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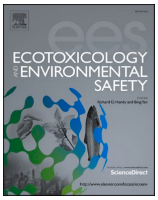

Microbiome analysis of groundwaters in the Slovenian Dinaric Karst, a recognized biodiversity hotspot

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ABSTRACT

Groundwater is the most important source of drinking water supply, irrigation, and industrial use, as well as a habitat for a diverse range of (micro)organisms. Some of the first studies of underground environments were carried out in the Dinaric Karst region of Slovenia, which harbors one of the highest subterranean biodiversities in the world. Despite their ecological importance, groundwater microbial communities remain underexplored. In this study, we conducted a comprehensive microbiome analysis of karst groundwater, investigating the abundance and diversity of viruses, archaea, bacteria, fungi, and protozoa. We detected a high diversity of both prokaryote- and eukaryote-infecting viruses, indicating the connection of subterranean environments with surface waters. In the archaeal community, seven different phyla were identified, dominated by members of "*Candidatus* Woesearchaeota" and *Nitrososphaerota* (syn. *Thermoproteota*). In the bacterial community of majority of the samples, the *Pseudomonadota* was the most abundant phylum, followed by *Bacteroidota*, *Actinomycetota*, and *Verrucomicrobiota*. Fungal abundance and diversity differed greatly between cave water samples, with the Ascomycota families Cladosporiaceae, Didymellaceae, Aspergillaceae and saprotrophic Basidiomycota detected in all samples. Besides fungi, microscopic eukaryotic diversity consisted of ciliates, copepods and golden-brown algae present in all samples. Differences in microbial communities between the sampling sites highlight the need for tailored site-specific groundwater conservation and monitoring strategies. Moreover, the identification of microbes associated with anthropogenic activities underscores their potential as source-specific indicators of groundwater pollution. As the first holistic overview of microbial diversity of subterranean water, the study establishes a valuable baseline for future research and conservation of these unique karst ecosystems.

1. Introduction

As the largest liquid freshwater resource in the world, groundwater is an important source for drinking water supply, irrigation, and industrial use (Griebler and Avramov, 2015). Over time, it became severely threatened by anthropogenic activities including climate change, overexploitation and pollution (Burri et al., 2019). As a subterranean environment, groundwater is generally characterized by the absence of light, low or no seasonal temperature fluctuations and scarcity of organic matter due to the absence of light-driven primary producers (Culver and Pipan, 2019). The low level of nutrients is reflected in lower abundance of organisms and shorter food chains of microorganisms and animals (Culver and Pipan, 2019).

On a global scale, the areas with the highest known biodiversity of subterranean organisms are located in temperate climates, such as Dinaric Karst in southeastern Europe (Zagmajster et al., 2014). The northwest part of the Dinaric Karst in Slovenia is a biodiversity hotspot of subterranean organisms with a world-leading number of exclusively subterranean, predominantly invertebrate species (Bregović et al., 2019). Their diversity and relative abundance, among the others, sustain

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the world’s largest exclusively cave-dwelling tetrapod, the European blind cave salamander known as the olm (*Proteus anguinus*). The presence of this endemic and highly endangered amphibian therefore reflects the stability of food chains, the vitality and health of groundwater communities at various trophic levels (Kostanjšek et al., 2023).

Although hidden beneath the surface, subterranean habitats are not completely isolated. Organic matter and minerals are transported from the surface, primarily by interstitial water seeping through porous carbonate rock (Culver and Pipan, 2019). In addition to nutrients that sustain most of the subterranean food chains, the inflowing water is also a source of chemical and microbiological pollutants. A wide range of microorganisms thrive in caves, originating from surface environments and/or playing important ecological roles in cave development and functioning (Tomczyk-Żak and Zielenkiewicz, 2016). The sustainability of subterranean ecosystems depends primarily on the lowest trophic levels (Tomczyk-Żak and Zielenkiewicz, 2016), which, due to the absence of photosynthesis, consist entirely of microorganisms. Knowing the composition of such microbial communities is the first step towards understanding the processes that maintain a stable ecosystem and groundwater quality (Griebler et al., 2014). Nevertheless, knowledge of underground microbiota is limited, mainly due to the inaccessibility, complexity of the communities, and methodological challenges. Most of the recent reports have focused on microbes in cave waters, larger groundwater bodies relatively close to the surface. These studies have primarily investigated the microbial indicators of water quality (Seman et al., 2015), microbial diversity (Couton et al., 2023; Krause et al., 2014), and metabolic properties of environment-specific microbes (Candiroglu and Dogruoz Gungor, 2017). Initial studies relied on cultivation of microorganisms, while latter harness the advantages of amplicon sequencing (Alonso et al., 2019; Langille et al., 2013) to detect bacterial and archaeal (Gios et al., 2023; Fillinger et al., 2019), fungal (Retter et al., 2024), protist (Risse-buhl et al., 2013; Cukusic et al., 2025) and viral diversity (Malki et al., 2020; Pratama et al., 2025).

