viruses, and that the endemic species in the sinkhole are supported by microbial chemosynthesis. Here, we characterised the metabolic and biogeochemical cycling potential of the microbial communities inhabiting Bundera Sinkhole. We found that the microbial communities, largely represented by novel taxonomic lineages, display depth-dependent metabolisms. Key metabolic genes group into three depth-specific clusters that reflect distinct phases along the dissolved oxygen and salinity gradients. In particular, chemotrophic metabolisms that couple nitrogen and sulphur cycling appear to be characteristic of the dominant members in this ecosystem. These data support the idea that microbial chemosynthesis is sustaining the higher trophic levels in the sinkhole. The viruses too are highly novel (<2% described) and abundant (comprising 12% of microbial intracellular DNA). They largely infect prokaryotic taxa that drive key metabolic processes in the sinkhole. To the best of our knowledge, this is the first whole metagenomic analysis of an anchialine ecosystem, and thus presents key findings that contribute to our understanding of ecosystem functions in subterranean estuaries. Understanding the diversity of metabolic strategies utilised by anchialine microbial communities can provide important insights into how trophic webs are supported in these unique ecosystems. This is particularly important given the high endemism of anchialine species and the potential vulnerability of these ecosystems to global environmental change and other anthropogenic influences and elsewhere to coastal development. Identifying the key microbial members and biogeochemical process is critical for the conservation of anchialine ecosystems.



Unraveling hidden relationships: Dynamics and evolution of *Thiothrix*-crustacean symbioses in sulfidic cave environments

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Mutualism and commensalism are prevalent symbiotic relationships in aquatic extreme environments such as water bodies in sulfidic caves. These habitats are characterized by chemosynthesis-based food production, darkness, low oxygen levels, and the presence of hydrogen sulfide. Sulfidic caves offer a unique opportunity to study these interactions due to their easier accessibility compared to e.g. deep-sea vents. Specialized bacterial strains, such as those from the genus *Thiothrix*, are engaged in symbiosis with amphipods species in these habitats. Notably, *Thiothrix* bacteria have been found in symbiosis first in 2004 with the marine amphipod *Urothoe poseidonis* and later in 2009 with the cave dwelling *Niphargus ictus* in the Frasassi caves complex, in Italy. These discoveries highlight the widespread occurrence of *Thiothrix*-amphipod symbioses across diverse ecological settings, primarily associated with the presence of hydrogen sulfide and suitable hosts. Further research has identified these associations in other *Niphargus* species within the Frasassi caves and in other sulfide-rich caves, such as Movile Cave in Romania (2014) and Melissotrypa Cave in Greece (2019). Previous studies suggested multiple independent evolutionary occurrences of these symbioses, with certain amphipod clades and *Thiothrix* strains exhibiting a predisposition for establishing and maintaining such relationships. Despite these findings, empirical data on *Thiothrix*-crustacean symbioses in sulfidic cave environments remain limited, hindering comprehensive evolutionary reconstructions. To address this, we sampled diverse sulfidic environments and investigated them using both Sanger and Nanopore sequencing. Our aim is to elucidate the dynamics of *Thiothrix*-crustacean symbioses in the Palearctic ecozone, uncovering their evolutionary intricacies and ecological significance, particularly regarding host adaptation to toxic environments.



What's inside a *Niphargus*?

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Host-associated microbiomes play a pivotal role in maintaining the health and fitness of diverse animal and plant species. While certain microbiomes exhibit phylogenetic relationships (phylosymbiosis) driven by evolutionary relatedness and ecological constraints, others show high flexibility, primarily influenced by environmental conditions. Furthermore, not all animals require a microbiome, underscoring the complexity of host-microbe relationships. Despite recent mainstream interest in microbiome research, our knowledge of host-microbe interactions in subterranean environments remains incomplete. Recent studies on subterranean species have highlighted the crucial role of gut microbiota in adapting to subterranean environments. However, we still don't know to which extent phylogenetic relatedness and environmental parameters influence the microbiome composition of groundwater animals. In this study, we addressed these questions using groundwater crustaceans of the amphipod genus *Niphargus*, alongside detecting signals of human disturbance within the microbiome. We analysed the microbiomes of 46 individuals from three caves in Abruzzo (central Italy). These caves cover a gradient of human disturbance, ranging from minimal human access (Val Cordora cave) to moderate speleological activity (Grotta a Male), and finally to a show cave (Stiffe cave) with daily visits (ca. 45,000 tourists a year) and seasonal infiltration of wastewater. Our results showed high variability in microbiome diversity across samples and no evidence of phylosymbiosis. We found that caves and microhabitats significantly affect microbial community composition, but accounting for just 11.9% and 8.8% of the variance, respectively. These findings suggest that environmental factors play a stronger role in shaping the microbiome. Despite the variability in overall microbiome diversity, we identified a stable 'core' (a set of taxa present at $\geq 1\%$ abundance in $\geq 75\%$ of samples) within the microbial communities across the caves. *Pseudomonas* was the most dominant genus (70% of the core), followed by an unidentified genus within the order Rickettsiales (15.6% of the core). Seven other genera were also part of the core but at lower abundances, collectively contributing less than 20%. Notably, a specific Amplicon Sequence Variant (ASV) within the genus *Pseudomonas*, affiliated with the species *P. korensis*, was a consistent core member, dominating all samples. It constituted approximately 25% of the reads and 60% of the core, highlighting its ecological importance in driving host-microbe interactions in these subterranean environments. The dominance of a single taxon in the microbiota of *Niphargus* of three different caves suggests a strong habitat filtering for specialists. We further investigated the occurrence of the class 1 integrons (intI1/16S gene), a marker known as a proxy for anthropogenic pollution and antimicrobial resistance genes. The abundance of intI1 was lower in the individuals of Val Cordora Cave, confirming a limited disturbance. This study highlights the potential for examining subterranean animals from 'their inside' to gain novel insights into the essential role of microbes in contributing to their fitness. Furthermore, it suggests the possibility of analysing host-associated microbial communities to detect potential human-induced alterations. However, our current understanding of these dynamics remains limited, akin to opening a Pandora's box that could change our perspective on studying subterranean species.



Unravelling the diversity and metabolic strategies of microorganisms colonizing quartzite subterranean environments through in-lab analyses and in-field sequencing

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Although it is generally estimated that subterranean environments host more than 80% of the Earth's microorganisms, most of the bacteria thriving in such peculiar and, in some cases, hardly reachable sites are still unknown. Caves represent windows into the subsurface through which we can directly access and explore the genetic and metabolic diversity of subterranean microbes. In caves, microbes organize themselves into collective structures with composition and genetic functions that are driven by the cave characteristics and heterogeneity in terms of mineral and rock substrates, pH, temperature, availability of nutrients, humidity/water content and possible external vegetation. Microbial communities inhabiting caves in quartz (the crystalline form of silica)-rich rocks are still underexplored as compared to carbonatic caves and lava tubes. The importance of studying microbial interactions with silica resides in the fact that silicon is the seventh most abundant element in the universe and the second most abundant element on Earth, after oxygen. The knowledge of the microorganisms colonizing quartzite subterranean environments and their interaction with silica is of great interest for understanding the formation of ancient natural quartz-rich environments and for the study of potential analogs of silica-rich rocks detected on Mars. The aim of this work was to investigate the composition, metabolism, and biotechnological potentials of microbial communities and single strains of biofilms and biodeposits from the orthoquartzite caves in the Venezuelan Tepui mountains. These are considered one of the most ancient, remote, and pristine subterranean environments on Earth that present unique silica (stromatolite-like) formations associated with still unknown microbial activities. Because of this, quartzite caves in tepuis represent an excellent natural laboratory to investigate microbe-mineral interactions in dark, low-temperature Si-rich environments. Microbial analyses were conducted using cultivation-based approaches (using microbial isolation strategies, bacterial mutant construction and screening) and metagenomic analyses (for functional gene analyses and for metagenome-assembled genomes (MAGs) reconstruction) both in the laboratory and in situ by setting a temporary laboratory in the cave and optimizing experimental and bioinformatic procedures for limited resources and minimal laboratory settings. Microbial communities colonizing quartzite caves showed a high diversity of bacteria adapted to moderate acidic conditions and oligotrophy. Novel lineages of Ktedonobacterales (Chloroflexota), Terriglobia (Acidobacteriota), and Alphaproteobacteria (Pseudomonadota) were dominant in biofilms representing first and advanced stages of microbial colonization of quartzite subterranean rock and silica solubilization. Specific genetic functions and microbial phenotypes were found by analysing around 60 metagenome-assembled genomes (MAGs) and a strain collection of 90 culturable microbial strains associated with chemolithotrophic activities, enzymatic functions, antimicrobial production and stress resistance. These results indicate that bacteria establish cooperation (syntrophy) and competition (antimicrobial production) relationships, and some of them have the capacity to oxidize atmospheric trace gasses (CO and H₂) and to fix CO₂, leading to the development of complex microbial communities able to interact with the quartzite rock and to colonize dark, oligotrophic and silica-based environments.



