Subterranean Biology 56: 29–49 (2026)
doi: 10.3897/subtbiol.56.189049
https://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

Comparative study of testate amoebae biodiversity in surface and subterranean karst habitats – example from Dinaric karst (South-Eastern Europe)

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Academic editor: Rosaura Mayén-Estrada | Received 18 February 2026 | Accepted 7 April 2026 | Published 22 April 2026

https://zoobank.org/CE0D56AC-9D35-409A-BDE2-0F2D3B4918E6

Citation: Baković N, Baković R, Siemensma F, Pipan T (2026) Comparative study of testate amoebae biodiversity in surface and subterranean karst habitats – example from Dinaric karst (South-Eastern Europe). Subterranean Biology 56: 29–49. https://doi.org/10.3897/subtbiol.56.189049

Abstract

The Dinarides of Bosnia and Herzegovina are exceptionally rich in surface and subterranean geomorphological and hydrological karst features which make them an ideal setting for ecological studies of microorganisms. This work presents the results of a seven-year study of testate amoebae, polyphyletic eukaryotic microorganisms, across various karst habitats. The main objectives were to collect data on testate amoebae biodiversity and to deepen our understanding of the transformation of their assemblages across surface and subterranean habitats. More than 50 taxa of testate amoebae were identified, including the notable finding of *Psammonobiotus dinarica* – a species originally described from caves – in a pristine surface karst river. A Jaccard resemblance-based cluster analysis of overall testate amoebae biodiversity revealed a clear separation of lake assemblages from river, spring, soil and cave assemblages. These results were confirmed by site-based cluster analysis and NMDS ordination, which showed strong separation (SIMPROF test) of lake, soil and cave assemblages, but also distinct assemblages of hydrologically interconnected karst features (sinking river Šuica → sinkhole cave Ponor Kovači → the resurgence Vrilo Spring). This study confirms a high similarity between river, spring, cave, and soil assemblages but reveals a previously unknown distinction of lake assemblages. It also showed that spring assemblages have higher similarity to river assemblages than to those in caves, implying a possible impact of primary production due to diffuse light in springs during summer droughts. This study emphasizes the need for further research of microbial assemblages in relation to environmental factors in order to support science-based protection of both surface and subterranean karst ecosystems.

Keywords

Cave microorganisms, cave protists, cave protozoa, caves, karst hydrological systems, protist biogeography

Introduction

Testate amoebae are a polyphyletic group of single celled eukaryotic organisms (Protists) named after their main identification feature – test (shell) that encloses an amoeboid body (Todorov and Bankov 2019). Their highest diversity is recorded in organic matter rich and humid habitats like bogs, mires, peatlands but also soil (e.g. De Graaf 1957; Jauhiainen 2002; Mazei et al. 2017; Saldaev et al. 2023). Freshwater habitats, such as rivers and lakes, are less studied though they are also rich habitats for testate amoebae (Nicholls 2003; Mazei and Tsyganov 2006a, 2006b; Costa et al. 2016; Sysoev et al. 2024). Some species are adapted also to brackish habitats such as marine littoral interstitial habitats and anchialine caves (Golemansky and Todorov 2004; Hengstum et al. 2009), but also to karst freshwater habitats – caves (Golemansky and Bonnet 1994).

The extensive literature review on freshwater cave testate amoebae worldwide was conducted by Mazei et al. (2012) with some new data from Italy and Russia. These data can be supplemented by research from Russia (Chibisova 1967), Croatia (Baković et al. 2019, 2022, 2023a, 2023b; García-Bodelón et al. 2024), Hungary (Bereczky 1970), Spain (Soler-Zamora et al. 2021) and Guinea (Decloitre 1955). Data on cave testate amoebae in Bosnia and Herzegovina were mostly derived from the results of the International Expedition „Ponor Kovači-Izvor Ričine“ (Baković 2017; Baković et al. 2019, 2023b), research at the UNESCO site Vjetrenica Cave (Georgijevski et al. 1956; Baković 2021) and several other caves along the Bosnian-Herzegovinian karst poljes (Georgijevski et al. 1956). Some new species of testate amoebae from caves have been described from Dinaric karst and they have, to date, only been found in caves (Baković et al. 2019, 2023b). Although not connected to the caves, research performed by S. Luketa on species from epigenous mosses and *Sphagnum* mosses (Luketa 2016, 2017a, 2017b, 2021) provided valuable morphometric data on several insufficiently known and rare taxa in Bosnia and Herzegovina.

The literature on comparative studies of subterranean and surface karst habitats usually relies on general comparison of known surface assemblages with those in caves and does not provide detailed analysis (Golemansky and Bonnet 1994) or includes in comparison only soil habitats and moss habitats (Mazei et al. 2012). One notable study was conducted by Georgijevski et al. (1956), which investigated aquatic fauna and, to a lesser extent, protists at 39 karst localities across Bosnia and Herzegovina, Croatia, and Montenegro. Although the document provides partial data on the biodiversity of testate amoebae, results were obtained using artificial growth media, making them difficult to compare with other findings. Molecular study of Arcellinid testate amoebae by García-Bodelón et al. (2024), performed on two cave habitats and rivers entering and exiting these caves, shows that their communities are more diverse outside the caves, but also that cave habitats with guano host unique communities that significantly differ from all other communities in the same study.

Main objectives of this research are: a) to contribute new data on testate amoebae diversity from the Dinaric karst area of Bosnia and Herzegovina; b) to compare surface and subterranean assemblages of testate amoebae in karst landscapes; c) to fill the gap regarding the transformation of testate amoebae assemblages along karst system consisting of: karst spring → karst river → sinkhole cave → karst resurgence spring → artificial lake.

Study location

The study area belongs to the Outer Dinarides, underlain predominantly by Jurassic and Cretaceous limestones and dolomites (Ozimec and Radoš 2013) at elevations ranging from 716 to 1414 m a.s.l. Administratively, it is located within Hercegbosanska and Zapadnohercegovačka counties in Bosnia and Herzegovina. The study site encompasses multiple springs, caves, the Šuica River and its sinkhole, two lakes, as well as soil sampling locations (Figs 1, 2).

