MICROBIAL COMMUNITIES IN PERCOLATING WATER IN CAVES OF PĂDUREA CRAIULUI MOUNTAINS (NW ROMANIA)

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SUMMARY. Due to the absence of light belowground, and thus the consequent lack of photosynthetic plants, microorganisms and dead organic materials transported from the surface provide the basis of any groundwater food web. We aim to relate the abundance of microbial assemblages to the presence and density of groundwater fauna in the epikarst of Pădurea Craiului Mountains (NW Romania), and to determine whether the water dripping from the unsaturated zone of karst can be regarded as microbiologically and chemically clean. For that, we determined the density of aerobic heterotrophic bacteria, coliform microorganisms, and various microbial physiological groups (i.e. iron reducing bacteria, ammonifying bacteria, and denitrifying bacteria) in samples of water dripping in three caves. Our analyses revealed that the sampled groundwater is not contaminated by surface microflora, \textit{i.e.}, it is free of coliform microorganisms (\textit{Enterobacteriaceae}). Although the estimation of the abundance of aerobic heterotrophic bacteria showed relatively low numbers of viable cells, other physiological tests revealed relatively larger numbers of iron reducers, ammonifying and denitrifying bacteria. The relative large number of microorganisms involved in the nitrogen cycle can be related to ammonification and denitrification processes occurring in the above-karst soils through which percolating water passes. In general, the abundance of microorganisms was larger in locations where there was a larger population density of groundwater fauna, and conversely, at locations with lower density of groundwater fauna, fewer microorganisms were detected.

\textbf{Keywords:} cave, groundwater fauna, microorganisms, Pădurea Craiului Mountains, unsaturated zone

\textbf{Introduction}

Microorganisms and dead organic materials transported from the surface provide the basis of any groundwater ecosystem. Microorganisms were only recently detected in the upper-most layer of karst, but their role is poorly understood in this habitat. Besides the decomposition of organic matter processes, which occur in all natural habitats, in karst, microorganisms represent food for larger organisms, such as

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groundwater fauna. The upper part of the unsaturated zone of karst is often seen as an ecotone between soil and karst ecosystems (Gibert et al., 1997). It contains thus elements of both surface and subsurface environments. As microbial densities are in general larger and microbial activity more intense in soils than in underground habitats, this ecotone can be a good source of microorganisms and other organic materials, and thus energy, which are transported by percolating water to lower levels of karst.

In contrast to microorganisms, groundwater fauna in the unsaturated zone is better documented, from the largest amphipods to the smallest copepods (Delay, 1968; Galassi, 2001; Moldovan et al., 2007; Pipan and Brancelj, 2001; Pipan, 2003; Pipan and Brancelj, 2004; Rouch, 1968; Sket et al., 2004). On the contrary, the only indication of the presence of microorganisms in epikarst, the upper part of the unsaturated zone (Klimchouk, 2004), is that of Gerič and coworkers (2004), where bacteria of various types were detected, and a relation was established between the abundance of bacteria and the number of collected meiofauna.

The aim of our research is to relate the abundance of microbial assemblages to the presence and density of groundwater fauna in the epikarst of Pădurea Craiului Mountains (NW Romania). Our aim is also to determine whether the water that percolates through the epikarst in the belowground environments, such as the caves, is chemically and microbiologically clean.

**Materials and methods**

*Site description and sampling.* Pădurea Craiului Mountains are located in NW Transylvania (Romania); they are mainly formed of carbonatic rocks and largely karstified with many caves (Rusu, 1988). Physico-chemical measurements and sampling of fauna and microorganisms were carried out in three caves, in one sampling point in each cave. Two of the caves (*i.e.* Peştera de la Vadu Crişului and Peştera Ungurului) are located at an altitude of 305 m in the gorges formed by Crişul Repede River, in the vicinity of the village of Ţuncuiuş, at 3.75 km distance from each other (Fig. 1). Peştera cu Apă din Valea Leşului is located at an altitude of 650 m on Leşului Valley, a left tributary of Iadului River (Fig. 1). The water used for microbiological analyses was sampled directly in sterile bottles. Sampling of fauna in percolating water was performed according to the method described by Brancelj (2004). Basically, the water dripping from the cave ceilings was collected during one month via a large funnel into buckets that were foreseen with 100 μm mesh-sized planktonic nets, allowing the excess of water to pass through, but retaining the debris with the animals.