Can Microbes Inform about the Vulnerability of Springs — Assessment of Microbial Communities and Spring Water Quality across Austria

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Springs, as crucial sources of water, are increasingly susceptible to environmental shifts, especially those induced by climate change. The ECOSPRING research project, supported by the Austrian Academy of Sciences, is dedicated to evaluate microbial communities and water quality trends in selected springs across Austria. Our primary focus is to assess the springs reactions and susceptibilities to hydrological phenomena and climate change. The project initial objective is to categorize the springs based on their temporal fluctuations in physicochemical properties. An in-depth examination of discharge, temperature, pH, EC, stable water isotope signature, nutrients and major ions dynamics, as well as the composition of dissolved organic matter will offer significant insights into the geological influences, prevailing hydrological conditions, and the imprint from the catchments. Microorganisms play a crucial role in aquatic ecosystems and can act as sensitive markers for environmental conditions. We aim to analyze the correlation between the composition of microbial communities and the hydrogeological and physicochemical conditions. Linked to a current national groundwater fauna biodiversity project, we sample the springs invertebrate fauna with an emphasis on stygobiont species. Our research efforts span two spatial scales: a local scale, where we study three springs in Styria monthly, and a regional to national scale. For the large scale, we selected about 100 springs across Austria. The timing of our visits to each spring is determined by its discharge minima and maxima. Each spring is sampled twice. This approach ensures a comprehensive understanding of the diverse spring ecosystems across the country in relation to their unique discharge patterns. Our preliminary findings suggest a link between the dynamic physicochemical conditions and the microbiome of spring waters. The mechanisms responsible for these observed patterns are not yet fully understood and await further exploration. In conclusion, this research project aims to improve our understanding of spring waters' vulnerability to human-induced pressures, such as climate change. The results will form a knowledge foundation for future water resources management and promote the protection and sustainable use of these unique ecosystems.



Effects of acute salinity changes to stygobionts in anchialine systems of the Yucatan Peninsula

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Climate change and the corresponding rise in sea level have the potential of changing physical and chemical properties of groundwater, which could modify community structure and ecological functions. There is a recent growing effort in studying the impact of climate change and anthropogenic pollutants on groundwater species around the world. Groundwater habitats in the Yucatan Peninsula are the only freshwater source in this karstic region. Stygobiont crustaceans in the Yucatan Peninsula are mainly separated into fully marine or fresh groundwater species. However, the isopod *Creaseriella anops* and three *Typhlatya* species are widely distributed and co-occur in the anchialine habitats of these region. Thus, they constitute optimal models to study differences in tolerance to environmental changes both under climate change scenarios and anthropogenic contamination. In this work, we investigate the response to acute salinity changes in these species using indicators of aerobic and anaerobic metabolism, antioxidant system and cellular damage and compare them to their native environmental conditions. Our results show that close phylogenetically related species differ in tolerance to salinity and suggest different degrees of vulnerability to groundwater salinization. We discuss our findings in the context of dynamic energy budget and the energy-limited tolerance to stress, characterize the observed responses and place them in ordered categories of physiological stress.



One cave, multiple worlds: cave zonation and habitat differentiation determining cave fauna distribution in a new Hotspot in Neotropics

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Despite the importance of understanding the factors that influence invertebrate communities within caves around the world, studies are still scarce. To understand ecological aspects influencing the fauna associated with the Gruta do Padre cave, the 5th-largest cave in Brazil, we performed the spatial and niche-related analysis at micro- and mesoscale to assess how terrestrial invertebrate communities respond to microhabitat characteristics along the cave floor. We explore variables related to physical, trophic, and microclimate conditions, such as substrates, shelters, and organic debris availability and diversity in different cave zones. Considering the whole Padre Cave (samplings along transects (3x10m), in quadrants (1x1m), and other cave areas), 115 species were counted, grouped into 31 orders, and at least 61 families. The richest taxa were Araneae (22 spp.), Diptera (15), and Coleoptera (14). At least 25 obligate cave species of Hexapoda (8 spp.), Arachnida (6), Crustacea (5), Myriapoda (2), Mollusca (1), Nemertea (1), Annelida (1) and Osteichthyes (1) were recorded, pointing out Padre Cave as a new hotspot of subterranean biodiversity. Distance from the nearest entrance and the cave zonation were the main factors determining cave fauna distribution. In addition, species richness and composition respond to substrate and shelter diversity, temperature, and organic debris diversity and availability. Humidity and distance from the nearest entrance were identified as the most significant contributors to the differentiation in the average niche conditions of the cave zones. Ecological studies aiming at understanding the distribution and niche requirements of subterranean fauna can be useful in determining conservation strategies, especially in areas of high diversity, such as hotspots. By identifying key ecological interactions and dependencies, researchers can better predict the impacts of environmental change and human activities on subterranean ecosystems. In biodiversity hotspots, where risks are particularly high, such informed strategies are essential to preserve unique and often fragile subterranean biodiversity.



Investigating the effect of microclimate variability and habitat heterogeneity on functional diversity of cave-dwelling arthropod communities

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The drivers of functional trait variation within communities are not well understood, particularly for communities in less-studied environments such as caves. Microclimate variability may decrease the strength of environmental filtering, resulting in communities supporting different amounts of trait variation. Additionally, habitat heterogeneity can filter species according to specific functional traits, narrowing the trait variation. We quantified the drivers of trait variation in arthropod communities across a microclimate and heterogeneity gradient naturally delineated along a 100-meter transect from a cave entrance inward. We measured the abundances of species in six important orders (Coleoptera, Oribatida, Opiliones, Collembola, Araneae and Isopoda) and classes (Chilopoda and Diplopoda) and the distributions of eight functional traits related to arthropod morphology, ecology, activity, feeding, and subterranean habitat specialization. For this study, we selected eight caves situated in the Banat region of Romania. To measure arthropod abundance and characterize the environment within each cave, we sampled arthropods and measured environmental variables (i.e., wall heterogeneity, gallery maximum width and height, light intensity, air temperature, and relative humidity) along a 100-meter transect divided into ten 10-meter sectors from the entrance of the cave. Sampling was carried out from August to September 2017 using three techniques: opportunistic searches, timed searches, and quadrat sampling on both cave walls and floor. For all analyses, we pooled samples from the left and right cave walls per species per sector. This resulted in 160 sampling units: eight caves × 10 sectors per transect × 2 substrates (floor and wall). We analyzed patterns along the gradients of within-community trait dissimilarity using the RaoQ index and the community-weighted-mean (CWM) index with specifically designed null models. We found that both microclimate variability and habitat heterogeneity drive functional trait variability, but their importance varied depending on the trait. Our study highlights the importance of small-scale environmental conditions in driving community trait variation and suggests that small-scale environmental and spatial heterogeneity should be considered for better prediction of functional diversity.



Habitat-specific disparity in mandible morphology of Leptodirini beetle (Leiodidae: Cholevinae)

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Karstic landscapes encompass a variety of surface and subterranean habitats. From the surface downwards, karstic profile typically includes leaf litter, soil, epikarst and deep subterranean habitats. Studying species inhabiting these habitats holds the key to understanding their adaptations to their preferred habitat. Understanding the ecological function of a particular morphological trait and its variation along a continuum of several selective factors is crucial for comprehending its adaptive value to the specific habitat, but has been insufficiently studied in subterranean animals. One such understudied issue is the morphology of the mouthparts in beetles which refers to consuming various types of food. Adaptation to different habitats includes diversification of mouthparts, which mirrors differences in trophic niches. Leptodirini is a Leiodidae tribe with the most diverse subterranean representatives in the West Palearctic, inhabiting habitats across the entire karstic vertical profile but their trophic ecology remains significantly understudied. Most subterranean Leiodidae are scavengers linked to allochthonous resources in oligotrophic habitats and have weakly attracted researches to study their mouthparts and trophic niches comparatively. In this study, we analyzed the mandible morphometry in 16 Leptodirini and two Catopini species, mostly from Slovenia, along the karstic profile to comprehend the diversity in morphology of their mandibles. We confirmed that, on the one hand, there exist divergence of mandible shape and size across habitats in the karstic vertical profile, and on the other hand, convergence in representatives inhabiting the same habitat. Divergence refers to mandible gradual elongation and slenderizing with increasing depth of preferred habitats, which improves general food-collecting effectiveness of mandibles. In these saprophagous beetles, this trend perfectly coincides with a logarithmical decline in organic matter amounts across the karstic vertical profile. Convergence additionally refers to modification of mandible structures, such as relatively straight mandibular tip and prostheca brush in terrestrial vs. perpendicularly medially bent tip and prostheca comb in semiaquatic species. The litter-, epikarst- and deep terrestrial subterranean habitat-dwelling guilds can be distinguished among terrestrial representatives, and moonmilk-browsing and water-filtering guilds among semiaquatic representatives. We conclude that shape and size of mandibles in Leptodirini is environmentdriven, the substrate organic matter being the most important adaptation driver.



Understanding distribution and interactions among European subterranean spiders

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Understanding biodiversity patterns and how they will be affected by global change is a major global challenge. Based on projected negative trends of biodiversity change, conservation plans have been developed to protect the distributional ranges of threatened species. However, caves and other subterranean habitats have been largely overlooked in global conservation plans, and we still have a poor understanding of subterranean species distribution, their biotic interactions, and how they respond to different anthropogenic threats. A common practice to understand species distributions is the use of species distribution modelling. However, these methods usually predict the potential distribution of species based on a set of abiotic factors, ignoring in most cases the possible positive or negative species interactions or other biotic responses that can shape species distributions. Here, we propose using joint species distribution models to understand the distribution of European subterranean spiders and biotic interactions among them. These models estimate the distribution of multiple species simultaneously and allow the decomposition of species co-occurrence patterns into components describing shared environmental responses and residual patterns of co-occurrence, the latter being possibly imputable to biotic interactions. By considering climatic variables along with other abiotic and biotic factors, we present the most up-to-date and accurate species richness map of European cave spiders and shed light on how biotic interactions may have shaped the distributions of this target group. Stemming from these predictions, we will be able to evaluate if the Natura 2000 network of protected areas adequately covers subterranean spiders, and how we can move forward to improve the situation within the “30x30” agenda of the European Union.