The Šuica River (ŠUI-R) (total length: 48.5 km) emerges from the Veliki Stražanj spring-cave (STR-C) and the Mali Stražanj spring on an elevated plateau between the karst fields Kupreško Polje and Šuičko Polje. Water from Veliki Stražanj flows into a natural amphitheatre-shaped pool named Badanj (ŠUI-SP), from which the surface stream develops. During dry periods, the upper course of the Šuica River dries out, and surface water appears only downstream of the Volarac Spring. The river flows through the Šuičko Polje, partly within canyons, and then enters the Duvanjsko Polje karst field. Duvanjsko Polje covers an area of 112 km^2 and is subject to periodic flooding (Radoš et al. 2012; Ozimec and Radoš 2013). The Šuica River sinks into the sinkhole-type cave Ponor Kovači (KOV-C), after which it continues underground towards the Vrilo Spring (VRI-SP) (syn. Ričina Spring), located at the southeastern margin of the Livanjsko Polje karst field. The hydrological connection between Ponor Kovači and the Vrilo Spring was confirmed by dye tracing (at an aerial distance of 5150 m) (Božičević 1984). To date, 2 km of Ponor Kovači and 2.3 km of the Vrilo Spring have been mapped (Speleological Society Mijatovi Dvori 2025). The Vrilo Spring is a complex spring system with four entrances that are hydrologically active only during part of the year. During active periods, the spring forms a short surface stream Ričina which flows into the artificial Buško Blato Lake (BUŠ-L). At the time of investigation, discharge was not noticeable, and the riverbed contained standing water. Buško Blato Lake (surface area of 55.92 km^2 and a maximum depth of 17 m) is an artificial reservoir located in the southeastern part of Livanjsko Polje (which has a total area of 402 km^2) (Ozimec and Radoš 2013).

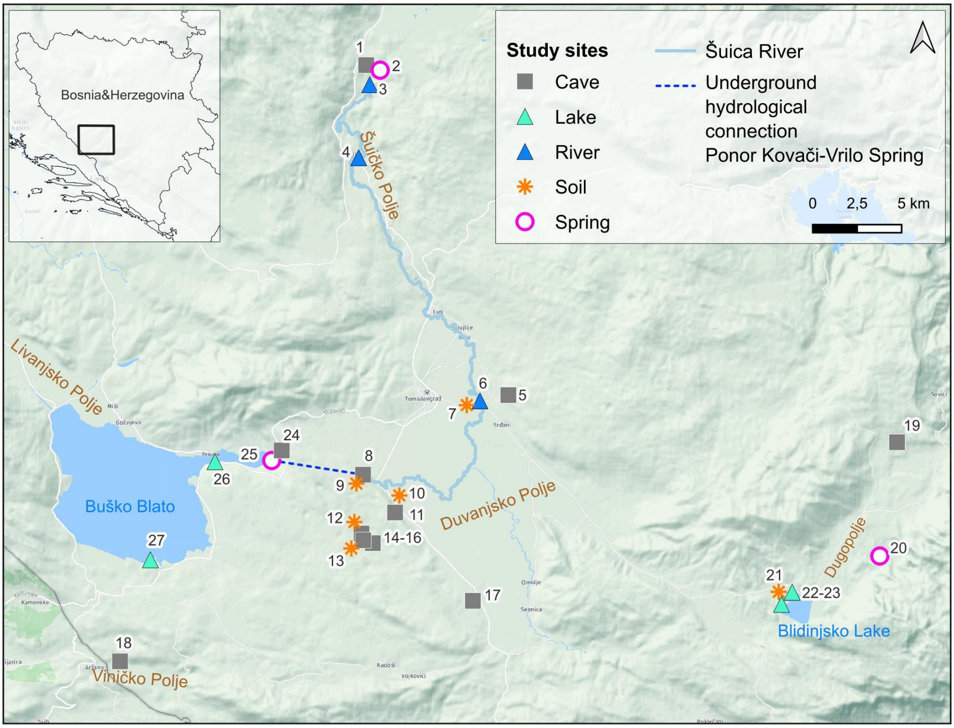

Figure 1. Study location: Caves: 1 – Veliki Stražanj; 5 – Krstovnjača; 8 – Ponor Kovači; 17 – Velika Bukovačka; 18 – Bilobrkova Cave; 19 – Bošnjakuša; 15–17 – Vranjevača, Listvača, and Lisičja Cave; 24 – Dobra. Springs: 2 – Šuica Spring at Badanj; 20 – Jasle Spring; 25 – Vrilo Spring. Lakes: 26–27 – Buško Blato Lake; 22–23 – Blidinje Lake. River Šuica: 3 – upper course; 4 – in Šuičko Polje; 6 – in Duvanjsko Polje. Soil: 7 & 10 – along Duvanjsko Polje; 9 – above Ponor Kovači; 12 – above Vranjevača Cave; 13 – above Listvača Cave; 21 – near Blidinje Lake.

Blidinje Lake (BLI-L) (lake surface between 2.5 and 6 km^2 with an average depth of about 1 m) is situated in the Dugopolje karst field, formed by artificial sealing of sinkholes along its eastern margin (Radoš 2017). Nearby lies the Jasle Spring (JAS-SP), which emerges from a semi-cave and flows into a small, intermittent wetland lake.

The investigated caves and pit caves include: Dahna (DAH-C) (hills above Duvanjsko Polje), Lisičija špilja 1 (LIS-C), Listvača (LTV-C), and Vranjevača (VRA-C) (all three on the Grabovica Mt. plateau), Krstovnjača (KRS-C) (Ljubuša Mt.), and Bošnjakuša (BOŠ-C) (Vran Mt.). These are small speleological sites (length < 100 m) of simple morphology, except for Dahna Cave (length 1190 m), which exhibits somewhat more complex morphology. These caves are hydrologically inactive, although some contain small water pools or lakes. Periodically active caves investigated include Velika Bukovačka (BUK-C) (at the edge of Duvanjsko Polje) and Bilobrkova Cave (BIL-C) (at the edge of Viničko Polje), which are also small (length < 100 m) and simple, while Veliki Stražanj (STR-C) (on the elevated plateau between the Kupreško Polje and Šuičko Polje) is somewhat larger (229 m) (Speleological Society Mijatovi Dvori 2025). Only aphotic habitats outside the hydrologically active period were investigated.