*Physico-chemical measurements.* Before sampling, physico-chemical parameters (*i.e.* pH, temperature, electrical conductivity) were measured in the field using a Multiparameter Hanna Combo instrument (Hanna Instruments Inc., Woonsocket, RI, USA).
Culture-based quantification of bacteria. Cultivation of microorganisms was initiated within 24 h of sampling.

The medium used for quantification of aerobic heterotrophic bacteria had a pH of 7.5 and contained (g/l) peptone (5), yeast extract (1), FePO₄ (0.1) and agar (12). For each cave, 0.2 and 0.4 ml of percolating water were inoculated in triplicates onto Petri dishes. The cultures were incubated in the dark at 30°C and the colonies grown onto medium were counted after 7 days of incubation. The number of viable cells was calculated as colony forming units (CFU) by averaging the numbers obtained in each of the inoculated plates.

The density of Fe-reducing bacteria, ammonifying bacteria and denitrifying bacteria, was calculated by estimating the most probable number (MPN) according to the method described by Lorch et al. (1995). The medium used had a pH of 7.0 and contained per liter of water 1 g K₂HPO₄, 0.8 g KH₂PO₄, 0.2 g KCl, 0.2 g Fe₂O₅×3H₂O, 0.5 g yeast extract, 5 g peptone and 20 g glucose. A tenfold serial dilution was made for each sample in this medium up to a dilution of 10⁻³ in triplicates. The cultures were incubated 48 h in the dark at 30°C. The formation of Fe²⁺ ions, and thus the
presence of iron reducing bacteria, was detected by adding 1 ml of the iron chelator \( \alpha-\alpha' \) dipyridyl to 9 ml of culture. The cultures containing iron reducers colored in red after the treatment with \( \alpha-\alpha' \) dipyridyl.

The medium used for determining the density of ammonifying bacteria was the peptone water (pH 7.9), which contained 2% peptone and 0.5% NaCl. A tenfold serial dilution was made for each sample in this medium up to a dilution of \( 10^3 \) in triplicates. The cultures were incubated 48 h in the dark at 30°C, and MPN was calculated like in the case of iron reducing bacteria. Ammonifying bacteria decompose the amino acids present in peptone and produce ammonia. After incubation, 1 ml of Nessler reagent (0.09 M solution of potassium tetraiodomercurate in 2.5 M potassium hydroxide) was added to 1 ml of culture. The Nessler reagent colors the cultures where ammonia formed in yellow or orange.

The density of denitrifying bacteria was determined by estimating the MPN like in case of iron reducing and ammonifying bacteria. The medium used for the growth of denitrifying bacteria had a pH of 7.2 and contained per liter of water 2 g KNO\(_3\), 5 g CaCO\(_3\), 10 g glucose and 10 ml of Winogradsky solution. After 48 h of incubation in the dark at 30°C, the presence of nitrates formed as a result of nitrates reduction was detected by adding 0.1 ml Griess II reagent (a solution of 0.5 g sulfanilic acid in 150 ml acetic acid 30%) to 1 ml of culture. Griess II reagent colors the cultures where nitrates formed, and thus denitrifying bacteria were present in red.

After determining the four physiological groups of microorganisms (i.e. aerobic heterotrophic bacteria, Fe-reducing bacteria, ammonifying bacteria and denitrifying bacteria), the bacterial indicator of water quality (BIWQ) was calculated according to formula proposed by Muntean (1995-1996):

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\text{BIWQ} = \frac{1}{n} \sum \log_{10} N
\]

where \( n \) is the number of analyzed physiological groups of bacteria and \( N \) is the number of bacteria in each physiological group.

For determining the density of coliform microorganisms, a presumptive and a confirmation test were performed. The medium used for performing the presumptive test was the lauryl sulfate broth (LSB), simple and double concentrated. The simple-concentrated medium (LSBs) contained (per liter of water) 20 g peptone, 2.75 g Na\(_2\)HPO\(_4\), 2.75 g NaH\(_2\)PO\(_4\), 5 g NaCl, 5 g lactose and 0.1 g sodium lauryl sulfate [\( \text{CH}_3(\text{CH}_2)_{11}\text{OSO}_3\text{Na} \)]. The double-concentrated medium (LSBd) contained the same ingredients as for LSBs but in double the amounts.