Assessing the drivers behind variation in alpha and beta diversity of stygobitic copepods inside and outside European protected areas

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Despite hosting thousands of metazoan species, groundwater ecosystems are still poorly considered when designating new protected areas or reshaping existing ones. The Natura 2000 (N2k) network of protected areas (PAs), established under the Habitats and Birds Directives of the European Union (EU) and including more than 27000 sites, represents the largest transnational network of PAs worldwide. Nonetheless, subterranean biodiversity is likely to be poorly represented within the N2k network, as less than 9% of N2k sites have been designated to directly protect underground habitats. In subterranean aquatic habitats, copepods (Crustacea Copepoda) represent one of the most diversified taxa, showing a huge variety of morphological and ecological adaptations to darkness, oligotrophic conditions, and diversified habitats (e.g., pools and gours within caves, fissures in the bedrock, groundwater-fed springs, hyporheic habitats). Further, they are a key node of subterranean aquatic food webs and provide essential ecosystem services such as bioturbation and recycling of nutrients. Finally, many lineages of obligate groundwater-dwelling (i.e., stygobitic) copepods represent the remnants of ancient phylogenetic lineages which went extinct in surface aquatic habitats. Therefore, assessing the degree to which the biodiversity of groundwater copepods is covered by existing PAs is mandatory to preserve the precious ecological functions and unique evolutionary histories they convey. To this aim, we first refined an existing dataset including more than 7000 occurrence records of European stygobitic copepods, by fixing some scientific binomia according to the latest research on copepods' systematics and adjusting the coordinates (and related uncertainty) for hundreds of records through GIS techniques and web searches. Then, we applied ecological modelling techniques to assess whether the observed patterns of species richness (i.e., alpha diversity) are related to the degree of N2k coverage. To reduce the risk that confounding factors due to the non-random localization of N2k sites may bias our estimates, we applied propensity score matching to select environmentally matching pairs of 10-km European grid cells with either high (i.e., protected) or low (i.e., unprotected) N2k coverage. Subsequently, we tested to what extent a set of predictors related to current and historical climatic patterns, groundwater habitat types and N2k coverage can explain observed compositional dissimilarity of copepods' assemblages within and between biogeographic regions. The fitted models suggest that the extent to which a certain area is covered by N2k PAs does not influence the richness of stygobitic copepods, with most of observed alpha diversity being explained by a mix of climatic and habitat-related variables. Differently, the same environmental predictors explained a relatively low portion of species turnover between grid cells. As for species richness, differences in N2k coverage do not seem to influence compositional dissimilarities between areas hosting stygobitic copepods. Our results suggest that the N2k network does not effectively protect areas being rich in groundwater-dwelling invertebrates, pointing to the need of expanding European protected areas to preserve groundwater biodiversity. However, the consistency of the highlighted trends beyond the N2k network will be assessed by including also nationally designated PAs and the Emerald Network in the modelling workflow.



Diversity of oribatid mites (Acari, Oribatida) along environmental gradients of the cave entrances

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At the transition between the caves and the surface environment, sharp microclimate gradients may develop, depending on topography and morphology of the cave entrances. These gradients are followed by differences in the soil type and composition of the vegetation. The aim of the present contribution was to reveal the changes in the composition of oribatid mite communities, as a model group of soil animals, at sites located along slopes stretching from the cave entrances towards the surrounding forests. This research was carried out in the Slovak Karst, the largest karst area in Slovakia, during the period 2005–2008. We investigated the model communities at the entrances of two caves, the Silická ľadnica Cave and the Snežná diera Cave. A total of 6 and 7 sites were selected along a microclimate gradient in the Snežná diera Cave and the Silická ľadnica Cave, respectively. Soil samples were taken from the sites several times during the study period. The mites were extracted from the soil samples using a Tullgren-type high-gradient extractor. Temperature of soil increased along the microclimatic gradient from the cave entrances towards the adjacent forests in both localities, while an opposite trend emerged for soil moisture. Despite the nature of the investigated cave entrances was slightly different (slope length, inclination and orientation, vegetation structure), we found similar results in the composition of oribatid communities and their abundance. Oribatid abundances, species richness and Shannon diversity indices increased from colder towards warmer sites of the gradient. Communities recorded at the cave entrances comprised not only tolerant forest species, but also mountain-specialists which did not appear at warmer forest sites. At the same time, we found that the sites markedly differed in composition of dominant species, thus revealing a strong segregation of the communities along the gradient. Our results suggests that in the relatively warm karst landscape, the cave entrances can serve as refuges for cold-adapted species. A wide range of microhabitats along microclimatic gradients between the cave entrances and the surrounding landscape offers suitable conditions for highly diversified oribatid communities, thus increasing local biodiversity. Therefore, the protection and conservation of these microhabitats should become a priority. The study was supported from the Slovak Research and Development Agency, project APVV-21-0379, and from the Slovak Scientific Grant Agency, project VEGA 1/0438/22.



Sardinia (Italy) as a hotspot of diversity for subterranean terrestrial isopods (Crustacea, Oniscidea)

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Italy is the country with the highest diversity of terrestrial isopods (suborder Oniscidea). At present 383 species are known, of which 231 are endemic (60%). Sardinia is the second largest island of the Mediterranean (24,090 km²) with a complex geology. Karst formations cover 9% of the total island surface, ranging from sea level up to 1,550 m altitude, with 4,275 caves presently known. The island is very rich in woodlice species: investigations in recent years have increased the number of known Oniscidea from 83 to 115, with many new species under description. A total of 49 species can be considered as subterranean, i.e., troglolithic (25 species), stygobiotic (3 species) or endogean (21 species). Only 4 species are considered trogliphilic. These numbers will probably increase in the future when more caves will be sampled. Most of the subterranean species of Oniscidea from Sardinia belong to the lower Oniscidea, the Synocheta: family Trichoniscidae (*Alpioniscus* 6 species, *Catalauniscus* 4, *Nesiotoniscus* 9, *Oritoniscus* 4, *Scotoniscus* 3) and Styloniscidae (*Cordioniscus* 1). The subterranean species among the higher Oniscidea, the Crinocheta, are less numerous, belonging to the family Spelaeoniscidae (*Spelaeoniscus* 3 species), Platyarthridae (*Platyarthrus* 2), Cylisticidae (*Cylisticus* 1), Agnaridae (*Tritracheoniscus* 1), and Armadillidiidae (*Paraschizidium* 2; *Trogleluma* 2 and *Alloschizidium* 8). From a biogeographical point of view, it is important to point out the Tyrrhenian species of the genera *Catalauniscus*, *Oritoniscus*, *Nesiotoniscus* and *Scotoniscus*, which demonstrate the faunistic affinities of Sardinia with the north-eastern Iberian Peninsula and southern France, to which Sardinia was connected during the Oligocene. Moreover, of particular interest are the three stygobiotic species of the genus *Alpioniscus*, occurring in subterranean waters of the karst areas in central-eastern Sardinia. One of these species, *Alpioniscus kuheni* (Schmalfuss, 2005) was originally included in a distinct genus (*Utopioniscus*) and considered to be a very primitive taxon still living in an aquatic environment. A molecular analysis showed that all the three aquatic species of Trichoniscidae are included in the same clade with the other troglolithic species of *Alpioniscus*, indicating that they are not primitive Trichoniscidae but rather species of *Alpioniscus* secondarily adapted to an aquatic way of life.



Fig. A, *Alpioniscus fragilis* (Budde-Lund, 1909) (Trichoniscidae) from Grotta Su Fummu, San Nicolò Gerrei, Sud Sardegna; B, *Spelaeoniscus* n. sp. (Spelaeoniscidae) from Grotta del Cane, Narcao, Sud Sardegna (photos by C. Onnis).

Salinity and temperature increase impact groundwater crustacean metabolism and behaviour

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Predictions of drought in the Mediterranean region suggest the potential for saltwater intrusion from the sea, leading to increased salinity of continental waters. Recently, it has been highlighted that climate change may severely impact even the relatively isolated subterranean aquatic ecosystems and its stygofauna, especially in the Mediterranean area. Many aquatic species in the caves of the Mediterranean area already inhabit brackish waters and have thus developed physiological adaptations to cope with temporary variations in salinity and temperature. The Pace of Life Syndrome (POLS) posits that closely related species or populations experiencing different ecological conditions should exhibit differences in a suite of energetic (i.e. metabolic), physiological, and behavioural traits. Investigating the implications of POLS on stygofauna facing varying levels of water temperature and salinity regime (e.g., variation in behaviour, resource and space use) can enhance our understanding of subterranean ecosystem responses to ongoing climate change. Within the Apulian endemic stygofauna, the Mysidacea *Spelaeomysis bottazzi* can serve as a valuable model for studying how animals regulate their metabolic rate and behaviour under different salinity levels and temperature conditions due to its widespread distribution within the Apulian ecoregion. Here, we present a research plan to measure *S. bottazzi* physiological responses using open flow respirometry and analyse their behaviour with movement analysis software such as EthoVision XT15. Additionally, we will examine whether individuals from different populations, already accustomed to higher salinities, exhibit osmotic stress that affects their metabolic rate, behaviour, and survival. Specifically, we will test the hypothesis that climate changes will imply a shift in physiology and behaviour via their effect on organism physiology, energy and behaviour. Our goal is to develop a predictive model that illustrates how salinity and temperature individually impact the metabolism and survival of animals differently than when these factors interact, which is actually what is happenings in nature



Predator Protection Measures at Bat Rock Roosts

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Natural or man-made rock shelters are important for many European bat species as they provide essential habitats. However, these habitats are exposed to various disturbances. One notable example of these disturbances is the presence of predators such as raccoons (*Procyon lotor*), red foxes (*Vulpes vulpes*), martens (*Martes martes*), and wildcats (*Felis silvestris*), which infiltrate these bat habitats and can negatively impact bat populations. To protect many bat species and their populations from such predators, protective measures are implemented. Commonly, bars are used as gates at the openings of rock shelters. However, the use of bars alone is not always sufficient for bat protection. Cunning predators like raccoons can still prey on bats by waiting at one spot in front of the opening. Hence, special construction measures are necessary to enhance the protection of bats from these threats. For instance, in rock roosts in Freyburg, located in the Saxony-Anhalt region of Germany, the local population of the rare Lesser Horseshoe Bat was in decline. Therefore, several special construction measures were introduced by the end of 2019 at the most crucial hibernation roosts of the Lesser Horseshoe Bat. As a result, the population increased from 776 individuals in the winter of 2019/20 to 1152 individuals in 2020/21. The installation of special slippery inclined, or angled boards, known as "jumping boards" and "landing boards", at the entrance of the rock shelter effectively prevented predators from accessing the bat roosts. Specifically, "ear boards" were constructed to deter raccoons, known for their ability to grip and climb over solid edges. Another example is the tunnel system in the FFH-site (flora-fauna-habitat) at "Büchenberg", also located in the Saxony-Anhalt region of Germany. A large number of bats enter the tunnel through a specific, small gap at the mining shaft head. At this gap, predators could easily prey on the bats when they flew in and out. To address this issue, a special "mouth-like" steel structure was constructed over the flight gap, measuring 4 meters in length and 50 centimeters in height. This design effectively prevented predators from catching the bats. In conclusion, special construction measures and innovative designs play a crucial role in protecting bat populations from predators, thereby significantly contributing to the conservation of these endangered species.