Figure 2. Study sites: **A** Duvanjsko Polje and meanders of the Šuica River during the high water period **B** Listvača Cave **C** Šuica River sinking into Ponor Kovači during the high water period **D** Ponor Kovači during the dry period **E** Vrilo Spring during the high water period **F** Vrilo Spring during the dry period. Photos: **A, C, E** by Domagoj Madunić; **B, F** by Damir Basara; **D** by Robert Baković.

Soil samples were collected at the Grabovica Mt. plateau (VRA-S, LTV-S), in Duvanjsko Polje (DUV-S), on a hill near Ponor Kovači (KOV-S), and on a hill near Blidinje Lake (BLI-S).

Methodology

A total of 107 samples were collected at the end of August or the beginning of September over 7 years (2013, 2014, 2018, 2019, 2021, 2023, 2024) as part of the International Caving Expedition „Ponor Kovači-Izvor Ričine“ (Bosnia and Herzegovina). Part of the results of this study (data from 26 samples) were published in Baković (2017) and Baković et al. (2023b, 2019). Samples of aquatic sediments were taken from ten caves (aphotic habitats), three springs (diffuse-light habitats), two lakes (surface-photic habitats), one sinkhole (aphotic habitat) and soil at five locations. Number of samples per site is shown in Table 1.

Table 1. List of species per site* and number of samples per site.

Short abbreviation	**BIL-C**	**DAH-C**	**DOB-C**	**LIS-C**	**LTV-C**	**KOV-C**	**STR-C**	**BUK-C**	**VRA-C**	**BOŠ-C**	**KRS-C**	**VRI-SP**	**ŠUI-SP**	**JAS-SP**	**BLI-L**	**BUŠ-L**	**ŠUI-R**	**VRA-S**	**LTV-S**	**BLI-S**	**KOV-S**	**DUV-S**
Site name	**Bilobrkova Cave**	**Dahna**	**Dobra**	**Lisičja Cave 1**	**Listvača**	**Ponor Kovači**	**Veliki Stržanj**	**Velika Bukovačka**	**Vranjevača**	**Bošnjakuša**	**Krstovnjača**	**Vrilo Spring**	**Šuica Spring (Badanj)**	**Jaslo Spring**	**Blidinje Lake**	**Buško Blato Lake**	**Šuica River**	**Soil above the Vranjevača Cave**	**Soil above the Listvača Cave**	**Soil near the Blidinje Lake**	**Soil above the Ponor Kovači**	**Soil along Duvanjsko Polje**
Arcella crenulata Deflandre, 1928																	▲					
Arcella hemisphaerica Perty, 1852																▲						
Arcella vulgaris Ehrenberg, 1830																▲						
Arcella spp.							■										▲					✱
Assulina muscorum Greeff, 1888				■																		
Centropyxis aculeata Ehrenberg, 1832												○		○	▲	▲	▲					
Centropyxis aerophila Deflandre, 1929	■			■	■	■	■	■	■	■		○		○			▲	✱	✱	✱	✱	✱
Centropyxis bipilata Bakovićet al., 2019	■			■		■			■	■												
Centropyxis cassis Wallich, 1864												○					▲					
Centropyxis constricta Ehrenberg, 1841														○	▲							
Centropyxis elongata Penard, 1890						■						○						✱			✱	
Centropyxis plagiostoma Bonnet & Thomas, 1955						■		■	■			○						✱	✱		✱	✱
Centropyxis spp.		■		■								○						✱		✱		
Cryptodifflugia oviformis Penard, 1902	■	■			■	■	■	■		■		○					▲				✱	✱
Cryptodifflugia cf. *sacculus* Penard, 1902	■																					
Cyclopyxis eurystoma Deflandre, 1929						■			■	■							▲	✱	✱		✱	✱
Cyclopyxis kahli Deflandre, 1929												○						✱	✱		✱	
Cyclopyxis spp.	■	■		■		■	■										▲		✱			
Cylindrifflugia elegans Penard, 1890												○				▲	▲					
Cyphoderia ampulla Ehrenberg, 1840		■			■	■		■				○		○		▲	▲					
Corythion dubium Taranek, 1871																				✱		

Short abbreviation	**BIL-C**	**DAH-C**	**DOB-C**	**LIS-C**	**LTV-C**	**KOV-C**	**STR-C**	**BUK-C**	**VRA-C**	**BOŠ-C**	**KRS-C**	**VRI-SP**	**ŠUI-SP**	**JAS-SP**	**BLI-L**	**BUŠ-L**	**ŠUI-R**	**VRA-S**	**LTV-S**	**BLI-S**	**KOV-S**	**DUV-S**
Site name	**Bilobrkova Cave**	**Dahna**	**Dobra**	**Lisičja Cave 1**	**Listvača**	**Ponor Kovači**	**Veliki Stržanj**	**Velika Bukovačka**	**Vranjevača**	**Bošnjakuša**	**Krstovnjača**	**Vrilo Spring**	**Šuica Spring (Badanj)**	**Jaslo Spring**	**Blidinje Lake**	**Buško Blato Lake**	**Šuica River**	**Soil above the Vranjevača Cave**	**Soil above the Listvača Cave**	**Soil near the Blidinje Lake**	**Soil above the Ponor Kovači**	**Soil along Duvanjsko Polje**
Difflugia cf. *brevicolla* Cash & Hopkinson, 1909						■																
Difflugia oblonga Ehrenberg, 1831						■						○					▲					
Difflugia pyriformis Perty, 1849						■																
Difflugia pristis Penard, 1902	■							■	■													
Difflugia spp.	■	■				■		■		■		○		○	▲	▲	▲				✱	
Diplochlamys sp.						■																
Euglypha bryophila Brown, 1911						■		■														
Euglypha filifera Penard, 1890																▲						
Euglypha cf. *laevis* Ehrenberg, 1845		■																	✱			
Euglypha cf. *rotunda* Wailes & Penard, 1911		■				■		■		■		○		○			▲	✱	✱		✱	✱
Euglypha tuberculata Dujardin, 1841	■									■												
Euglypha spp.																		✱	✱			
Frenopyxis laevigata Penard, 1890												○					▲					
Galeripora artocrea Leidy, 1876																					✱	
Hyalosphenia insecta Harnisch, 1938																					✱	
Heleopera petricola Leidy, 1879						■			■			○									✱	
Heleopera sp.																				✱		
Microchlamys patella Claparède & Lachmann, 1859	■				■	■	■	■			■	○		○		▲	▲			✱	✱	
Netzelia corona Wallich, 1864												○										
Netzelia gramen Penard, 1902												○				▲						
Netzelia lithophila Penard, 1902						■																
Paramphitrema sp.		■										○										
Paraquadrula irregularis Wallich, 1863									■	■				○		▲					✱	
Phryganella acropodia Hertwig & Lesser, 1874																▲						

