Guidelines for sampling invertebrates in caves and springs

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1. Characteristics of subterranean habitats

Subterranean habitats differ from the surface ones in several important aspects, i.e. absence of light, paucity of organic matter and lack of short term (seasonal/diurnal) variability in climatic conditions. Subterranean habitats, consisting of interweavement of crevices and voids of different sizes, are present in different types of bedrock, and can be filled with water. We, humans, can access a small portion of subterranean habitats directly, through caves, using proper equipment and speleological knowledge. Another access points are springs, contact zones between subterranean and surface realm, where aquatic species can be found. All other types of subterranean habitats remain inaccessible to us for most of the time.

Different kinds of species can be found in subterranean habitats: some species finish there accidentally, others may use caves seasonally (like bats). Apart from these, there are also many highly specialized and cave-adapted species that live exclusively underground. The latter are usually rare, small and hard to detect. In order to find them, we need to employ different sampling techniques and repeat sampling visits to the same site.

The fieldwork guidelines in front of you focus on how to sample animals in caves and springs, but are not including monitoring techniques to sample subterranean fauna in other subterranean habitats or detect and identify bats. The guideline is organized in a walkthrough manner, from preparatory activities for the field work followed by presentation of the sampling techniques, and concluding with post-fieldwork sample processing (Figure 1). It is intended to help students and other researchers, naturalists - who wish to participate in improving the knowledge on subterranean species distribution. Although field sampling is just a first step, it is very important to execute it correctly, as it affects all post interpretations of the findings.

Figure 1: Each fieldwork is preceded by preparatory work, and followed by post-fieldwork sample treatment. It ends with properly sorted and stored samples, which can then go into detailed morphological and/or molecular analyses, leading to identification of collected individuals. All mentioned makes a solid ground for further statistical analyses and interpretation of the results in the form of reports or articles.

2. Preparatory actions

2.1. Applying for permits

Subterranean habitats and species are often endangered, and subjected to protection by national and international legislation. It is, therefore, of vital importance to consider this aspect and apply for the permission to perform sampling of subterranean species. Each researcher should respect the laws protecting subterranean habitats and fauna in the research area, saying otherwise, it should be done with a valid permit.

The researcher also has a moral commitment to be respectful to the habitats he/she is sampling, and should work in the way to leave no or least signs of its presence. Also, it is advised to collect only as many specimens as relevant for the research and avoid unnecessary disturbance of fauna and devastation of cave habitats.

All cave fauna in Bosnia and Herzegovina is protected by the entity-level nature protection laws. Appliance for sampling permission is coordinated by:

- *Republički zavod za zaštitu kulturno-istorijskog i prirodnog nasljeđa in Republika Srpska (https://nasljedje.org/)*
- *Federalno ministarstvo okoliša i turizma in Federacija Bosne in Hercegovine (https://www.fmoit.gov.ba/)*

2.2. Defining aims of the research

Before executing the fieldwork, consider the objectives of the research. Those can be general sampling of a site, a more detailed inventory of subterranean fauna or, perhaps, a targeted sampling of a specific taxon. Defining the aims to the greatest possible extent is beneficial to arrange where, how often and using which methods one shall sample.

2.3. Locating sampling site

Gaining as much available information about the sampling localities is very important and can save a lot of time. Access to caves and springs can sometimes be very hard and time-consuming, especially if you are not familiar with its accurate location. Prior to the field work, gather the following information:

- the exact location of the site, using geographical coordinates or description of the access to it by the locals who know it;
- for the caves, plans and information on the presence of vertical pits, lakes or water bodies inside the cave;
- the existing data on animals collected during previous studies.

Many parts of Bosnia and Herzegovina are, unfortunately, still covered with mines from past wars. It is of vital importance to consider whether such minefields exist in the area that is planned for sampling - and avoid the mined areas.

For the SubBIOCODE project area, all existing information on subterranean species can be found online. Scan the QR code and explore the current knowledge on subterranean biodiversity in the SubBIOCODE Database (db.subbiocode.net)!

2.4. Preparing the materials and equipment

2.4.1. Personal gear

When visiting springs, the usual hiking gear is needed, with addition to the rubber boots to enable stepping into the water if necessary.

For the caves, the suitable personal equipment is a must. Caves cannot be safely visited without a helmet and a headlamp, an additional light and the extra battery pack. Necessary personal equipment also includes overall, rubber or hiking boots and rubber gloves. Vertical pits can be overcome only by trained speleologists, who are familiar with the caving harness, ropes and other equipment that enables a safe visit to the cave.