Fig. An object with a "mouth-like" steel structure was installed to prevent predators from catching bats in the FFH site (Flora-Fauna Habitat) at Büchenberg (Saxony-Anhalt, Germany).

Conservation genetics of Olms (*Proteus anguinus*): baseline for their future

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The olm (*Proteus anguinus*), one of the most charismatic cave species, is the subject of various fields of biological research and is an ambassador for the conservation of subterranean biodiversity. It is strictly protected by national and international legislation, classified as priority species for conservation by the European Union Habitats Directive and considered as vulnerable on the IUCN Red List of Threatened Species. Despite all this attention, baseline knowledge for its conservation is still lacking. Recent insights into its evolutionary history have shown that there are nine highly divergent evolutionary lineages of olms along the Dinaric Karst. Two of them have an extremely small distribution range and are therefore highly vulnerable and endangered. A set of available polymorphic microsatellite markers enabled us to assess the structure and genetic diversity within lineages and to identify each individual. In repeated sampling individuals were genotyped and genetically marked aiming to estimate the size of their respective population in four different lineages in Slovenia by mark release recapture method. Due to limited access to individuals at most sites, a sample size ranged from 45 to 96 individuals per site. An exception was Planina Cave, where almost 800 samples were collected in one year. Recapture in populations of all lineages was low, ranging from 0 to 18 % of recaptured animals. The highest recapture and the largest sampling interval was in one population of Dolenjska lineage where 28 individuals were marked in 2016 and 5 were recaptured on the same site in 2021. The results in different populations and within different lineages suggest that only a smaller proportion of individuals show high site fidelity and that recaptured individuals are predominantly adult animals according to their body size. The low recapture rate in the lineage with the smallest distribution range (Stična) is encouraging news from the perspective of conservation, as it shows that one of the most endangered and isolated lineage is hiding more individuals than we are able to observe. However, this does not diminish the necessity of conservation actions to safeguard these disparate lineages and populations.



Bue Marino cave: A new global hotspot of subterranean biodiversity on Sardinia island (Mediterranean Sea)

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In a communication presented in 2007 at the 20th Italian Congress of Speleology, it was pointed out that the Bue Marino cave (a huge karstic system formed by 70 km of accessible galleries, located on eastern Sardinia Island in the Mediterranean Sea) was a symbolic place in the history of biospeleology. As early as 2007, as many as 45 terrestrial and aquatic animal species were found in 50 years of opportunistic surveys in the cave. To complete the species richness assessment of metazoan fauna using morphological and molecular techniques and taking the occasion of an environmental impact assessment study carried out in 2021 for the planned work of securing and reopening a small gallery to the public, a new series of studies was carried out in the last three years. Owing to the complexity of the cave environment, a stratified sampling approach was adopted. Habitat sampling techniques varied in different habitats (percolating waters, small freshwater lakes, large siphons, anchialine lakes, flooded galleries, and terrestrial habitats on sand, gravel, and rocky substrata). The methods adopted (from filtering percolating waters, cave diving into large siphons and lakes taking substratum samples and using baited traps both in terrestrial and aquatic sites) cannot give comparable results in the whole cave system but were designed to collect the maximum number of species in the largest number of sites. Species identification was confirmed in selected cases using DNA sequencing to detect cryptic species; barcoding of the entire fauna is still under development. The sampling survey confirmed the presence of all 45 species cited in 2007, and led to the discovery of additional crustacean species, some of which were new to science. The results of the study increased the list of known species to 79, 33 of which troglobionts or stygobionts. These numbers are quite high, considering that caves with more than 25 strictly subterranean animal species are considered worldwide biodiversity hotspots, and Bue Marino cave must be added to the list of the 28 hotspots documented thus far at the global scale. Moreover, only 3 km of galleries out of 70 km of the cave system have been investigated so far, suggesting that the species richness of this cave is still highly underestimated. Determinants of biodiversity hotspots were identified in the complex habitat structure and long paleogeographic history of the area. Sardinia has a long and complex history that began around 20 Mya when the island, together with Corsica, detached from the continental plate and migrated to reach its current position, carrying a large number of species that became endemic to the island, due to the high fragmentation of the karstic massifs; the Bue Marino cave system is the richest of these karstic fragments investigated so far. Unfortunately, during our research, the presence of an invasive alien species, the so-called “blue crab” (*Callinectes sapidus*), was observed in a flooded anchialine gallery 500 m from the entrance, raising questions about the threats to the conservation of this complex subterranean ecosystem.



Subterranean fauna associated with Mesovoid Shallow Substratum in Iron Formations from southeastern Brazil: invertebrate biodiversity of a highly threatened ecosystem

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Iron Formations (IF) globally and in Brazil are under significant threat from extensive mining activities, particularly the Mesovoid Shallow Substratum (MSS), an understudied subterranean environment. Although caves are somehow protected by the Brazilian legislation, outcrops of IF without caves are not. Therefore, evaluating the IF MSS fauna could enhance our understanding of troglobiotic biodiversity and inform putative conservation measures. This study utilized a comprehensive and spatially explicit database to analyze subterranean fauna within the MSS of iron duricrust (canga) in two localities of the Iron Quadrangle and one locality in the Southern Espinhaço Range in southeastern Brazil. Data were compiled from three biospeleological monitoring projects (BRU, ITA, and CMD), encompassing 108,005 individuals and 1,054 morphospecies from seven phyla, globally the largest dataset yet on MSS in IF. These organisms were sampled monthly between July 2014 and June 2022 using five trap types, [classic MSS traps (N=49 sampling points), MSS traps with leaf litter (N=65), depth traps (N=18), flexible leaf litter traps (N=82), and drip traps (N=17)], which allowed us to infer trap method effectiveness and possible biases. Arthropoda dominated the sampled invertebrates, representing over 97% of all invertebrates sampled in each locality. We identified 31 morphospecies of troglomorphic fauna, primarily Arthropoda and Platyhelminthes. Classic MSS traps were the most efficient method, capturing almost 80% of all invertebrates, followed by depth traps (15.4%). Unique morphospecies were more prevalent in each locality than shared among localities, with BRU exhibiting the highest uniqueness (38%). β -diversity partitioning revealed species replacement as the main contributor to spatial differences. Over time, we found a variable pattern among localities for the whole community and a decrease of total dissimilarity and importance of species replacement from BRU, ITA to CMD for troglomorphic organisms. We observed a positive correlation between spatial distance and compositional dissimilarity of invertebrates found in each sampling point. This correlation was evident when considering all trap types and specifically classic MSS traps in the Iron Quadrangle, but no correlation was found in the Southern Espinhaço Range. Mining poses a significant threat to Brazilian IFs, with their biological significance not fully understood but highly endangered due to their limited distribution and the high economic demand associated with the country's iron ore deposits. Conservation efforts require a comprehensive understanding of both biotic and abiotic factors shaping the entire IF ecosystem.



Application of the latest Guide to IUCN SSC Membership in SSC Cave Invertebrate Specialist Group

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The International Union for Conservation of Nature (IUCN) is the first global environmental union, with more than 1,400 governmental and non-governmental member organizations and the input of more than 16,000 experts, cooperating to promote nature-based solutions as key to the implementation of international treaties for conserving the integrity and diversity of nature. In September 2020, the latest Guide to IUCN SSC Membership (2021-2025) was adopted, presenting the introduction to IUCN and Species Survival Commission (SSC), their structure and outlining what is expected from SSC Members, including dealing with external communications. It includes a new Vision, Objectives and Species Conservation Cycle and Species Strategic Plan for the period 2021-2025. The plan is structured around 13 Key Species Results, each corresponding to one of the objectives. New guidelines for Chairs and Members are given, promoting a stronger engagement from members. The Guide also presents the online tools for members and their management, the IUCN Commission System and Union Portal. The IUCN SSC Cave Invertebrate Specialist Group (CISG) is one of the youngest of all SSC groups, established in 2014, focusing mainly on facilitating Red List assessments. The Group faced unexpected challenges, but has reached a number of 80 members since 2018. The changes presented in the latest guide have called for a revision and adaptation of the current membership system to CISG. In this talk, we will present these changes, discuss challenges for old and new members and propose the approach to the presented issues.



Culture and science beget action: developing the first protection and restoration strategy for Hawaiian anchialine ecosystems

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Hawai'i boasts a high density and variety of anchialine pools with pronounced levels of endemism, plus cultural and socio-economic value. Yet the function and status of these ecosystems are beset by numerous threats, including invasive species, pollution, development, climate change and other human impacts. The geographic isolation of Hawaiian anchialine ecosystems, coupled with their sensitivity to external stressors, can result in rapid and acute responses, further underscoring an urgent push for protecting and restoring these habitats. Here we highlight a critical step that the Hawai'i Department of Land and Natural Resources and partners are taking in developing a statewide protection and restoration strategy to promote on-the-ground management actions. We developed a decision support tool to optimize protection and restoration efforts for marshes and anchialine ecosystems, guided by the U.S. Environmental Protection Agency's Core Elements Framework, grassroots restoration efforts already in progress, decades of research and monitoring, and generations of traditional and place-based knowledge systems. This iterative and collaborative process included numerous consultations with resource managers, partner agencies, nonprofits, researchers and cultural practitioners that will inform statewide management priorities. This tool has since been beta-tested by staff and partners. Once finalized and implemented, the Hawai'i Wetlands Protection and Restoration Strategy will result in measurable improvements to the state's valuable anchialine resources and co-management capacity.