Short abbreviation	**BIL-C**	**DAH-C**	**DOB-C**	**LIS-C**	**LTV-C**	**KOV-C**	**STR-C**	**BUK-C**	**VRA-C**	**BOŠ-C**	**KRS-C**	**VRI-SP**	**ŠUI-SP**	**JAS-SP**	**BLI-L**	**BUŠ-L**	**ŠUI-R**	**VRA-S**	**LTV-S**	**BLI-S**	**KOV-S**	**DUV-S**
Site name	**Bilobrkova Cave**	**Dahna**	**Dobra**	**Lisičja Cave 1**	**Listvača**	**Ponor Kovači**	**Veliki Stržanj**	**Velika Bukovačka**	**Vranjevača**	**Bošnjakuša**	**Krstovnjača**	**Vrilo Spring**	**Šuica Spring (Badanj)**	**Jaslo Spring**	**Blidinje Lake**	**Buško Blato Lake**	**Šuica River**	**Soil above the Vranjevača Cave**	**Soil above the Listvača Cave**	**Soil near the Blidinje Lake**	**Soil above the Ponor Kovači**	**Soil along Duvanjsko Polje**
Plagiopyxis declivis Bonnet, 1955						■	■			■		○						✱	✱	✱	✱	✱
Psammonobiotus dinarica Baković & Siemensma, 2023 (=cf. *Conicocassis* sp.)	■			■	■	■		■									▲					
Pseudodifflugia sp.															▲							
Pseudawerintzewia sp.																						✱
Sphenoderia lenta Schlumberger, 1845												○										
Tracheleuglypha dentata Vejdovsky, 1882		■																			✱	
Tracheleuglypha acolla Bonnet & Thomas, 1955								■		■												
Trinema complanatum Penard, 1890																		✱	✱	✱	✱	✱
Trinema enchelys Ehrenberg, 1838						■						○						✱	✱		✱	✱
Trinema lineare Penard, 1890	■	■	■			■	■	■	■	■		○					▲	✱	✱	✱	✱	✱
Unidentified testate amoebae		■		■	■	■			■	■						▲				✱		
Total number of species	**11**	**11**	**1**	**7**	**6**	**23**	**7**	**12**	**9**	**12**	**1**	**23**	**0**	**8**	**4**	**12**	**17**	**12**	**12**	**9**	**18**	**11**
Number of samples per site	**5**	**14**	**1**	**5**	**5**	**9**	**2**	**5**	**5**	**2**	**1**	**11**	**2**	**2**	**4**	**4**	**10**	**4**	**4**	**4**	**4**	**4**

Legend: ■ - cave (aphotic habitat); ○ - Spring (habitat with diffuse light); ▲ - surface lake (habitat with permanent daylight); ▲ - surface river (habitat with permanent daylight); ✱ - soil (habitat with light present only in surface layer of soil, and aphotic in deeper layers).

*This annex includes check list published in Baković (2017), Baković et al. (2023b, 2019) (from 26 samples) and new (unpublished) data (from 81 sample).

Diverse microhabitats were selected for sampling at each site to allow for a more comprehensive assessment of biodiversity. Samples from caves and springs were collected and examined in accordance with the methodology presented in Baković et al. (2019). Samples from lakes and streams were collected by scooping the surface layer of aquatic sediment with a plastic bottle and were refrigerated upon arrival at the laboratory. All samples from caves, springs, and surface aquatic habitats were examined within 48 hours of sampling. Soil samples were collected in plastic bags and soaked with water for 12–24 hours at room temperature prior to examination. Triplicates of 0.2 ml of sample containing both water and sediment were examined. All observed species of testate amoebae were recorded.

Data on the presence/absence of testate amoebae identified to the species and genus level were used in the statistical analyses. Species occurrence across different habitats was analysed using an UpSet plot, with the R packages “UpSetR”, “dplyr”, and the “upset” function in RStudio version 2025.05.1 Build 513 (Posit Software, PBC).

Similarity among individual habitats (cave, river, lake, spring) was analyzed using hierarchical cluster analysis based on the Jaccard resemblance (Jaccard 1901). Clusters were grouped using the average linkage method.

Similarity among sites was analyzed using hierarchical cluster analysis and non-metric multi-dimensional scaling (NMDS) (Kruskal 1964) based on the Bray-Curtis similarity index (Bray and Curtis 1957). Only sites with more than three samples (seven caves, one spring, two lakes, one stream, and five soil sites) were used in cluster and NMDS analyses to increase the statistical power. Clusters were grouped using the average linkage method. The statistical significance of clusters was tested using the 5% similarity profile routine (SIMPROF test) (Clarke et al. 2008). NMDS results were presented as two-dimensional plot with marked stress value based on Kruskal Stress Formula I and minimum stress of 0.01. The stress value was assessed according to the scale of fit proposed by Clarke (1993). Trajectory on the NMDS plot is indicating only the direction of water flow and does not represent a statistical test.

Analyses of Jaccard resemblance, Bray-Curtis similarity, cluster analyses and NMDS were performed in PRIMER 6 (PRIMER-E Ltd.).

Results

A survey of surface and subterranean habitats revealed 45 taxa of testate amoebae identified to the species level, along with more than 10 additional taxa identified at the genus level (Table 1, Figs 3, 4). Several individuals could not be confidently assigned even to a genus because detritus in the apertures obscured their structure—often an important identification characteristic—or because other key features were also hidden by attached, non-removable detritus. Certain individuals had distinct and easily observable morphology, yet their diagnostic features did not align with any currently known species, indicating that they may represent potential new species. Among the recorded species were two originally described from cave environments – *Centropyxis bipilata* and *Psammonobiotus dinarica* (Baković et al. 2019, 2023b). *Centropyxis bipilata* was found exclusively in caves in this study (Figs 3C, 4B), whereas live individuals of *P. dinarica* (Fig. 4D) were recorded in the upper course of the Šuica River (a surface habitat), approximately 200 m downstream of the spring.