The objective of the present study was to provide a baseline diversity catalogue of natural microbial communities in groundwaters sampled from karst springs and cave waters in Slovenia with the confirmed presence of olm populations as indicators of a well-preserved water environment with limited human impact. Analysis of the complete microbial communities was carried out by high-throughput sequencing (HTS), and viral diversity by shotgun sequencing. To the best of our knowledge, this is the first simultaneous analysis of the subterranean microbiome comprising DNA and RNA viruses, archaea, bacteria, fungi, and protozoa, giving insight into microbial dynamics in Slovenian groundwater Karst systems.

2. Materials and methods

2.1. Site description and sampling

We collected water samples from seven different locations of karst springs and subterranean rivers in Slovenia with confirmed presence of olm populations (Fig. 1, Table 1). We sampled water from two karst springs, Jelševniščica (45°34´20″N, 15°08´58″E, sampling location CW5) and Vir (45°56´51″N, 14°49´02″E, sampling location CW2) at the location where water emerges to the surface. Two samples were collected from accessible spring caves before water emerges to the surface, namely in Kompoljska jama (45°47´58″N, 14°43´51″E, sampling location CW6) and in Krška jama (45°53´24″N, 14°46´15″E, sampling location CW3). We collected two water samples from the underground Pivka River deep in Postojnsko – planinski jamski sistem (Postojna – Planina Cave system) in the part Črna jama (sampling location CW1) and Planinska jama (sampling location CW4). The Pivka River is the main allogenic recharge for the cave system (45°46´57″N, 14°12´13″E), which includes several caves. We collected another ground water sample from the Reka River, 100 m deep after its ponor in Škocjanske jame (Škocjan Caves) (45°39´57″N, 13°59´24″E, CW7).

We conducted sampling in the period avoiding extreme hydrological conditions of high and low water discharges (Table 1). Three replicates of cave groundwater samples were aseptically collected, stored at 4 °C and processed further within two days. *On-site* measurements of temperature, pH, electric conductivity (EC) and dissolved oxygen (DO) were performed by a Multi-parameter portable meter (WTW Multiline Multi 3620 IDS, WTW Oxi 3310 IDS). Nitrate concentrations (NO_3) were measured using a Perkin Elmer Lambda 25 UV/Vis spectrophotometer according to the standard method ISO 7890–3 (ISO, 7890–3, 1988).

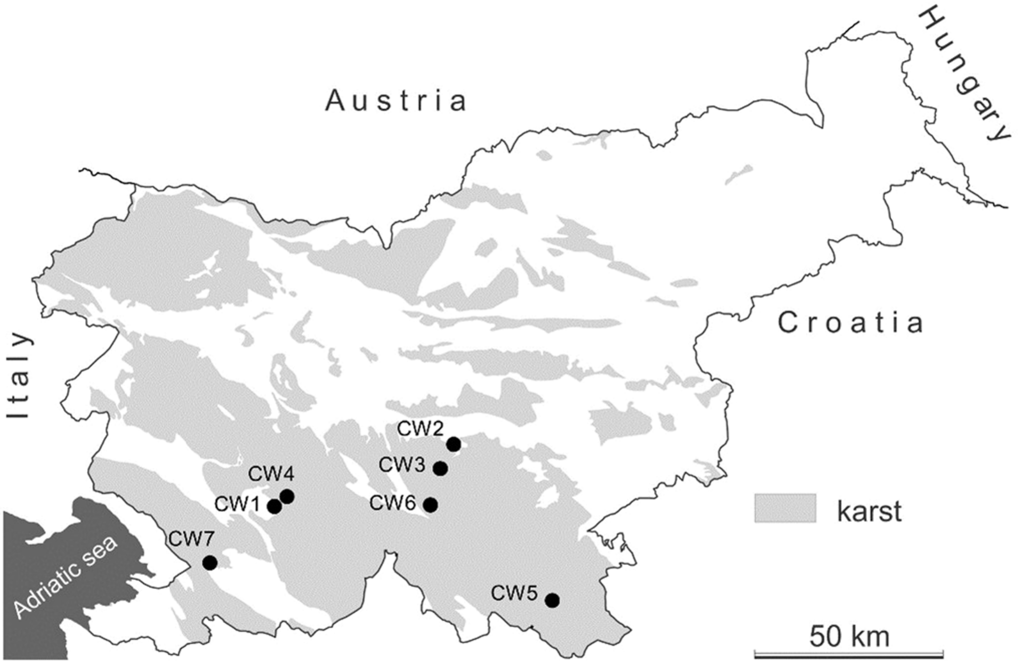

Fig. 1. Sampling locations in Slovenia: Črna jama (CW1); Vir (CW2), Krška jama (CW3); Planinska jama (CW4); Jelševniščica (CW5); Kompoljska jama (CW6); Škocjanske jame (CW7).