2.4.2. Basic sampling equipment

- forceps (soft ones are best for subterranean animals)
- soft paintbrush (to collect tiny animals)
- vials of different size (some with alcohol)
- aspirator
- tracing paper for labeling
- pencil

Figure 2: Basic sampling equipment.

The collected individuals are usually stored in ethanol. For the morphological analyses it is best to store the collected specimens in 70% ethanol; yet, for the molecular studies, it is best to use 96%. This is only a general way, please be aware that some taxa have to be stored in a unique way.

2.4.3. Equipment for terrestrial traps

The equipment for setting terrestrial live-catching pitfall traps includes;

- Plastic cups,
- vial with the bait,
- labels with the collector's contact or a permit information (optional but useful as coincidental finder of the trap has information to whom do they belong),
- small shovel,
- metal net (optional but useful as it prevents small mammals (mice, voles or dormice) to destroy the traps),
- plastic tray (for collecting animals from the traps).

Baits are frequently made of cheese, fish, livers, etc., which have been left for a couple of days or weeks at room temperature to rot. This mixture is put into the vials and a cotton gauze is put on the top of it, instead of a cap, to prevent animals from accessing the bait.

2.4.4. Sampling equipment for aquatic fauna

When sampling water (lakes/rivers in the cave or springs), additional equipment is needed:

- hand water net
- spoon
- “turkey baster”
- plastic tray
- thermometer or multimeter;
- dropper.

Figure 3: Equipment for sampling aquatic fauna.

2.4.5. Equipment for aquatic traps

The material for setting aquatic traps includes:

- plastic bottle
- rope
- bait

3. Sampling of terrestrial fauna

3.1. Direct collecting

To find terrestrial animals we have to carefully examine all the surfaces and hand-picking them using forceps, brush or aspirator. As animals can be tiny, only couple of mm big, proper attention is needed not to deform them. Check the cave walls and the floor, turn the stones and check the muddy sediments. Pay special attention to any kind of organic debris, like plant remains, feces and carcasses, as the animals usually stick around the food resources. Terrestrial animals can also be found on the surface of water puddles or on the cave walls covered with a thin water film (the so-called cave hygropetric).

3.2. Sampling with live-catching pitfall traps

Pitfall traps are commonly used when sampling in the caves, as they are usually more efficient than visual inspection. At least when it comes to taxa such as cave beetles. The best practice is to use live-catching traps as they do not affect the ecosystem as the ones with preserving media, meaning that animals remain alive after they fall into the trap. When using live-catching traps, upon checking them, a researcher can take only the specimens relevant for the research and release all the others to minimize the negative impacts on the cave fauna.

When in a cave, the first task is to find a suitable spot for setting the traps, avoiding completely dried-out places and sites where the trap can be flooded. Decide how many traps to set - this depends on the size of the cave, the microhabitats in it, and the research aims. Setting a trap approximately every 10 meters is suggested for a thorough sampling of a cave.

The main steps of setting up a trap include:

1. Digging a hole, large enough to fit a plastic cup - the trap.
2. Putting the plastic cup in the hole.
3. Adding small stones/mud in a cup, to enable animals to hide from the potential predators that also fall into a trap.
4. Putting in a bait, facing it upwards so that it is inaccessible to the animals that fall into the trap.
5. Labeling it with the information about the collector/permit.
6. Placing small stones and mud around the cup and leveling it, so the edge of the cup is leveled with the surrounding. This enables even the smallest animals to reach the edge of the cup and fall into it.
7. Setting a metal net over the top of the cup and place some stones on the edge of it. This prevents smaller mammals from destroying the trap.
8. Arranging several larger stones in a way that protects the trap from the dripping water (like a roof).
9. Marking the traps on a cave map and adding the numbers, as all traps must be found upon the next visit. You can use reflecting tape or aluminum foil, to easily locate the traps. Marking the traps by individual signs/numbers is important also to follow what was caught in each of the traps.

Figure 4: Left: When setting a trap, the edges of the cup should be leveled with the surroundings. Right: After putting a metal net over the trap, arrange the stones over it.