This study recorded the highest overall biodiversity of testate amoebae in cave habitats, with 33 identified species. High species richness was also observed in soil (26 species) and spring habitats (23 species) (Fig. 5). The greatest number of unique species was recorded in caves (11 species), followed by soil (7 species) and lakes (6 species). A notable similarity in species composition was observed among soil, spring, and cave habitats, which shared a total of six species.

The species count per site did not necessarily correlate with the total number of samples (Table 1). For example, 11 species were recorded in 14 samples from

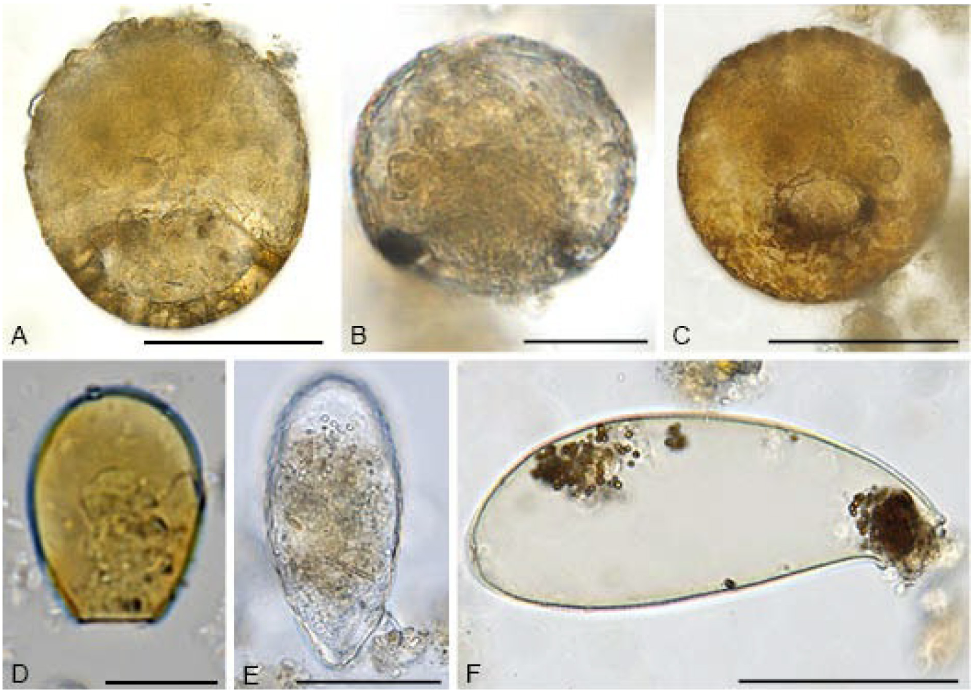

Figure 3. Testate amoebae found in this study in cave habitats: **A** *Centropyxis aerophila* **B** *Cyclopyxis eurystoma* **C** *Centropyxis bipilata* **D** *Cryptodifflugia oviformis* **E** *Trinema enchelys* **F** *Cyphoderia ampulla*. All specimens shown are from live preparations. Scale bars: 50 µm (**A, C, E, F**); 25 µm (**B**); 10 µm (**D**).

the Dahna Cave, whereas 12 species were recorded from only two samples at Bošnjakuša Cave. The highest site-specific biodiversity of testate amoebae was observed at Ponor Kovači and Vrilo Spring (23 species each), soil above Ponor Kovači (18 species) and Šuica River (17 species).

Cluster analysis using Jaccard similarity (Fig. 6) resulted in a clear separation of lake assemblages (14.46) from a second cluster (32.69), which further divided into two subclusters. The first subcluster contained cave and soil assemblages (similarity = 43.90), while the second contained spring and river assemblages (42.86).

Given that the studied caves are under variable influences of surface waters (e.g., the Šuica River sinks into the Ponor Kovači sinkhole, some caves are hydrologically inactive, while others occasionally discharge water, lakes differ in their hydrological input, and soils vary in moisture levels), a more detailed site-level analysis was conducted to better elucidate the relationships among habitats. Cluster analysis of individual sites based on Bray-Curtis similarity (Fig. 7) revealed four statistically significant clusters (SIMPROF test, 5% significance level). Clusters A (26.67), B (36.74), and D (62.91) showed a clear grouping by habitat type: lakes, caves, and soil, respectively. Cluster C (57.09) grouped the sinkhole-type cave Ponor Kovači (KOV-C), Vrilo Spring (VRI-S), and the Šuica River (ŠUI-R). The similarity between the Vrilo Spring and Šuica River assemblages (60.00) was slightly higher than their similarity