On art, caves, and subterranean conservation

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Caves, with their unique geological, biological, and cultural features, have long captivated human imagination. From photography to movies, from comics to painting, from dance to poetry, caves and their biota have been prominently featured in various forms of artistic expression. In a short, thought-provoking paper dating back to 1998 (*Journal of Cave and Karst Studies*, 60: 67), Dan L. Danielopold argued that combining artistic perception with scientific facts could significantly enhance cultural education. Danielopold suggested that integrating artistic perception with scientific knowledge could effectively convey the significance of the unique organisms inhabiting karstified areas as public goods with amenity value, thus emphasizing their importance in our cultural heritage. However, this idea by Danielopold remained silent in the years ahead. In this presentation, we will discuss realized and unrealized connections between scientific disciplines and artistic endeavors. We will illustrate different art forms depicting subterranean ecosystems, how each of them fits within conservation science and action, and how artists could benefit from interactions with researchers in subterranean biology, and *vice versa*. We will then ask two questions: How can we effectively break the cultural divide between scientists and artists? How can we test the effectiveness of art in enhancing conservation of subterranean biota? By exploring these questions, we may develop a future where art and science intertwine to the common goal of safeguarding a rich and diverse subterranean biological heritage for future generations.



Subterranean fauna conservation requires data

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Approaches to conservation fall into two broad groups: management of habitat in selected areas such as nature reserves or management of species across their range in multiple types of land use. Subterranean habitat management can, to some extent, be driven by broad geological principles but management of species require substantially more data about where the species occur, their ecology and tolerance of disturbance. While there may occasionally be a complication in terms of species delimitation, obtaining distributional and ecological data for species in caves is comparatively straightforward, except for determining the importance of mesocaverns in the matrix close around a cave. In contrast, acquiring information on species ranges and ecology in subterranean habitats of the broad landscape, where increasingly it appears most subterranean species occur, is challenging. This talk discusses some general issues related to collecting subterranean species and associated data in broad landscapes and presents some information on species ranges and salinity occurrence for several groups of stygofauna and troglifauna in the Pilbara region of Western Australia. Some of the difficulties in use of CO1 for species delimitation are mentioned, as well as the differences in stygophiles/troglophiles in the broad landscape versus caves. Ranges of Pilbara stygofauna species fall loosely into two categories: stygophiles and stygoxenes with Pilbara-wide and sometimes near cosmopolitan distributions, or stygobites with linear ranges <50 km. In most surveys aiming to inventory the species of an area and document distributions > 20% of species will be recorded from only one site. Most of these species will have ranges <25 km. Nearly all Pilbara troglifauna species are trogllobites with ranges <30 km; only about 10% of species are widespread trogllophiles and troglloxenes. Linear ranges of only a few kilometres are common for troglifauna. Mine pits can extend tens of kilometres (although often interspersed with undisturbed areas) while groundwater drawdown can be almost a scale of magnitude larger. Recently large groundwater reinjection programs have begun to dispose of the groundwater from mine pit dewatering (to keep pits dry while mining deep below the water table) that result in groundwater mounding. The level of subterranean habitat disturbance is high. Salinity in the Pilbara is low and has relatively little variability in an Australian context but some palaeovalleys have hypersaline water at depth. The mean salinity at which stygofaunal ostracods and syncarids occur is usually <2,000 mg/L, for amphipods 3,000 mg/L and copepods 3,000-5,000 mg/L. The ratio between mean and maximum salinity at which species have been recorded is usually <2 for all groups, suggesting that reinjection of groundwater extracted from deep groundwater may negatively impact stygofauna. While DNA bar-coding and other techniques have enabled many advances in the delimitation of subterranean species, some underlying assumptions of these techniques are violated in subterranean work, particularly because of poor dispersal. This is less easy to deal with in inventory surveys than when undertaking detailed taxonomic studies.



Non-native species in subterranean ecosystems: Who are they, how do they get there, and what we can do about it?

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Non-native invasive species pose a significant threat to native biodiversity and human societies. Yet, some habitats may be less susceptible to biological invasions due to their unique abiotic and biotic characteristics, and only invaders with traits similar to native organisms may successfully establishing therein. Subterranean ecosystems are a quintessential example of habitats imposing such strong environmental filters, selecting for species with convergent adaptations to life in darkness. Therefore, subterranean ecosystems should predictably be less susceptible to biological invasions than surface ecosystems. Although records of non-native species in subterranean ecosystems have increased in recent decades, our knowledge about this topic remains scarce. We present the first global mapping of available evidence on non-native species in subterranean ecosystems. Our synthesis focuses on the taxonomic composition of the successful invaders, their biological traits, their centers of origin, and traveling routes. We also discuss knowledge about their potential impacts and advance solution on how to move forward in the study of subterranean biological invasions. Given the vulnerability of subterranean ecosystems and the lack of attention they have received in conservation policies, we recommend future studies to deepen our understanding of invasiveness in these habitats and to raise public and scientific awareness about preserving these fragile ecosystems.



Towards optimizing protected area coverage of subterranean biodiversity

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European Subterranean biodiversity within the European Union remains critically underprotected, with numerous species facing increasing threats from climate and land use change. To address these challenges and ensure the long-term conservation of subterranean habitats, a precautionary solution is to include them in protected areas to the greatest possible extent. However, given that resources for conservation are always limited, we confront an important question: which area should be prioritized for protection? One approach is spatial prioritization planning, namely using optimization algorithms to integrate different data sources to identify conservation hotspots to be protected. We tested this methodology using subterranean spiders within the Western Italian Alps as a case study. This is a coherent biogeographic area with comprehensively documented subterranean spider fauna. Our approach uses data on 31 subterranean spider species to construct spatial biodiversity layers based on taxonomic, functional, and phylogenetic diversity. These layers are further integrated with data on threats derived from SPECTRE, an open-source database with spatial explicit layers for different anthropogenic impacts (e.g. mining intensity, pollution, urbanization, climate change intensity and velocity), creating a multi-faceted conservation framework. By overlapping diversity patterns, threats, and the current network of protected areas within the Western Italian Alps, we can establish i) priority areas, considering how diversity patterns will vary in future climate change scenarios; and ii) conservation gaps and needs. Based on this, we can elaborate different optimal solutions to maximize the protection of the highest number of species, their evolutionary histories, and ecological functions in relation to existing threats, while accounting for both the minimal cost in conservation and two alternative protected areas targets (30% and 50% of total available surface). Being part of the project P.R.I.N. 2022 “DEEP CHANGE”, aiming to expose the vulnerability of subterranean environments and biodiversity to climate change, this approach offers a crucial instrument in subterranean biodiversity conservation strategies. For example, it has the potential to influence management and policies to include the protection of these overlooked ecosystems in the European Biodiversity Strategy for 2030 and other conservation agendas.



Molecular evolution of Circadian Rhythm genes in blind water beetles from the dark biosphere

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Circadian rhythms (CRs) are natural activity rhythms that are synchronised, usually to a 24-hour period, by external cues (zeitgebers) such as light or temperature. For species living permanently in the dark biosphere of groundwater and cave environments, it is unclear how their biological clock compensates for the lack of these cues and how this influences their physiology and behaviour. Past studies of cave fish have shown that some species maintain rhythmicity and light entrained CRs, while other species have become arrhythmic in their behaviour, and show loss of function mutations in key CR genes associated with light entrainment. We further explored the molecular evolution of CR genes using an invertebrate system based on independently evolved blind water beetle (Dytiscidae) species from island-like subterranean aquifers in Western Australia. We hypothesise that genes specifically associated with light entrainment of CRs in subterranean beetle species would evolve under neutral evolution and accumulate deleterious mutations. Using data from transcriptomes, exon capture, and newly generated Illumina and Nanopore data from 14 surface and 48 subterranean species of the genera *Paroster* and *Limbodessus*, we identified 15 CR genes, including a *Drosophila*-like cryptochrome gene *cry1*, involved in light entrainment of CRs, and a mammal-like cryptochrome gene *cry2*. Comparative sequence analyses revealed that *cry1*, and a non-visual opsin gene *Rh7*, showed evidence of pseudogenisation (insertions or deletions resulting in frameshift mutations or mutations introducing stop codons) in multiple subterranean species, in contrast to surface species and recently evolved subterranean species, which retained functional versions of these same genes (Fig. 1). The remaining 13 genes retained open reading frames in all beetle species (surface and subterranean) and are likely to be functional. Overall, these analyses suggest that the subterranean beetle species have maintained functional proteins associated with clock-like gene expression, but most species are likely to have lost the ability of light entrainment. Further research is required to determine whether subterranean beetle species have arrhythmic biological clocks and what features of their groundwater environment might entrain their activity levels.