Table 1
Physio-chemical parameters of sampled cave waters. EC - electrical conductivity; DO – dissolved oxygen; NO$_3$ – nitrate.

Sample	Location	Date dd/mm/yyyy	T (°C)	pH	EC (µS/cm)	DO (mg/l)	DO (%)	NO3 (mg/l)
CW1	Pivka River – Črna jama	08/05/2018	7.6	8.14	396	9.18	82.3	2.8
CW2	Vir	05/10/2018	12.6	6.70	604	10.40	95.6	11.7
CW3	Krka River – Krška jama	05/06/2018	12.1	7.61	532	6.07	57.7	5.2
CW4	Pivka River – Planinska jama	16/08/2017	10.8	8.03	422	10.58	100.3	18.3
CW5	Jelševniščica	05/10/2018	11.1	6.80	404	13.75	126.0	4.0
CW6	Kompoljska jama	05/10/2018	9.0	7.29	449	16.30	138.6	4.8
CW7	Reka River – Škocjanske jame	17/09/2018	16.1	8.08	364	9.21	96.0	1.6

2.2. Preparation of cave water samples for the characterization of microbiomes

The protocol used for concentration of viruses was described previously (Bačnik et al., 2020). Briefly, samples (5 L per sample) were filtered (filter paper and cellulose acetate membranes) and the concentration was done using convective interaction media (CIM) quaternary amine 8 mL monolithic column (BIA Separations, Ajdovščina, Slovenia) on a chromatography system AKTA Purifier 100 (GE Healthcare, Chicago, IL, USA). To concentrate archaea, bacteria, protozoa and fungi, 1 L of each water sample obtained from each of the 7 sampling sites was filtered in duplicate through nitrocellulose filters with a pore diameter of 0.22 µm (Merck Millipore, Burlington, MA, USA).

2.3. Nucleic acids isolation, preamplification and sequencing

For virome analysis, RNA and DNA were isolated from elution fractions using QIAamp MinElute Virus Spin Kit according to the manufacturer's instructions (QIAGEN, Germantown, MD, USA). To obtain sufficient DNA for library preparation, samples were randomly preamplified as previously described (Bačnik et al., 2020). Preamplification products were sent to Fasteris (Geneva, Switzerland) for library preparation and shotgun sequencing using Illumina MiSeq (Illumina, San Diego, CA, USA) in 2 × 250 bp mode. The negative control of isolation was spiked with luciferase RNA (Promega, WI, USA) and was also sent for sequencing.

Filters containing concentrated bacteria, archaea, fungi, and protozoa were transferred under aseptic conditions into bead tubes provided as a part of the Power Water Kit (QIAGEN, Carlsbad, CA, USA). Environmental DNA was extracted from the filters using Power Water Kit according to the manufacturer's instructions. Ready-to-use DNA was stored at −20 °C until further processing. For archaea, 16S rRNA gene was amplified using primers SSU1ArF (5′-TCCGGTTGATCCYGCBRG-3′) and SSU520R (5′-GCTACGRRYGYTTTARRC-3′) (Bahram et al., 2019), for bacteria, the hypervariable V3 and V4 regions of the 16S rRNA gene were amplified using the universal bacterial primers 341F_ill (5′-CCTACGGGNGGCWGCAG-3′) and 802R_ill (5′-GACTACHVGGGTATCTAATCC-3′) (Herlemann et al., 2011). For fungi, the ITS2 region of the ITS rDNA gene was amplified using the ITS4-Fun (5′-AGCCTCCGCTTATTGATATGCTTAART-3′) and 5.8S-Fun (5′-AACTTTYRRCAAYGGATCWCT-3′) primers (Taylor et al., 2016). For protozoa, primers 18S_616F (5′-TTAAARVGYTCGTAGTYG-3′) and 18S_1132R (5′-CCGTCAATTHCTTYAART-3′) were used to amplify 18S rRNA gene (Hugerth et al., 2014). The first step of polymerase chain reaction (PCR) was performed in-house using the Phusion® High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, MA, USA) according to the manufacturer’s protocol. Amplification was carried out in a PCR Mastercycler Ep Gradient (Eppendorf, Hamburg, Germany) with an initial denaturation of 2 min at 98 °C, followed by 20 cycles of 25 s at 98 °C, 30 s at 55 °C, and of 30 s at 72 °C, with a final step of 5 min at 72 °C. Amplification products were sent to Microsynth AG (Switzerland) for the 2nd PCR step and sequencing with Illumina Miseq V3 (300 bp paired-end sequencing).