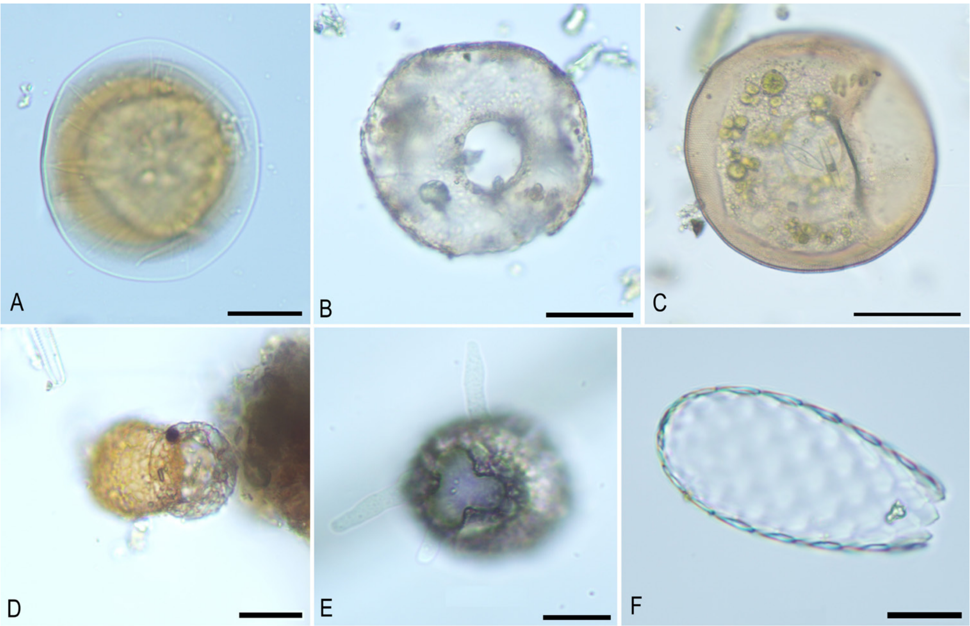

Figure 4. Testate amoebae found in this study in surface aquatic habitats: **A** *Microchlamys patella* **C** *Arcella* sp. **D** *Psammonobiotus dinarica* **E** *Netzelia gramen* with visible pseudopodia. Testate amoebae found in this study in caves: **B** *Centropyxis bipilata* **F** *Euglypha tuberculata*. All specimens shown are from live preparations. Scale bars: 20 μm (**A, D, E, F**); 30 μm (**B**); 50 μm (**C**).

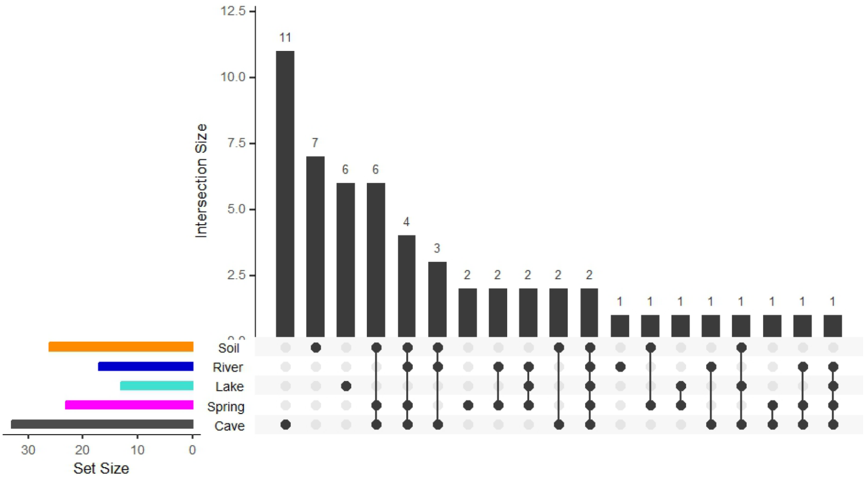

Figure 5. Biodiversity of testate amoebae per habitat (set size – total number of species per habitat; intersection size – number of unique species per habitat/habitats).

to Ponor Kovači. This relationship was further confirmed by NMDS analysis (2D stress = 0.13) (Fig. 8). A trajectory line was used to illustrate the flow direction of the Šuica River (surface-subsurface-spring-lake).

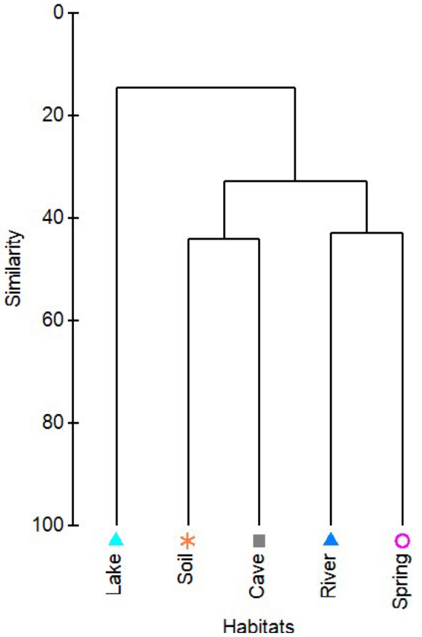

Figure 6. Cluster analysis of the overall biodiversity of testate amoebae across different habitats, based on Jaccard similarity.

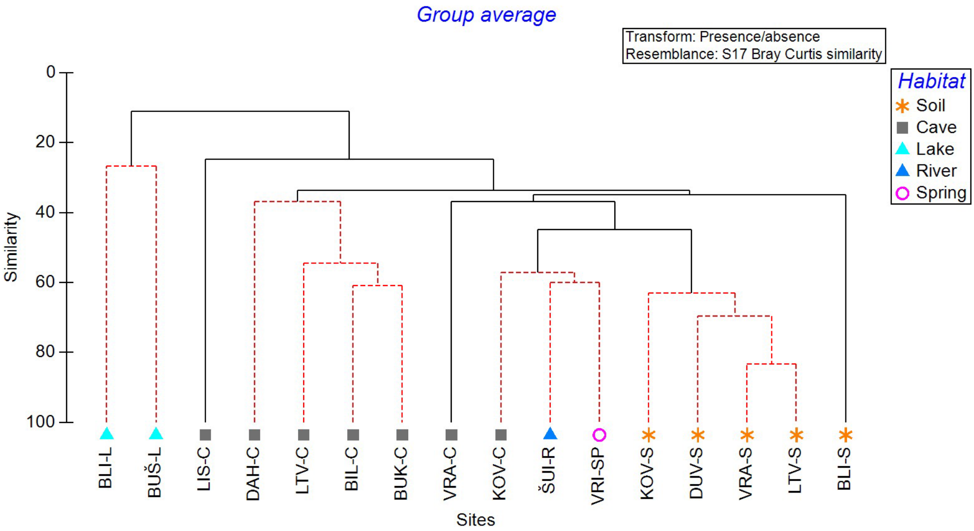

Figure 7. Cluster analysis of individual sites with 5% SIMPROF test (red branches) based on Bray-Curtis similarity.

Additionally, a notable cluster includes the sites BIL-C and BUK-C (Fig. 7), both of which are caves with regular water discharge outside the dry season.