Evolution of chemosensation in subterranean beetles from a phylogenomics spyglass

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Chemical signals in subterranean habitats vary significantly from those above ground due to the contrasting environmental conditions. Absolute darkness, climatic stability and limited food availability inherently influence the abundance, volatility and distribution of chemical compounds, which may have consequently impacted the taste and smell capabilities of strict cave-dwellers. The broadly convergent loss of vision has been proposed to be compensated by enhanced mechanosensory and chemosensory capabilities. Evidence of extra-optic modifications in cave fauna include elongated antennae containing specific sensory organs in beetles, modifications in the taste buds and lateral line in fishes, and enhanced development of olfactory brain regions in crustaceans. Despite representing unique systems to understand biological adaptation, the genomic basis of chemosensation across cave-dwelling species remains unexplored from a macroevolutionary perspective, with only a few studies so far focusing on single species at a time. In this study, we used a phylogenomic approach to explore chemosensory gene repertoire evolution in Coleoptera, with focus on three beetle tribes (Bidessini, Hydroporini and Leptoridirini) that represent independent transitions to aquatic and terrestrial subterranean environments. Our results indicated that there is a dramatic variation in the chemosensory gene repertoire across different species. Overall, no parallel changes in the net rate of evolution of chemosensory gene families were detected prior, during, or after the underground transitions among subterranean lineages. Contrarily, we found evidence of lineage-specific changes within surface and subterranean lineages, which indicates a highly dynamic evolution for the chemosensory gene repertoire. Nevertheless, our results revealed key gene duplications and losses shared between some of the lineages transitioning to the underground, including the loss of sugar receptors and gene duplications of the highly conserved ionotropic receptors, involved in thermal and humidity sensing among other olfactory roles in insects. These duplications were detected both in independent subterranean lineages and their surface relatives, suggesting parallel evolution of these genes across lineages giving rise to cave-dwelling species. Overall, our results shed light on the genomic basis of chemoreception in cave beetles and pave the way for further approaches aiming to reveal the genomic underpinnings of adaptation to life underground.



Increase in copy number at multiallelic copy number variants facilitates rapid adaptation to subterranean habitats in Mexican *Astyanax* fishes

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Populations that evolve the same traits in parallel due to the same strong selective pressure provide excellent opportunities to study the molecular basis of adaptation. While it is well recognized that copy number variation contributes to phenotypic diversity, its involvement in ecological adaptation is not readily obvious. By identifying genomic regions whose copy numbers repeatedly diverge between ancestral and derived ecotypes in multiple populations, the association of such CNVs with adaptation to the novel environment can be inferred. We employed this approach to pinpoint CNVs associated with recurrent adaptation to the cave environment in the natural populations of the Mexican tetra fish (*Astyanax mexicanus*), a well-known model for studying troglomorphy. We detected CNVs from publicly available genomic data of 44 individuals from two ancestral (surface form) and three recently derived (cave form) populations. We discovered 102 genes and 292 genomic regions that comprise 0.8% of the reference genome and exhibit recurring copy number divergence between the two ecotypes. Functional analysis uncovered their association with processes relevant for adaptation to subterranean life, such as visual processing, immune response, metabolism, nervous system functioning, and oxygen consumption. Most divergent CNVs are multiallelic, showing higher copy numbers in cavefish than in surface fish. Our findings suggest that selection draws from standing genetic variation at multiallelic CNVs to increase their copy number, providing a fast route to ecological adaptation.



Hybridization in an adaptive radiation between and within extreme *Niphargus* ecomorphs

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Adaptive radiation consists in a rapid increase in the number of species originating from a common ancestor associated with ecological differentiation. How a single ancestor can provide all necessary genetic variation resulting in a multitude of ecologically divergent species remains a puzzle to scientists. Theoretical models predicting rapid depletion of genetic variation are not consistent with the pace and the magnitude of differentiation. It is therefore unclear how genetic variation can be maintained given rapid and continuous speciation. Increasingly, it has been recognized that genetic variation is enhanced via hybridization and introgression. We tested this hypothesis in the amphipod genus *Niphargus*, which experienced a series of adaptive radiations within the subterranean aquatic habitat. Within *Niphargus*, our recent research showed that the south-eastern Dinaric clade underwent an adaptive radiation about 15-10 Mya. Following this radiation, species within subterranean lakes diversified again into about five large and morphologically distinct species about 2 Mya, including the bizarre, long-legged and spiny *N. balcanicus*. Analyses of a fragment of the mitochondrial gene COI suggested a close relationship among the species, and unilocus molecular delimitation methods did not discriminate two morphologically distinct yet sympatric species, *N. balcanicus* and *N. vjetrenicensis*. Moreover, in the latter we detected high variation in nuclear but not in mitochondrial markers. A potential explanation for this incongruency is hybridization. To test for hybridization within the young adaptive radiation of lake ecomorphs, and to assess whether hybridization can explain the evolution of the highly differentiated morphology of *N. balcanicus*, we employed ddRAD sequencing. We sequenced 245 individuals belonging to the south-eastern Dinaric adaptive radiation consisting of 38 morphospecies from 69 localities. We obtained about 500,000 filtered SNPs used for phylogenetic maximum likelihood inference. The phylogenetic reconstruction suggested that species currently named as *N. vjetrenicensis*, *N. kusceri* and *N. balcanicus* are not justified. They represent polyphyletic lineages, comprised of several distinct populations or incipient species. Preliminary analyses suggested recent hybridization between and within distinct lake *Niphargus* lineages. Whether ancestral hybridization between distinct *Niphargus* lineages contributed to the onset of adaptive radiation is currently unknown, yet we show that hybridization between extant divergent *Niphargus* lineages is possible. Further analyses are needed to test whether hybridization and introgression may have resulted in novel genetic variants underlying the evolution of unusual morphologies. Overall, our results indicate that south-eastern Dinaric *Niphargus* comprises a mix of cryptic diversity, hybridization, and extraordinary morphological disparity.



Evolution of Hawaiian cave-adapted arthropod communities along the space-time continuum

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Islands have long been recognized as natural history laboratories for studies of the processes that generate biodiversity, although these processes have not generally been studied in island subterranean habitats. On volcanic islands, like the island of Hawai'i, lava tubes in flows of different ages additionally provide fine-scale time constraints on evolutionary process, allowing for estimates of the timing of speciation, cave adaptation, and community assemblage processes. We are investigating these processes by focusing on comparative evolutionary studies in ten core troglobiont taxa found in all lava tube habitats across three volcanoes on the island of Hawai'i. First phase studies focused on generating mitochondrial COI phylogenies to identify similar phylogeographic patterns in genetic diversity across species. Based on these results, phase two studies used ddRAD SNP datasets to estimate population demographic histories in two core lineages – the sap-feeding planthopper *Oliarus polyphemus* (Hemiptera: Cixiidae) and detritivorous millipedes from the genus *Nannolene* (Diplopoda: Cambalidae). Comparison of phylogeographic patterns across these two groups from different functional guilds suggests similar routes of evolution across the landscape and potentially supports the hypothesis that multiple members of the Hawaiian subterranean arthropod communities invaded younger lava tube habitats from older, underlying flows. Continuing studies will use genomics to test these hypotheses of community-level patterns of diversification and migration across the landscape in additional core lineages (e.g. beetles, crickets, moths) from Hawaiian lava tube communities.



Surviving in the heat: thermal range and behavioural adaptations of the groundwater obligate amphipod *Niphargus longicaudatus* (Costa 1851)

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Groundwater ecosystems host diverse and specialized invertebrates, many of which are sensitive to environmental changes. Among all, the *Niphargus* amphipod species, together with the copepods, holds primacy in European groundwater, with many representatives of the *N. longicaudatus* group. This study aimed to determine the thermal niche of the species *Niphargus longicaudatus* sampled from Stiffe cave in Abruzzo (Central Italy) using the chronic method. Additionally, the study examined the species' behaviour at two different temperatures: the average annual water temperature of the sampling site and the preferred temperature identified during the chronic method test. Differences in swimming behaviour were analysed through 3D video tracking analysis, studying up to eight swimming parameters: swimming speed, percentage of activity, average swimming activity, horizontal and vertical swimming speeds, time spent in the water column, trajectory convex volume, and path tortuosity. Our findings revealed that *N. longicaudatus* has a wide thermal niche with a natural performance range of 12-18°C, which strongly differs from the mean annual temperature of 9°C of the sampling site. This challenges the idea that obligate groundwater species are only adapted to narrow temperature ranges. In addition, behavioural tests showed that *N. longicaudatus* can cope with cold temperatures by reducing swimming activity and minimizing vertical migration. These results suggest that while the species can tolerate lower temperatures by reducing its locomotory activity, its optimal performance occurs at warmer temperatures. These findings go deeper to understanding the thermal adaptability of *N. longicaudatus* and have significant implications for the conservation of obligate groundwater species in the context of climate change. The ability to tolerate a range of temperatures indicates potential resistance to future climatic shifts and higher dispersal capability for species irrespective of relatively firm thermal barriers. In the era of climate change, dispersal is of primary importance due to the ability of generalist species to colonize vacant habitats.



Enlightened by a blind fish. Comparative analyses of cavernicolous and surface-dwelling populations of *Garra longipinnis*

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Garra longipinnis is a cyprinid freshwater fish endemic to Northern Oman and the UAE. Despite the harsh climatic conditions, this taxon is quite common and inhabits all kinds of water bodies available in this area. The particular interesting thing about this species is that, in addition to the many surface populations, one troglomorphic cave population is known inhabiting the Al Hoota cave in the midst of the Hajar mountains. We investigated the population structure between the cave and nearby surface populations by analyzing variable microsatellite markers. The results clearly show that the cave population is genetically isolated and gene flow can be detected only scarcely and unidirectional from the cave to the surface populations. Besides, phenotypic plasticity (exposure to light) seems to play an important role when it comes to the degree of troglomorphic traits (pigmentation, eye size). To further study the effects of light exposure on the phenotype, we conducted breeding experiments under controlled light regimes. We will employ histological analysis and μ CT scans to compare the degree of degeneration between specimens reared under different light regimes from both morphotypes and different developmental stages. Further on, in the course of an extensive phylogeographic framework, we investigated the genetic diversity of *Garra* specimens from the Northern Oman including samples from 56 sampling sites. We received concordant results from analyzing three mt markers as well as 18 nuclear microsatellite markers which show five genetically and geographically clearly differentiated groups, which we subsequently address as distinct species from different geographic regions. However, the cave population belongs to the same species as the nearby surface populations, specifically *Garra longipinnis*. *Garra* from northern Oman is currently listed as “Least Concern” (IUCN Red List). Hence, the newly discovered *Garra* species-complex in northern Oman is in urgent need for reevaluation, as desertification, habitat destruction as well as water shortage and potential chemical pollution pose serious threats to organisms occupying extreme habitats. A Species description for the newly discovered taxa was conducted and will provide the basis for a conservation reassessment.