2.4. Bioinformatic analyses of cave water samples

For virome analysis, trimmed, size- and quality-filtered read subsets were exported from CLC Genomics Workbench 22 (Qiagen Bioinformatics, CA, USA) and compared for similarity to a complete protein NCBI nr database using sequence aligner software DIAMOND (Buchfink et al., 2021) with default parameters. DIAMOND results were used as an input for the taxonomic classification of the reads using MEGAN Metagenome Analyzer (v. 6.21.12, July 2020 database) (Huson et al., 2016). The obtained MEGAN output, in the form of summarized reads, was used to provide an overview of the taxonomic classification of the sequencing reads. Host associations of viral sequences were based on taxonomic classification at the level of viral families and their common host associations as identified by International Committee on Taxonomy of Viruses (ICTV) (Lefkowitz et al., 2018).

Bacterial, archaeal, fungal and protozoan microbiomes were analyzed using the QIIME2 2022.8 software package (Bolyen et al., 2019). Trimming of primers, adapters and regions with quality score < 20 was performed. To avoid bias introduced by suboptimal assembly of paired-end reads due to different amplicon lengths (particularly for ITS), only single-end forward reads were used for the analysis. Reads were denoised using dada2, representative sequences were aligned using MAFFT software and the midpoint-rooted tree was inferred using FastTree, a program to efficiently infer approximately-maximum-likelihood phylogenetic trees from large datasets. All amplicon sequence variants (ASVs) represented by less than 50 reads were removed. Taxonomic assignment of the ASVs was performed with the vsearch classifier within QIIME, which performs a global alignment between the representative ASV sequence and the database sequences and then assigns the taxonomy based on best matches in the database for each query. Two databases were used: Silva release 132 (bacterial 16S, archaeal 16S and eukaryotic 18S ribosomal DNA; (Quast et al., 2013)) and dynamically clustered UNITE ITS database version 8.0 (fungal ITS; (Nilsson et al., 2019).

Distance-based redundancy analysis (dbRDA) was used to assess the relationship between microbial community composition and environmental variables. Community matrices for each taxonomic group were constructed from Hellinger-transformed sequence abundance tables. dbRDA was performed using Bray–Curtis dissimilarity as implemented in the capscale function (Legendre, 1999) of the *vegan* R package. Collinearity among the environmental predictors was examined using variance inflation factors (vif.cca), which confirmed low multicollinearity (all VIF < 2). The significance of the global model and of individual environmental terms was assessed using permutation tests (anova.cca). Ordination figures were generated using the ggplot2 package (Wickham, 2016).

3. Results

3.1. Physio-chemical parameters

Environmental conditions varied among sampling sites (Table 1), with temperature of the groundwater ranging from 7.6 °C at Pivka River – Planinska jama (CW4) to 16.1 °C at Reka River – Škocjanske jame

(CW7). This highest temperature at the point of sampling, which was 100 m deep in the cave, reflected the external environmental conditions of the river sinking into the cave. Sampled waters were well-oxygenated (> 80 % dissolved oxygen, DO), except the Krka River in Krška jama (CW3) (57.7 % DO, 6.07 mg/l). To assess the influence of environmental variables on community compositions, we performed distance-based redundancy analysis (dbRDA; Suppl. Fig. 1; (Bačnik, 2026)). Owing to the limited number of samples, the measured environmental variables did not explain a detectable proportion of community variation for archaea, bacteria, fungi or ether eukaryotes and viruses, as indicated by adjusted R^2 values close to zero (Suppl. Table 1).

3.2. *Viromes of groundwaters*

To examine virome composition in cave waters, we analysed data obtained by shotgun metagenomic sequencing of concentrated water samples. Each sample yielded between ~2.2 and 3.0 million reads, with viral reads ranging from 0.4 % to 3.7 % of total reads (Suppl. Table 2). Based on short read classification, we detected 99 viral families belonging to *Monodnaviria, Duplodnaviria, Varidnaviria* and *Riboviria* realms (Suppl. Table 3–4). Among sequences belonging to *Monodnaviria* realm**,** ubiquitous ssDNA bacteriophages of *Microviridae* family were most abundant in all samples, followed by Rep-encoding single-strand (CRESS)-DNA viruses (e.g. *Circoviridae, Geminiviridae*). The viromes of cave waters contained a high abundance of dsDNA bacteriophages from *Duplodnaviria* realm. Reads classified into *Varidnaviria* realm mostly represented sequences of nucleocytoplasmic large DNA viruses (NCLDV, e.g. *Mimiviridae, Phycodnaviridae*) and reads of non-tailed phages from *Tectiviridae* family (Suppl. Table 4, Fig. 2B). We detected sequences of ssRNA viruses from *Riboviria* realm infecting plants (e.g. *Solemoviridae, Tombusviridae, Virgaviridae)* (Suppl. Table 4, Fig. 2B). A large proportion of reads were assigned to uncultured/environmental viruses (Suppl. Table 3).