Before inoculation, Durham tubes were inserted upside-down in the test tubes containing the media. From each sample, 10 ml of percolating water was inoculated in five test tubes, each containing 10 ml LSBd; 1 ml of water was inoculated in five test tubes, each containing 10 ml LSBs; 0.1 ml of water was inoculated in five tubes, each containing 10 ml LSBs, and five test tubes were not inoculated and used as controls. The cultures were incubated 48 h in the dark at 37°C. After incubation, the test tubes
where visible gas bubbles formed inside the Durham tubes, and thus the lactose was fermented by supposedly present coliform microorganisms were noted as positive and used latter for estimating the density of coliform microorganisms according to the statistical table of McCrady (McCrady, 1937).

The cultures in all positive tubes were subjected to a second test in order to certify whether the microorganisms responsible for the fermentation of lactose with gas production in the Durham tubes belonged to coliform bacteria. The medium used for the confirmation test (the eosin methylene blue medium) had a pH 7.0 and contained (g per 100 ml of water) peptone (1), lactose (1), K₂HPO₄ (0.2), soluble eosin (0.04), methylene blue (0.0064) and agar (2).

IMViC tests. The IMViC tests are biochemical assessments used for differentiating the coliform bacteria, members of the Enterobacteriaceae. This family includes many pathogen bacteria that normally form the gut flora of humans and animals, and have in the environment a fecaloid origin.

**Indole test.** The medium used to perform this test was the peptone water (pH 7.9) that contained 1.5 % NaCl and 6.0 % peptone. From each sample, 1 ml of water was inoculated in 10 ml of peptone water. After 24 h incubation in the dark at 37°C, 0.25 ml of Kovács reagent (indole reagent) was added to each test tube in order to test whether indole formed following tryptophan hydrolysis. Kovács reagent was prepared by dissolving 5 g of p-dimethylaminobenzaldehyde in 75 ml amyllic alcohol, followed by the addition of 25 ml of concentrated HCl.

**Methyl red reaction.** This test follows the capacity of certain microorganisms to metabolize glucose with stable acids production. The medium used for this test (Clark and Lubs medium) had a pH 7.5 and contained (per 1 l of water) 5 g peptone, 5 g glucose and 5 g K₂HPO₄. From each sample, 1 ml of water was inoculated in 10 ml of medium. After 24 h incubation in the dark at 37°C, 0.25 ml of methyl red reagent was added to each test tube. Methyl red reagent was prepared by dissolving 0.1 g methyl red in 300 ml ethylic alcohol followed by the addition of 200 ml of water. The methyl red reagent will color all cultures where acids formed (pH < 4.4) in red.

**Voges-Proskauer reaction.** Some microorganisms are capable of metabolizing glucose, but do not produce sufficient amount of stable acids needed to lower the pH under 4.4. For this type of microorganisms, the final product following glucose metabolism is the acetyl methylcarbinol (CH₃-CO-CHOH-CH₃). In presence of alkaline compounds and oxygen, the acetyl methylcarbinol oxidizes to diacetyl (CH₃-CO-CO-CH₃). The subsequent reaction of dyacetil with the residual peptone in the medium will lead to a fluorescent-pink color. The medium used to perform this test was, like for the methyl red reaction, the Clark and Lubs medium. From each sample, 1 ml of water was inoculated in 10 ml of medium. After 24 h incubation in the dark at 37°C, each test tube was amedned with 50 μl of 2 % solution of FeCl₃ in order to accelerate the reaction. 5 ml of 10 % KOH solution was then added to each test tube. After another incubation time of 1 h in the dark at 37°C, a pink or red color will indicate the presence of acetyl methylcarbinol.
Citrate test. The aim of this test was to determine whether the microorganisms present in percolation water are capable of using the citrate as unique carbon source, as members of Enterobacteriaceae are known to have this capacity. The Simmon’s medium (pH 6.8) used to perform this test contained (g to 1 l of water) NaCl (5), MgSO₄ (0.2), NH₄H₂PO₄ (1), K₂HPO₄ (1), sodium citrate (2.8) and agar (30). After dissolving these ingredients, the medium was colored with 4 ml solution of bromothymol blue (pH indicator). 0.1 ml from each sample was inoculated on Petri dishes containing Simmon’s medium, and the cultures were incubated 72 h in the dark at 30°C. If the citrate was used, alkaline (pH > 7.6) or acid (pH < 6.0) products will form, and the medium (initially green) will turn into blue or yellow, respectively.

Results and discussion

Physico-chemical measurements. The physico-chemical parameters of percolating water had comparable values in all three caves (Fig. 2). The groundwater temperature ranged between 6.9 and 7.1°C, the pH had values between 8.9 and 8.94, and electrical conductivity between 163 to 169 μS/cm.