Evolution of plasticity and plastic traits during adaptation to life in caves

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According to „plasticity-first“ hypothesis, successful colonization of novel environments can occur rapidly due to phenotypic plasticity which is subsequently genetically assimilated. Genetic assimilation involves refinement of the initial plastic response and subsequent loss of plasticity, meaning that initially plastic traits become insensitive to environmental cues and constitutively expressed in the population. Therefore, part of the process of adaptation to new habitats and the evolution of new traits involves the evolution of plasticity itself. However, our understanding of mechanisms, especially molecular ones, that generate plasticity, its refinement, and, ultimately, genetic assimilation is largely lacking. Cave colonizations represent an ideal system to fill this gap, as the selection pressures in cave environments are well understood, the optimal phenotypes in cave-dwellers are known, and, importantly, the direction of evolution in caves is clear. Also, some species have both the surface and cave forms extant, which provides access to both the early plastic responses in ancestral surface populations and the evolved responses in their conspecific cave populations. Previously, we found extensive plasticity in ancestral epigeal populations of several cave dwelling species in many relevant phenotypic traits including pigmentation, eye size, and fat content. Importantly, plasticity was revealed when surface populations were exposed to ecologically relevant environmental cue –constant darkness in controlled laboratory conditions. This indicates that phenotypic plasticity might play a critical role in adaptation to cave environments and the evolution of cave-specific traits. However, there is a missing link in understanding the connection between the initial plastic response in surface ancestors and the evolution of derived cave forms. We will present our results on the evolution of plasticity in fish species *Astyanax mexicanus* and *Telestes karsticus* as well as isopod *Asellus aquaticus*. In addition, we will show how traits change over several generations of surface individuals of *A. mexicanus* and *A. aquaticus* exposed to complete darkness as well as which molecular and epigenetic processes might be involved in producing these plastic responses. Our research shows that initial plastic responses can be both adaptive and maladaptive. However, some plastic traits are refined already in the subsequent generations. In some cases, plasticity can be reduced or lost rapidly, which may explain how surface colonizers adapt swiftly to drastically different environment upon entering caves.



Evolutionary history of cave spiders of the Rhodinae subfamily (Dysderidae)

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The first cave spider in the world was found in the Dinarides back in 1847 and belongs to the Rhodinae subfamily of the family Dysderidae. The family Dysderidae numbers 644 species distributed around the Mediterranean, and is divided into three subfamilies, Dysderinae, Harpacteinae, and Rhodinae. Rhodinae is the smallest, and the majority of its 18 species and five genera are troglobionts. The genus Rhode, numbering nine species, is the only genus with surface representatives and its five species are distributed in Italy, Spain, and North Africa are surface-dwellers. The other four species are endemic to the Dinarides, one of them is surface dweller, and three are troglobionts. The other four Rhodinae genera (Stalita, Parastalita, Mesostalita and Stalitella) are endemic to the Dinarides, and all their species are troglobionts. To explore the relationships within the subfamily, and to provide an explicit phylogenetic hypothesis of the group, we gathered Rhodinae samples from the whole Dinarides plus two Rhode species from the western Mediterranean. The Dinaric dataset included all nominal species except for Stalita inermifemur and Rhode subterranea, which have not been collected since their descriptions. We amplified 5 genetic markers commonly used in spider phylogenies (COI, 16S, 18S, 28S, H3) and inferred phylogenetic relationships on a concatenated dataset by performing Maximum likelihood and MrBayes analyses. All nominal genera within Rhodinae were recovered as monophyletic, with Rhode being sister to a clade formed by all Dinaric endemic genera (“Stalita clade”). The very long stem branch of the Stalita clade might indicate high extinction levels or long period of isolation in the history of this group. We applied several single locus species delimitation methods (ABGD, GMYC, PTP) which recognized all nominal and eight new species, some of which were previously identified morphologically. Of the eight new species, three are in the genus Rhode, three in the genus Mesostalita, and for two the position is not clear. They either belong to a new, still undescribed genus, or in the genus Stalita, in which case morphological characters currently used to define Stalita and other genera in the Stalita clade need to be redefined.



Phylogeography and genetic diversity of water scorpions (*Nepa* spp., Insecta: Hemiptera)

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Water scorpions are predatory aquatic insects present in shallow freshwater habitats across the Northern hemisphere. There are currently five accepted species: the widely distributed *Nepa cinerea* (Eurasia and North Africa), *N. anophthalma* (endemic to Movile Cave), *N. sardiniensis* (Sardinia and Corsica), *N. hoffmanni* (East Asia), and *N. apiculata* (North America). Mitochondrial (COI) and nuclear (28S) markers indicate a congruent geographical structure of local diversity and a cryptic diversity in the Western Mediterranean basin. Species delimitation tests point to a larger extent of *N. sardiniensis* distribution, being sympatric with *N. cinerea* in Northern Africa and Eastern Spain. Varying degrees of gene flow are present between surface and cave populations of these two species. Although *N. cinerea* is sympatric with *N. hoffmanni* in mainland East Asia, there is a large genetic distance between them. *Nepa apiculata* and *N. hoffmanni* are distantly related to Eurasian species, suggesting their assignment to new genera. *Nepa anophthalma*, the single species currently considered stygobiotic, is genetically closest related to *N. cinerea*, hinting to the latter as the possible ancestor. The whole genome assembly of *N. anophthalma* is currently underway and we hope that it will provide information about the evolutionary history and adaptations of this species to the sulfidic conditions of Movile Cave.



Morphological divergence and variation in the *Asellus aquaticus* species complex

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The *Asellus aquaticus* species complex is an eco-evolutionary model system in subterranean biology. Its widespread surface populations paired with several cave populations that colonised caves independently create a great natural setup for comparative studies. Although many morphological changes related to cave life are obvious, numerous questions remain to be answered: How does cave colonisation affect morphology? Does sexual selection differ between the cave and the surface environment? Does the more stable and more isolated cave environment affect morphological variation and is variation further affected by sex? To get a better understanding, we measured 17 functional morphological traits of 825 individuals from eight cave and nine surface localities. We analysed the data with three different statistical approaches. 1) Multivariate analysis (principal component analysis and linear discriminant analysis) to unfold the general pattern in our data. 2) Univariate linear mixed models to understand how sex, habitat, and functionality interacts. 3) Bayesian hierarchical generalized linear models followed by generalised linear mixed models using a meta-analytical toolkit to compare within- and among-population variances between the two habitat types. Both the multi- and univariate models showed clear morphological divergence between cave and surface populations. While surface populations from a large geographic area formed one cluster, we found two morpho-groups within the cave habitat. Although the latter result is highly affected by certain morphological traits. We also found evidence for habitat-dependent sexual dimorphism. Our third approach showed no general effect of habitat on within-population morphological variation but revealed that between-population morphological variation is higher in caves. Our results clearly show that morphological differences are present between the habitats. It is also evident that there is an interplay between environmental and sexual selection affecting locally adapted populations' morphology. Larger between-population morphological variation in the cave when compared to the surface habitat might suggest substantial environmental differences between distinct caves, stochastic effects of genetic drift during cave colonization, or both. As quantification of variance in morphology is rare, we encourage reporting such information in large dataset studies as it can elaborate the understanding of cave colonisation, adaptation, and speciation. Although some of our results contradict widely accepted concepts in cave biology, there are various possible explanations to be explored in future studies.



Rampant convergent evolution in pseudoscorpions from disparate cave systems: Hawaiian lava tubes and Europe's Dinaric caves

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Species inhabiting ecosystems with strong niche filtering tend to exhibit similar phenotypes, regardless of their phylogenetic relatedness. The pseudoscorpion tribe Tyrannochthoniini encompasses six genera, two of which are endemic and share a unique morphological character: the presence of three spine-like setae on the chela. These two genera are *Vulcanochthonius* Muchmore, 2000, described from the lava tubes on the Big Island of Hawai'i, and *Troglochthonius* Beier, 1939, from the Dinaric Karst in Europe. The taxonomic boundaries and even the monophyly of the genera within the tribe have been questioned but never tested in a quantitative framework. We conducted a combined, morphological and multi-target gene phylogenetic analysis of the group to determine whether the common trait evolved independently or, conversely, if the two genera are closely related and inherited the trait from a common ancestor. We generated a matrix of 30 morphological characters and utilized two mitochondrial (*COI* and *16S* rRNA) along with three nuclear markers (*28S* rRNA, *18S* rRNA, and *H3*). Terminals represent all genera classified within the tribe. Clade ages were estimated using fossils and geological calibrations. Our results recover the monophyly of both the tribe and its constituent genera. Moreover, we demonstrate that the shared character between Hawaiian *Vulcanochthonius* and Dinaric *Troglochthonius* does not indicate a common ancestry but rather independent evolution, likely due to adaptation to harsh subterranean environments.



Variation of activity levels in cave crustaceans suggests retention of circadian rhythm

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Cave environments are characterized by an absence of a light-dark cycle, a relatively stable temperature, and constant levels of dissolved inorganic and organic matters in water. Despite that, previous studies have suggested that many cave animals retain a circadian rhythm, but this issue is still under-investigated. We monitored 16 anchialine caves and associated springs located in the Salento peninsula (Apulia) looking for cave invertebrates and by using visual surveys and minnow traps in two different seasons in 2024. Each site was monitored 4 times per season. We collected data on abundance for 4 cave crustaceans (*Typhlocaris salentina* Caroli, 1923; *Spelaeomysis bottazzii* Caroli, 1923; *Niphargus* spp.; *Salentinella gracilima* Ruffo, 1947). Then we focused on the most common species, *Spelaeomysis bottazzii*, to assess the activity level using a site occupancy model. Our results show that the abundance of active animals varies between day and night, being higher during nighttime in both cave and spring habitats. We hypothesize that the maintaining of a circadian rhythm might allow cave animals to exploit spring habitats under certain circumstances during nighttime.