While most viral reads belonged to prokaryote-infecting viruses, family level richness was higher among eukaryote-infecting families (35 plant/fungi/protist and 40 animal-infecting families vs. 22 prokaryote-infecting ones) (Suppl. Fig. 2; Suppl. Table 4). Ubiquitous bacteriophage taxa (*Microviridae, Caudoviricetes*) were shared across locations (Fig. 2B), while eukaryotic viral diversity varied more notably between sites (Fig. 2B).

3.3. *Archaea and bacteria in groundwaters*

To characterize archaeal and bacterial communities in cave water, we analysed partial 16S rRNA sequences obtained by amplicon sequencing. ASVs were assigned to seven archaeal and 13 bacterial phyla, plus one bacterial superphylum (Suppl. Table 5, Fig. 3A–B).

Among archaea, the dominant phyla were "*Candidatus* Woesearchaeota" (17.9–83.8 % of ASVs) and *Nitrososphaerota* (2–58.5 %). *Nitrososphaerota* were most abundant in samples Jelševniščica (CW5, 58.5 %) and Pivka River – Planinska jama (CW4, 57.7 %), while "*Ca.* Woesearchaeota" prevailed in Pivka River – Črna jama (CW1, 70.5 %), Vir (CW2, 61.7 %), and Kompoljska jama (CW6, 56.6 %), and especially in Reka River – Škocjanske jame (CW7, 83.8 %).

Among bacteria, the most abundant phyla were *Pseudomonadota* (15.6–67.6 % of all ASVs), *Bacteroidota* (9.4–34.5 %), *Actinomycetota* (3.1–20.3 %), and *Verrucomicrobiota* (2.4–12.7 %). Betaproteobacteria dominated within *Pseudomonadota*, particularly genus *Limnohabitans*, followed by *Rhodoferax* and *Methylotenera*. *Bacteroidota* ASVs were dominated by ubiquitous *Flavobacterium* (up to 25.9 % in CW2). Among ASVs identified as *Actinomycetota,* the most abundant genus was *Mycobacterium* (0.03 % – 4.7 %). Notably, members of *Bacillota* were most abundant in Krka River – Krška jama (CW3, 32 %), with vertebrate gut-associated genera *Romboutsia* and *Turicibacter* dominating. ASVs identified as superphylum "*Candidatus* Patescibacteria", were detected in all cave water samples (0.02–5.4 %).

3.4. *Diverse fungal communities, protozoans and other eukaryotes in cave waters*

The highest abundance in terms of fungal ASVs was observed in the samples Kompoljska jama (CW6) and Pivka River – Planinska jama (CW4) (Suppl. Table 5). Basidiomycota were the most abundant phylum, especially at Pivka River – Planinska jama (CW4), while Ascomycota were more variable in abundance, being the highest in Kompoljska jama (CW6) and Jelševniščica (CW5) and the lowest in Pivka River –