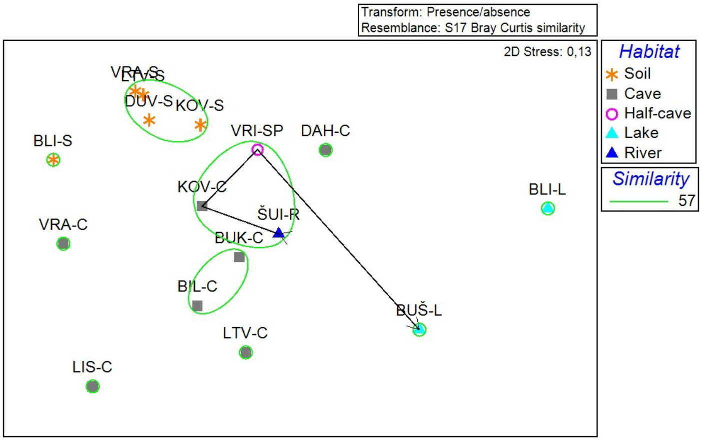

Figure 8. 2D projection of non-metric multi-dimensional scaling (NMDS) based on sites (trajectory line showing travel direction of waters of Šuica River from surface through sinkhole cave, spring and then inflow into Buško Blato Lake).

Discussion

The biodiversity of testate amoebae detected in this study, despite the relatively small sample volumes examined and the exclusive focus on the summer season, indicates a notable diversity within the investigated habitats. The success of detecting testate amoebae and assessing their diversity depends largely on the applied methodology. The approach used in this study enables direct comparison among the investigated habitats. However, the limited amount of processed material strongly suggests that the total biodiversity of testate amoebae is greater than documented in this study (Table 1). Methods relying on the processing of large sample volumes to achieve more efficient isolation of individuals from sediments are applicable for surface habitats but cannot be applied to caves, which are often extremely poor in suitable sediment.

Although the overall biodiversity of testate amoebae recorded from caves is higher than in other habitats (Fig. 5), this probably results from the greater number of samples collected in caves. In summer, drought-induced desiccation can drastically reduce habitat availability in caves, in some cases eliminating suitable conditions for testate amoebae altogether. Differences in biodiversity among caves can be primarily attributed to habitat heterogeneity – caves with a higher diversity of microhabitats (e.g. Ponor Kovači) are more likely to support diverse testate amoebae assemblages than caves that have low habitat diversity (e.g. caves Lisičja špilja 1 and Dahna).

Most of the species identified in caves correspond to taxa originally described from surface habitats, consistent with reports that caves are primarily colonized by surface-derived species (Golemansky and Bonnet 1994). An exception is *Centropyxis bipilata*, described from caves (Baković et al. 2019) and not detected in any surface habitats in this study. This supports the hypothesis that *C. bipilata* represents a cave-adapted species (troglobiont), a status so far primarily attributed to cave metazoans

(Culver and Pipan 2019). The testate amoeba *Psammonobiotus dinarica*, also originally described from caves (Baković et al. 2023b), was likewise recorded in this study (Table 1) in several caves and in the upper course of the Šuica River which represents the first reliable record of this species outside of a cave environment. Its absence from the middle and lower sections of the Šuica River, which are impacted by municipal wastewaters and agrochemicals (Ozimec and Radoš 2013) and are characterized by higher water temperatures and conductivity (Table 2), suggests potential sensitivity to these environmental stressors. Many testate amoebae are recognized as valuable bioindicators (e.g. Freitas et al. 2022; Evans et al. 2025a, 2025b), and further research should address whether *P. dinarica* could be regarded as an indicator species of good ecological conditions in karst waters.

A particularly significant outcome of this research is the repeated detection of testate amoebae, across nearly all studied habitats, that could not be assigned to any known species despite well visible and distinct morphological features. These taxa may represent new species for science, but for their formal description more data must be collected (Table 1). Karst environments of the Dinarides remain insufficiently explored, as reflected in the recent description of several new protist species from this region (Primc-Habdija and Matoničkin 2005; Udovič et al. 2018, 2023; Baković et al. 2019, 2023b, 2025; Siemensma and Holzmann 2023; Gligora Udovič et al. 2025). Further research is therefore essential, particularly in caves, which represent highly promising habitats due to their considerable evolutionary potential for speciation that is driven by strong natural selection (Trontelj 2019).

Table 2. Physio-chemical parameters in studied sites.

Short abreviation	Site name	Date	Water temperature (°C)	Water conductivity (µS/cm)	Air temperature (°C)	Relative air humidity (%)	Air CO_2 (ppm)	Air O_2 (%)
BIL-C	Bilobrkova Cave	8/2024	12.0–14.3	350-1082	16.1	88.3	1134	20.5
LIS-C	Lisičja špilja 1	8/2024	na	na	14.6	68.3	795	20.4
KOV-C	Ponor Kovači	8/2023	9.8	355	na	na	2020	na
BUK-C	Velika Bukovačka	8/2024	10.2	237	10.4	91.9	4472	20.0
VRA-C	Vranjevača	8/2024	na	na	11.0	85.1	1784	20.3
VRI-SP	Vrilo Spring	8/2023	17.2–26.6	285–335	na	na	na	na
JAS-SP	Jaslo Spring	8/2023	7.4	250	na	na	na	na
BLI-L	Blidinje Lake	8/2023	25.7	119	na	na	na	na
BUŠ-L	Buško Blato Lake	8/2023	27.7–32.1	180–299	na	na	na	na
ŠUI-R	Šuica River	8/2023	10.7 (upper course) 18.2 (Šuičko Polje) 23.3 (Duvanjsko Polje)	235 (upper course) 371 (Šuičko Polje) 482 (Duvanjsko Polje)	na	na	na	na

na – data not available.

The result of clustering based on overall testate amoebae diversity across habitats (Fig. 6) represents a rare comparison of cave and lake assemblages. Despite the literature data implying that cave assemblages are composed of aquatic and soil species (e.g. Gittleson and Hoover 1970; Golemansky and Bonnet 1994), our results indicate that this does not include lake assemblages, at least in this study. This conclusion was statistically confirmed by the separation of lake assemblages (SIMPROF test, 5% significance level) into a separate cluster (Fig. 7) and also supported by NMDS analysis (Fig. 8). On the other hand, our results confirm high similarity between cave assemblages and assemblages of soil and river habitats, as well as spring habitats, which have not yet been comparatively studied in relation to caves. The similarity between caves and soil and river assemblages is in accordance with already mentioned published literature.