Culture-based quantification of bacteria. The density of aerobic heterotrophic bacteria estimated in the percolation water in the three caves had comparable values to those obtained in other similar studies (Geric et al., 2004; Mulec et al., 2002). The largest density of aerobic heterotrophic bacteria (Fig. 3a) was registered in Peștera cu Apă din Valea Leșului (294 CFU/ml), while the lowest density of this type of microorganisms was obtained for Peștera de la Vadu Crișului (12 CFU/ml). 110 CFU/ml were obtained for Peștera Ungurului.

The density of iron reducing bacteria (Fig. 3b) was largest in Peștera cu Apă din Valea Leșului and Peștera Ungurului (360 cells/ml). Only 1 cell/ml was obtained for Peștera de la Vadu Crișului.

The number of ammonifying bacteria (Fig. 3c) was highest in Peștera cu Apă din Valea Leșului (1.1 × 10⁵ cells/ml). The density values of this type of microorganisms were 1.5 × 10⁴ cells/ml for Peștera Ungurului and 4.3 × 10³ cells/ml for Peștera de la Vadu Crișului. The density of denitrifiers (Fig. 3d) was largest in Peștera cu Apă din Valea Leșului (1.1 × 10⁴ cells/ml). The same number of denitrifying bacteria (2.9 × 10⁴ cells/ml) was obtained for both Peștera Ungurului and Peștera de la Vadu Crișului. The large number of microorganisms involved in the natural cycle of nitrogen can be related to the ammonification and denitrification processes occurring in the soils above the caves, which water washes percolating towards lower layers of karst.

Bacterial indicator of water quality was calculated on basis of the obtained density values of the various bacterial physiological groups. BIWQ (Fig. 4) suggested that the cleanest water is in Peștera de la Vadu Crișului, while the largest microbial charge was registered in Peștera cu Apă din Valea Leșului.
Coliform bacteria. For determining the presence and density of coliform microorganisms, a presumptive and a confirmation test were performed. After the presumptive test, which was based on gas formation in the Durham tubes due to lactose fermentation by supposedly present coliform bacteria, only two test tubes were recorded as positive. The cultures in these two test tubes were inoculated onto Petri dishes containing the eosin methylene blue medium. This confirmation test revealed no coliform-like growth, suggesting that the water dripping in the three caves is free of coliform bacteria. The IMViC tests (i.e. indole test, methyl red reaction, Voges-Proskauer reaction and citrate test) were all negative, suggesting that members of Enterobacteriaceae (coliform bacteria) are absent in the water dripping from the upper part of the unsaturated zone of karst in the three caves.
Fig. 3. Density (in cells/ml) of different bacterial physiological groups (a - aerobic heterotrophic bacteria; b - iron reducing bacteria; c - ammonifying bacteria; d - denitrifying bacteria) determined in percolation water in Peștera de la Vadu Crișului (Ω₁), Peștera Ungurului (Ω₂) and Peștera cu Apă din Valea Leșului (Ω₃).

These tests revealed that the water dripping in the three caves is clean; it is free of microorganisms with a fecaloid origin, organisms that are generally responsible for the microbiological pollution of groundwater or other environments. Measurements of the electrical conductivity indicated also that the percolation water is rather clean, as organically polluted groundwater has in general higher electrical conductivity values (Christensen et al., 2001).
**Groundwater fauna.** Groundwater fauna was collected at the same locations in each cave where sampling of water for microbiological analyses was performed. The abundance of groundwater fauna (mainly crustaceans of the *Copepoda*) was larger in locations where a larger density of microorganisms was obtained and, conversely, fewer microorganisms were detected in places with lower density of groundwater invertebrates (Fig. 5). In groundwater food webs, microorganisms and dead organic matter are the basis of all food chains, because light and photosynthesizing plants are absent in the subsurface. Due to the scarcity of resources in the subsurface, groundwater animals are in general omnivorous or detritivorous (Gibert et al., 2009) and microorganisms are good part of their diet. Groundwater animals seem thus to agglomerate more in places where more food (microorganisms) is present.

The water in caves, such as that dripping from the unsaturated zone of karst, feed the aquifers below and must be protected against both surface and subsurface human disturbances. Clean drinking water sources, such as the caves, are desired in present times, when pollution of the environment in general, and contamination of waters in particular, are obvious phenomena due to increased human population density, causing an increase in water demand, agricultural practices and landscape alteration, industrial activities, mining, electricity production, increase in urban area and demand for public drinking water, tourism and climate change (Danielopol et al., 2003).
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REFERENCES