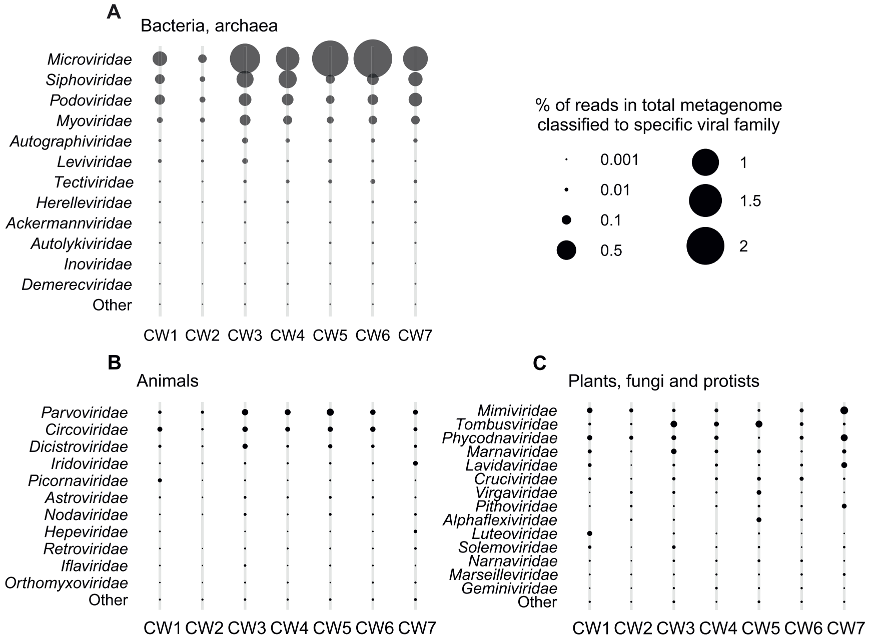

Fig. 2. Classification of virus reads for different cave water samples, at the family level. Bubble charts show virus families detected in each sample and their corresponding reads. The size of the bubble indicates the percentage of total reads assigned to each family based on DIAMOND blastx similarity search followed by MEGAN-LCA binning. Detected viral families are grouped by their common host category; bacteria/archaea (A), animals (B), plants/fungi/protists (C).

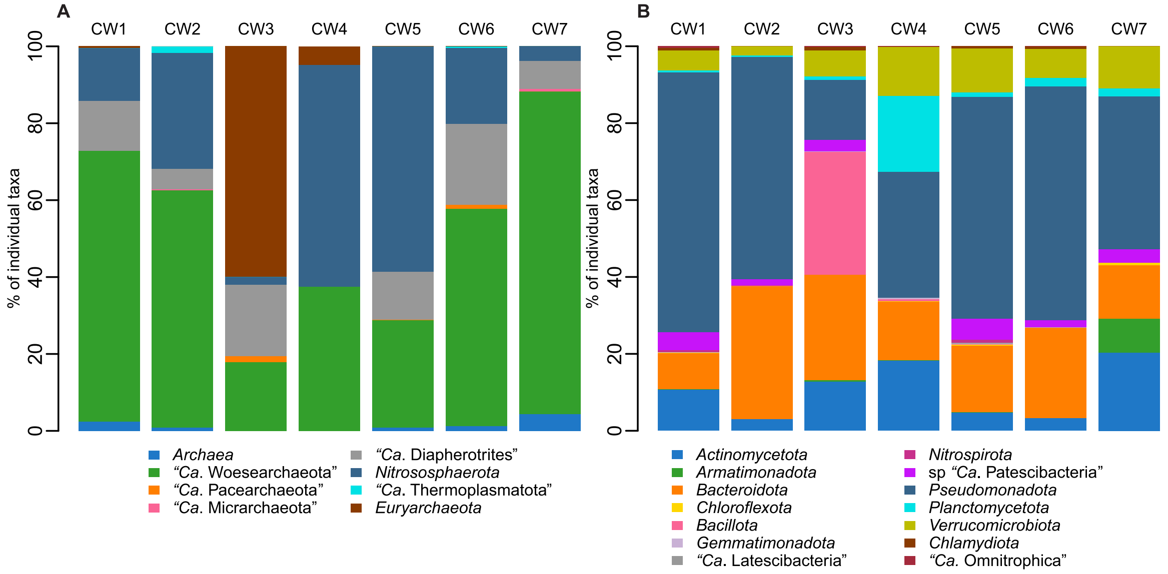

Fig. 3. Proportions of individual phyla in the archaeal (A) and in the bacterial (B) community of Slovenian cave waters.

Planinska jama (CW4) (Suppl. Table 6, Fig. 4A). Fungi belonging to taxa Mortierellomycota, Mucoromycota and Chytridiomycota were detected site-specifically (e.g., Mortierellomycota in CW6, Mucoromycota in CW3, Chytridiomycota in CW5) (Suppl. Table 6, Fig. 4A).

Fungal taxa present in all samples included ascomycetous genera *Cladosporium* and *Penicillium*, and saprotrophic Basidiomycota (e.g., genera *Russula, Coprinellus*). On the other hand, each cave had unique composition: Pivka River – Planinska jama (CW4) had the highest number of ascomycetous fungi (e.g. Lophiostomataceae, Phaeosphaeriaceae, Hyaloscyphaceae, Trichomonascaceae) and basidiomycetous genera (e.g. *Hypholoma, Trametes, Wallemia)*. ASVs belonging to the families Mucoraceae, Stereaceae, Malasseziaceae, and Sporidiobolaceae prevailed in Krka River – Krška jama (CW3). Kompoljska jama (CW6) showed the highest fungal ASV abundance and diversity, as well as the greatest number of genera found exclusively in this sample. Based on available literature, they were mainly associated with soil and plant material and animals (Fig. 4B).