A more detailed comparison of testate amoebae assemblages reviled more subtle differences between caves – most importantly the separation of Ponor Kovači (which was the only sinkhole-type cave in this study) from other studied caves. The most notable finding of this study is the statistically significant linkage (SIMPROF test, 5% significance level) of the system Šuica River → Ponor Kovači → Vrilo Spring based on testate amoebae assemblages (Fig. 7). These three localities are hydrologically connected as described by Božičević (1984): Šuica River sinks into Ponor Kovači and reappears after approximately 5.2 km at Vrilo Spring (resurgence spring). It seems that these hydrological conditions also play role in testate amoebae assemblages.

At the Ponor Kovači site, the studied habitat (a small lateral channel) was entirely aphotic and had remained hydrologically disconnected from the Šuica River for several months before sampling. Consequently, the community observed there underwent a habitat transformation: gradual disappearance of imported phototrophs passively introduced during flood events, the utilization of dissolved and particulate organic carbon, and a transition toward oligotrophic conditions (Culver and Pipan 2019). Hence, the data from Ponor Kovači well approximate the processes occurring in deeper sections of this sinkhole type cave. This is a main difference between Ponor Kovači and all other caves in this study. Distinct testate amoebae assemblages in the aphotic zones of sinkhole-type caves during dry periods, compared to hydrologically inactive caves, have also been reported from another study in Croatia (Baković et al. 2023b). Transformation of testate amoebae assemblages along hydrological gradients (sinking river → cave-river → river springing from the cave) was also confirmed using molecular methods (García-Bodelón et al. 2024).

An unexpected result was Vrilo Spring, that showed greater similarity to Šuica River than to Ponor Kovači. This spring is actually the entrance to a 2.3 km long cave system and habitats encompassed by this study contained water that came from deep cave channels during hydrological activity (Ozimec and Radoš 2013). The main difference between Vrilo Spring and Ponor Kovači is that studied habitats were under diffuse daylight. It was expected that the testate amoebae assemblages from Vrilo Spring would show higher resemblance to those from Ponor Kovači, but it was not the case. These data clearly demonstrate that light conditions (photic → aphotic → diffuse light → photic) could represent one important ecological factor influencing testate amoebae assemblages. Fully illuminated (river, lake) and habitats with diffuse light (spring) enable the development of phototrophic organisms such as algae, cyanobacteria and plants, which contribute to the overall availability of organic matter. Not only do microphototrophs represent food for some predatory testate amoebae (e.g. Arcellinida), but the entire ecosystem is affected through the presence of organic matter. The quantity of organic matter varies both across surface habitats (e.g. Bretschko 1990; Zhang et al. 2025) and subterranean habitats (Simon et al. 2010; Simon 2013). Caves are commonly perceived as extremely energy-poor habitats, but this is not the case for all caves. Bat guano, that is very common in caves included in this study, has a high proportion of organic matter (Sridhar et al. 2006; Reis et al. 2023). Studies from several karst hydrological systems demonstrated that surface water-groundwater interaction has a strong impact on the quantity of organic matter, but also emphasized the contribution of subsurface sources of available carbon (Simon et al. 2010; Barry-Sosa et al. 2024).

Regarding hydrological activity, it is also worth mentioning two geographically distant caves – Bilobrkova Cave (on the border of Viničko polje) and Velika Bukovačka (on the border of Duvanjsko polje) (Fig. 1). Both investigated sites were exclusively aphotic habitats during prolonged dry periods (i.e., they were hydrologically inactive). Nevertheless, they formed a distinct subcluster with a relatively high similarity (Fig. 7) and were positioned close to the Šuica → Ponor Kovači → Vrilo Spring hydrological system in the NMDS ordination (Fig. 8), suggesting a certain resemblance. Although these two sites lack concentrated surface inflows, the results indicate that the lotic character during hydrologically active periods creates a specific pattern of testate amoebae assemblages.

Conclusion

Our research contributed to the comparative knowledge of testate amoebae in different karst habitats with respect to general hydrology and light availability. The input for this study consisted of a large number of data (>100 samples) collected over seven years, but for a better understanding of the role of testate amoebae in karst ecosystems, it is essential to include monitoring of abundance and more detailed environmental parameters to perform parametric analyses that would further shed light on the role of light, lotic character and other possible abiotic factors such as substrate structure and microhabitat characteristics. The advancement of knowledge of microbial communities, on which every ecosystem rests, also imposes the exciting task of better investigating trophic interactions in caves. This will help us better understand karst ecosystems and support future protection of caves and surface karst habitats in a holistic and scientifically based manner.

Acknowledgement

This research was done with the permissions of the Federal Ministry of Environment and Tourism of the Federation of Bosnia and Herzegovina (No.: 04-23-7-623/13 ZM, 17.05.2013; No: 04-23-1082/14 ZM, 26.08.2014; No.: 04-23-1057/17, 14.12.2017, No. 04-23-714/18, 23.8.2018, 04-19-366/21-22, Sarajevo, 29.6.2021, No:04-23-19-2-300/22-1, 9.5.2022 and No: 04-23-19-2-300/22-1, 9.5.2022).

The authors would like to thank: members of the Speleological Society Mijatovi Dvori (Tomislavgrad, BiH) and other societies that participated in the organisation of the expeditions, all the supporters of the expedition, the Krišto family from Prisoje and the Orlova Stina Mountaineering Society (Tomislavgrad) for providing us with a house and mountaineering lodge for laboratory work and residence, Korana Baković, Ana and Anđa Radoš, Josip Marković, Miro Šumanović, Dr. Sc. Stipan Dilber, Mirko Šarac, Domagoj Madunić, Prof. Dr. Sc. Lada Lukić Bilela, mr.sc. Roman Ozimec, Damir Basara, Jana Valentič, Dr. Sc. Rajko Slapnik, Željka Baković, Lidija Basara, and other expedition and art colony participants at the camp. NB would like to thank to prof. dr. sc. Biserka Primc and prof. dr. sc. Renata Matoničkin Kepčija for their support of her research in 2013. TP received funding from the following EU projects: eLTER ERIC, LifeWatch ERIC, and Implementation of the international infrastructure project LifeWatch + eLTER (I0-E016).

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