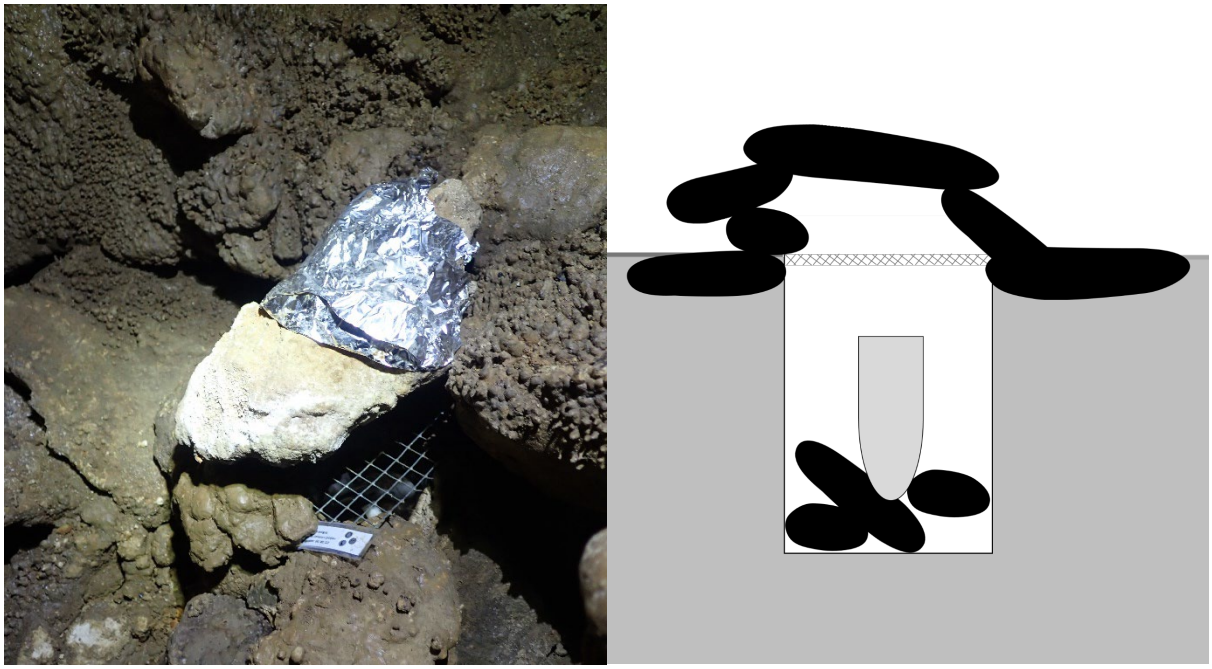

Figure 5: Left: The trap, after it has been completed. Right: Schematic representation of live catching terrestrial traps.

Live-catching traps need to be checked at the repetitive visit, which should not be longer than 3 weeks, so that individuals in the trap do not die or are not eaten by the predators in the trap.

When collecting the traps, please take care to follow the next steps:

- Remove the stones covering the trap and check them for the presence of small animals. Remove the net (if set), take the plastic cup out of the hole, and set it onto the plastic tray for inspection.
- Carefully take out and examine each stone from the trap separately; check also the vial with a bait. Put the animals into prepared vials with or without ethanol. Carefully check that all the animals of interest were collected from the trap and the rest released at the site.
- Take notes about the animals you collected and saw in each of the traps.
- Mark the vials with a label.

4. Sampling of aquatic fauna

Methods for sampling of aquatic habitats are similar, no matter if you are sampling the waters inside the cave or the waters coming out at the springs.

When arriving at the locality, take photos and coordinates of the spring. If there is an option, it is beneficial to measure physical-chemical parameters (temperature, nitrates, pH, dissolved oxygen, …). Measure parameters before disturbing water and note also the time of the measurement as parameters change during the day.

4.1. Direct sampling

The most simple method of sampling springs is turning stones in water and hand-picking animals on the stones or underneath them. The same can be done with leaf litter and other debris.

In order to get to the animals that hide in crevices at the bottom, sampling with a water net can be used. Before further steps, fill a plastic tray with about a centimeter deep clean water, and then preform a method called “kick sampling“. It includes shortly kicking the stones at the bottom of the stream/lake, disturbing the substratum, raising animals into the water column and then moving the net over the disturbed area in the form of a number eight. While doing this the mouth of the net should be a few centimeters above the bottom. In this way, the invertebrates, lifted in the water by kicking, will be caught in the net. If there is a strong current present, the net needs to be held against the current, so that the animals are flushed into the net.

To examine what animals were caught in the net, firstly flush out any fine sediment and remove larger parts e.g. water plants, and then turn the net inside out to put the content in a tray with clear water. Carefully check the sample and collect the specimens of interest into the vials. In case of general sampling, the whole net content should be washed into a bigger cup, and the water removed by filtering it through the net. The sample is then preserved in ethanol.

Again, all the samples need to be properly labeled at the sampling site.