To examine the protozoan community, a partial sequence of the 18S rRNA gene obtained by amplicon sequencing was analyzed. The abundance of protozoan ASVs varied considerably between the samples: low number of ASVs were detected in samples Krka River – Krška jama (CW3), Pivka River – Planinska jama (CW4) and Kompoljska jama (CW6) (Suppl. Tables 5 and 8). The low abundance in the samples was also reflected in considerable variation in eukaryote diversity between the samples, ranging from 12 to 59 taxa. These comprise a wide array of eukaryotic taxa besides protozoans, including algae, fungi, sponges, higher animals and plants (Suppl. Table 7). Obscured by the low abundance of ASVs, a comparison of eukaryote diversity between sampling sites showed that ciliates (Ciliophora, Spirotrichea), copepods (Arthropoda, Maxillopoda, Cyclopoida) and golden-brown algae (Ochrophyta, Chrysiophicaeae) were the only eukaryote phyla common to all samples (Suppl. Table 7).

4. Discussion

This study provides a comprehensive microbiome analysis exploring the diversity of viruses, archaea, bacteria, fungi and protozoa in subterranean cave waters in the Slovenian Dinaric Karst. Cave

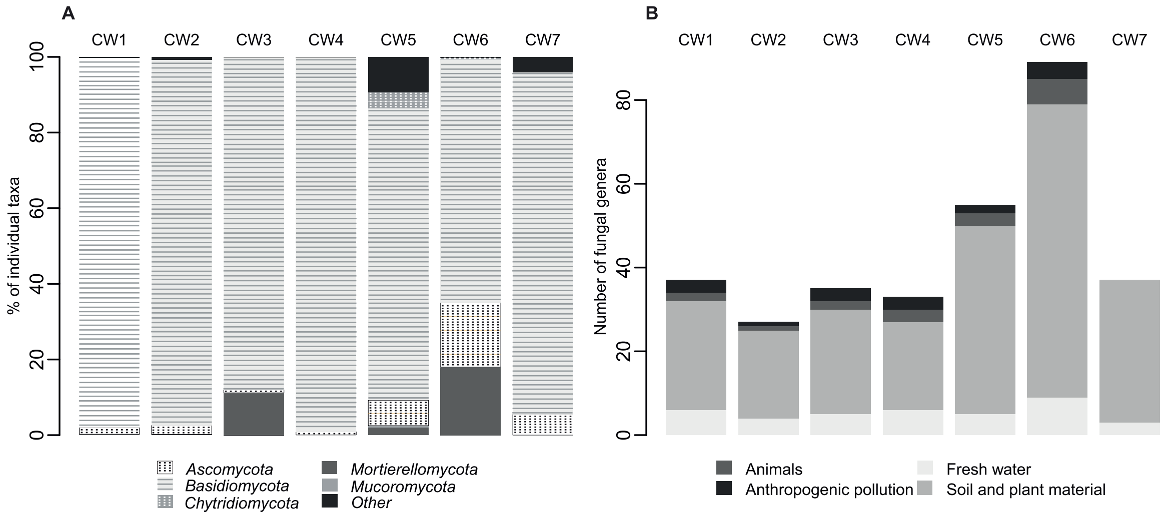

Fig. 4. (A) Comparison of abundance and diversity of fungal phyla in cave waters. (B) Number of fungal genera from cave waters associated with different origins. Figure summarizes the number of fungal genera in cave waters associated with soil and plant material (including plant pathogens and mycorrhizal fungi), animals (pathogens of bats, insects, fish), and anthropogenic pollution.

microorganisms, facing extreme conditions such as darkness and oligotrophy, play crucial roles in the biogeochemical cycles (Zhu et al., 2022). To thrive in these environments, which are bottom-up controlled, i.e., driven by the amount and bioavailability of organic carbon and major nutrients (Karwautz et al., 2022), they use various metabolic strategies (e.g. chemotrophy and mixotrophy) and form complex network of interactions, including competition, cooperation, and parasitism (Kosznik-Kwaśnicka et al., 2022).

We attempted to explore co-occurrence patterns among the detected taxa; however, no statistically supported associations were recovered, reflecting the limited number of samples relative to the high taxonomic richness (results not shown). Species richness and diversity, assessed by alpha diversity indices (Chao1, Shannon), varied among sampling sites and microbial groups (Suppl. Table 8). Our findings underscore that groundwater cave microbiomes harbor substantial, still largely unexplored microbial diversity. We discuss the results for individual groups of organisms, emphasizing their diversity, their implications for water quality assessment, and their potential for conservation of groundwaters.