The linkages between functional traits of Ostracoda (Crustacea) and their habitats in groundwaters and groundwater dependent ecosystems

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Functional traits are morphological, physiological, and phenological features, which affect an individual's growth, reproduction, and survival and form a basis for understanding the links between species' occurrence and environmental conditions. They provide insight into roles of organisms in their environments, elucidate ecological processes within ecosystems, and explain community response to environmental change. Groundwater delivers numerous ecosystem services, from supporting surface ecosystems to the provision of clean drinking water, all of which depend on the functional traits of groundwater biota. Therefore, it is important to understand the link between species traits and their environment. Functional traits of groundwater organisms are much less known and understood in comparison to their surface counterparts. Even less is known about the functional diversity of Ostracoda – a diverse and abundant group of small crustaceans ubiquitous in groundwater and groundwater dependent ecosystems. We mined existing databases and published literature to gather information on functional traits (e.g., pigmentation, eye development, body size, carapace shape, etc.) of ostracods found in groundwater and groundwater dependent habitats across Europe. We used the data to assess the changes in Ostracoda assemblage composition across groundwaters considering varying habitat structure and eco-hydrogeological characteristics (e.g., geology, void size, hydrology). We focused on traits related to the distance of groundwater habitat from the surface and degree of hydrological connectivity (i.e., eye development, pigmentation, body size), and the habitat type, such as interstitial environments, running or standing water (i.e., body size, carapace shape). These relationships between the species traits and habitat characteristics allowed us to identify which functional traits are associated with specific groundwater habitat characteristics. The preliminary analyses demonstrated that within habitats, species exhibited clustering based on traits studied, and across the larger scales, species distributions were correlated with their functional traits in a manner consistent with environmental filtering. For example, with increasing distance from the surface, the number of smaller species lacking pigmentation and eye increased. This analysis, when carried out on the full set of available data, may enable identification of traits useful for predicting the susceptibility of ostracod species to human pressures and environmental changes in groundwater and groundwater dependent ecosystems.



Acanthocephalus anguillae: An Evolutionary Misadventure of Parasite in Subterranean Environment

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Acanthocephalans are obligatory endoparasites that require two hosts to successfully complete their life cycle. Attached to the intestinal wall of vertebrates (final hosts) they shed eggs to the gut lumen and subsequently into the environment, where eggs are ingested by arthropods (intermediate hosts). These are then preyed upon by their final hosts, allowing a new generation of acanthocephalans to reach their site of sexual reproduction. *Acanthocephalus anguillae* is a widely distributed European acanthocephalan, typically found in surface freshwater rivers cycling between fish and isopod *Asellus aquaticus*. However, in the Postojna-Planina Cave System (PPCS) in Slovenia, the parasite was discovered in olms (*Proteus anguinus*) and cave populations of *A. aquaticus*. While this cave system theoretically provides both necessary host types to present the first case of a heteroxenous parasite restricted to the subterranean environment, the exact life cycle of the parasites and suitability of cave hosts remain unclear. In this study, we investigate the life cycle of *A. anguillae* in PPCS by characterizing gene flow with nearby surface populations and assessing the suitability of the olm as a final host through the search for fertilized parasitic eggs. We collected 88 individuals of *A. anguillae* from PPCS and seven surface locations across Slovenia. Using COI barcoding and ddRAD sequencing, we analyzed population structure and genetic diversity. The search for fertilized parasitic eggs was conducted on extensive collection of olms from the Chair of Zoology at the Department of Biology (University of Ljubljana) using a combination of μ CT and scanning electron microscopy (SEM), describing their size and form. Our results show low genetic differentiation between *A. anguillae* in PPCS and nearby surface populations, suggesting a panmictic population and significant gene flow between the surface and cave environment. Morphological investigation of acanthocephalan eggs in adult females did not provide evidence of fertilized eggs, as none of the observed eggs were distinctively elongated, which is an indication of fertilization. Our findings suggest that *A. anguillae* in PPCS does not form a distinct population, but is connected to the surface population. We assume this connection is enabled by the continuous inflow of acanthocephalan eggs via sinking karst river, which are ingested by the cave ecomorph of *Asellus aquaticus*. The infection of olms likely results from predation of these isopods. The confirmed connection to the surface acanthocephalan does not necessarily exclude the option that the parasite also cycles between cave hosts, but the unsuccessful search for fertilized acanthocephalan eggs in olms indicates that the inflow of eggs from the surface is the crucial mode of parasitic transmission within PPCS and presents the olm as an unsuitable, dead-end host.



Factors regulating the production of cocoons and the distribution of troglomorphic spiders of the genus *Meta* (C. L. KOCH, 1836) in alpine and pre-alpine areas.

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Among arthropods inhabiting subterranean habitats, troglomorphic spiders of the genus *Meta*, which are at the top of the reduced trophic chains characterizing hypogean environments, are commonly found at cave entrances. They are distinguished by a peculiar life cycle composed of a subadult and adult hypogean phase and an epigeal phase that occurs in the juvenile stages after hatching. In Europe there are two species, *Meta menardi* and *Meta bourneti*, both are present in Italy too. Our study had multiple objectives falling into three distinct categories: one related to population dynamics within caves, the second related to the reproductive success of spiders, and the last one of a purely methodological nature. From December 2022 to December 2023, samplings were conducted in twenty different caves focusing on alpine and pre-alpine areas of Piemonte and Lombardia regions in Italy, where ranges of *M. bourneti* and *M. menardi* partially overlap. Each site was divided into sectors of three meters in length. For each sector, microclimatic and morphological characteristics of the cave were recorded, and the number of cocoons, juveniles, males, and females found was counted, implementing the multiple observer method. Several morphometric parameters were collected for many of the cocoons and spiders. Regarding *M. bourneti*, only twenty total observations were made in three caves, and no cocoons were found. As for *M. menardi*, 769 total observations were made in seventeen caves; for this species, 106 cocoons were found considering both those laid in 2022 and 2023. Within the cave, adult spiders of *M. menardi* were associated with the wetter sectors; juveniles, males, and females were found to share the same sectors. Cocoons were more abundant in sectors closer to the exit and with a greater biomass of spiders. Across caves, altitude of the entrance and maximum number of females observed inside during the surveys positively affected the number of cocoons laid. We assessed relationships between the collected parameters using generalized linear mixed models. Results showed that among the environmental parameters, humidity is the main feature that affects occurrence of *Meta menardi* in the different sectors of the caves. In this species there is a shared utilization of optimal sectors in the different sites regardless sex and age of the individuals, thus not showing a clear intraspecific competition for optimal areas. Secondly, it emerged that choice of location for laying cocoons and their morphometric characteristics are influenced by morphological aspects of the caves and not by microclimatic conditions in the sectors. A concerning finding is that no cocoons were observed in the caves inhabited by *M. bourneti* in both 2022 and 2023. This fact might suggest a demographic decline of populations of cave-dwelling spiders inhabiting these isolated caves, which causes remain to be explored.



Reduced humidity affects thermal tolerance and compromises the survival of Pyrenean cave beetles

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To preserve subterranean biodiversity in the current context of climate change, it is essential to understand and predict how subterranean species will respond to changes in their environment. These species have evolved under extremely stable climatic conditions, characterized by constant temperatures and ambient humidity, close to saturation, which makes them potentially vulnerable to climate change. Such vulnerability has been documented by several studies, which show that cave insects have reduced thermal tolerances compared with most epigeal species. However, the potential effect of other limiting factors in subterranean environments on their thermal limits, such as humidity, has been overlooked. To address this question, we conducted physiological experiments to measure the upper thermal limit of four cave beetles at different humidity conditions. The study species were *Euryspeonomus eloseguiti*, *Troglocharinus impellitieri*, *Troglocharinus hustachei*, and *Stygiophyes ribagorzanus*, belonging to the tribe Leptodirini (Coleoptera, Leiodidae, Chovelinae) and distributed in different areas of the Spanish Pyrenees, from the Basque Country to Catalonia. Adults of these species were exposed to four temperatures (11, 20, 23 and 25°C) and two relative humidity conditions (100% and 75% RH) for each temperature, for 7 days, and their survival was checked every 24 hours. Survival analyses (Cox Proportional-Hazards models) revealed a significant interaction between temperature and humidity in all species. Across all species and temperatures, a reduction of humidity from 100% to 75% caused the death of all individuals in a timespan ranging from 24 hours to 6 days. None of the species survived at temperatures > 20°C after the 7 days of exposure at any humidity. However, the different species showed different survival patterns along the duration of the experiment. *E. eloseguiti* and *T. impellitieri* were most sensitive species to humidity reduction, as no individual survived more than 48 hours at 75% RH, at any temperature. *E. eloseguiti* was also the least thermotolerant species, even at 100% RH, as all individuals died within 4 days at 20°C-100%RH. In contrast, *T. impellitieri* had an 80% survival rate at 7 days in the same treatment. *T. hustachei* appeared to be slightly more tolerant to reduced humidity. At 3 days, it had a survival rate of 50% in 20°C - 75% RH, and 20% in 11°C - 75% RH, but no individuals survived longer than 4 days at 75% RH. *S. ribagorzanus* was found to be the most resistant species, with a survival rate at 6 days of 60% in 20°C - 75% RH and 20% in 11°C - 75% RH. This higher resistance could be linked to a lower degree of subterranean specialization of this species, reflected in its morphology (e.g. shorter appendages and a more rounded body shape). Our results show that a relatively small reduction in ambient humidity could increase the high thermal sensitivity of subterranean insects. These results have profound implications for the conservation subterranean biodiversity and highlight the importance of considering the effect of humidity in vulnerability assessments in a context of climate change.



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