4.2. Setting traps for aquatic fauna

Some of the aquatic cave taxa can also be sampled using specially designed traps. These are made from a plastic bottle cut at the upper third, and then the part with the bottle mouth is placed upside down into the lower part, forming a funnel. The rope is used to tighten the two pieces of bottle together and to retrieve the trap from the deeper waters. The bait, usually a paper napkin with yoghurt or some rotten meat, is placed inside the trap. The trap is set into the water and anchored with a rope to a place that can be easily reached. It should not be left in the water longer than a few days.

5. Sample storing and labeling

The specimens collected during sampling should be properly stored and labeled before leaving the sampling site. The best practice is to mark the samples on the spot, to avoid confusion with the other localities by labelling them. The label should include basic information: the name of the site, date, collector's name (sometimes referred to also as a legator) and, if using different types, information about the preserving medium. It is best to write the information on labeling paper with a pencil and put the label inside the container with the sample.
In a cave, fill in a recording scheme (check out the attachment), by inserting information about the conditions met and sightings of the animals within the cave
It is very useful to take a photo of the locality/entrance of a cave, to take the coordinates of the locality, and to make some notes about the best approach to the cave.

6. After fieldwork sample processing

At home or at the laboratory, examine and sort all the specimens collected. Place a sample from the vial into a petri dish and inspect it under a stereomicroscope. Try to determine animals as accurately as possible, even though that can often be only to a higher taxonomic category (genus, family, order, …). Put specimens recognized as one taxon from one cave (or one trap if more accurate information is needed) in a single vial. It happens very easily that one misses some animals, so it is important to carefully check the petri dish, the label, the vial and its lid for any small animals that could be attached to the surface.

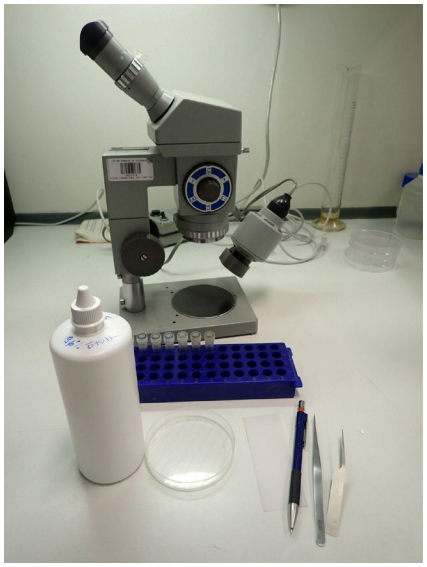

Figure 6: Equipment for sorting samples includes: stereomicroscope, vials, bottle with alcohol, petri dish, forceps, pencil and tracing paper.

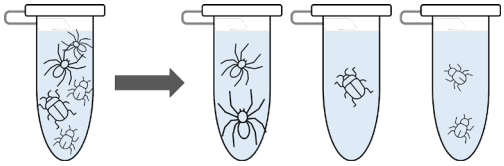

Figure 7: Schematic representation of sorting a sample from one cave by taxa.

Mark all the vials by putting in the label with the taxon name in them and making proper labeling for the locality. This includes: name of the locality, the closest settlement, larger city, country, coordinates (if not in WGS84, write the type of coordinates), date, legators. In addition, writing some additional information about the preserving media (96%, 70% or 40% ethanol, vinegar, etc.) or the exact locality within the cave can be useful. Make sure that all vials are filled with the media and properly sealed, otherwise the media, especially alcohol, evaporates rapidly. If storing for molecular analysis, keep the samples in the refrigerator for a shorter time or in a freezer for a longer time.

Vjetrenica pri Zavali, Zavala, Ravno,
Bosnia and Herzegovina; 17.9839, 42.8458;
7. 10. 2021; I. M. Zagmajster, T. Delić; 96% EtOH

Figure 8: Schematic representation of information for locality label, which should be put in a vial with sorted samples.

Import all collected data into the database or organize an appropriate excel table, where the data is stored and could be shared with other scientists. Include all relevant data and keep track of the storing place of your samples. Storing the samples properly enables to recheck them at any time or send them to specialists for the group.

Scan the QR code and learn about different taxonomic groups living in the subterranean habitats (https://www.hbsd.hr/podzemni_organizmi/).

7. Samples

The data on occurrences of the cave animals itself is very important as in most of the regions we do not have complete knowledge about species distributions. Properly stored and labeled specimens can be used for further morphological and molecular analysis, which gives us insight into the diversity and phylogeny of the taxonomic groups. Furthermore, in this manner, we can better understand the ecology of subterranean species and explain the reasons underlying their distributions. Appropriate interpretation of the results has important consequences for decision-makers and contributes importantly to adequate conservation measures for protecting cave habitats.

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