4.1. Subterranean water viromes as indicators of microbial diversity, surface inputs, and human activity

In all samples, *Microviridae* was the most abundant prokaryote-infecting viral family, consistent with ubiquity of its members in aquatic and sediment environments (Quaiser et al., 2015; Schweichhart et al., 2022). Similarly, the presence of dsDNA-tailed and non-tailed bacteriophages aligns with findings from other freshwater and cave environments (Malki et al., 2020; Pratama et al., 2025; Ulbrich et al., 2024). CRESS-DNA viruses (e.g., *Circoviridae, Geminiviridae*), often linked to zooplankton or sediments, were also detected (Eaglesham and Hewson, 2013), along with NCLDVs (e.g., *Mimiviridae*), which are known to infect protists and play crucial ecological role in the sea by accelerating the turnover of their unicellular hosts (Hingamp et al., 2013). We detected sequences of plant-infecting ssRNA viruses in cave water samples, with sequences belonging to members of known plant pathogens, possibly reflecting runoff from surface vegetation, soils, and animal feces, consistent with previous environmental virome studies (Mehle and Ravnikar, 2012). We also detected reads that belong to families from the *Picornavirales* order, a diverse group infecting eukaryotes, with many uncharacterized picorna-like viruses reported in the water environments (Zell et al., 2022).

The overall high viral diversity, including over 50 families, reflects both microbial richness and possible connectivity of subterranean environments with surface waters. Searching for sequences of viral fecal indicators, we detected some reads belonging to *Virgaviridae* family (Suppl. Table 4), with only a few reads classifying as pepper mild mottle virus (PMMoV), which has been proposed as an indicator of water pollution (Kuroda et al., 2015). In addition, in all analyzed samples, we detected reads classifying as crAss-like bacteriophages, among which sequences belonging to "crAssphage", associated with the human gut microbiome (Edwards et al., 2019), were most abundant in Krka River – Krška jama (CW3) and Pivka River – Planinska jama (CW4), both rivers with a large recharge area affected by agricultural and urban activities.

4.2. Archaeal and bacterial communities reflect nutrient conditions in cave waters

Ammonia-oxidizing archaea (AOA) from phylum *Nitrososphaerota*, the major nitrifying community relative to the total prokaryotic plankton (Ngugi et al., 2023), dominated in Jelševniščica (CW5) and Pivka River – Planinska jama (CW4) (Fig. 3A). Their presence highlights the role of archaea in nitrogen cycling in nutrient-limited environments and suggests low ammonium levels in those karst waters and is consistent with prior measurements of ammonium concentrations in some of the sampling sites (Mulec et al., 2019). Cave water samples with the highest proportion of *Nitrososphaerota* had the lower proportion of "*Candidatus* Woesearchaeota", which usually dominate in anoxic environments, are believed to have symbiotic or parasitic lifestyles and may contribute to anaerobic nutrient cycling, although their ecological roles remain poorly understood (Gios et al., 2023; Liu et al., 2021). Moreover, the detection of the class *Halobacteria* (*Euryarchaeota*) in the water sample from Krka River – Krška jama (CW3), (59.9 % of all ASVs) is likely a consequence of contamination during sample filtration, as these halophiles are not typical of freshwater cave environments. In fact, the research on extreme halophiles from brine is routinely performed in the laboratory where our water samples were processed.

The bacterial communities were dominated by *Pseudomonadota* (Fig. 3B). In caves, this phylum often dominates in water seepages, cave pools and cave sediments (Guan et al., 2023). Their ecological advantage is likely due to their metabolic versatility and ability to degrade diverse organic compounds, especially in nutrient-rich cave environments (Tomczyk-Żak and Zielenkiewicz, 2016). While *Gammaproteobacteria* are most often reported in caves (Hudoklin, 2004), they were only the third most abundant group in our samples. Instead, *Betaproteobacteria* predominated. The key genera were *Limnohabitans*, an important planktonic genus of inland freshwater habitats (Jezberová et al., 2017), *Rhodoferax*, a genus of phototrophic purple non-sulfur bacteria and *Methylotenera*, a genus of methylotrophs (Tomczyk-Żak and Zielenkiewicz, 2016). *Alphaproteobacteria* were the second most abundant group. It was best represented with the genus *Pseudomonas*. Species in this genus are associated with humid environments and can be abundant in tourist caves (Biagioli et al., 2023).

Among *Bacteroidota*, dominant genera comprise the ubiquitous *Flavobacterium*, *Pseudarcicella*, *Sedimibacterium*, and *Fluviicola*. The latter two are considered indicators of good water quality, whereas *Flavobacterium* and *Pseudomonas* indicate poor quality (Guo et al., 2020), and their lowest numbers were detected in Reka River – Škocjanske jame (CW7) and Pivka River – Planinska jama (CW4). Among *Actinomycetota*, the most represented genus was *Mycobacterium*, which predominated in Krka River – Krška jama (CW3) and Vir (CW2) (Fig. 3B). The high relative abundance of *Mycobacterium* was previously reported for anthropized caves, suggesting human inputs (Buresova-Faitova et al., 2022). The superphylum "*Candidatus* Patescibacteria", characterized by reduced metabolic pathways and likely parasitic or symbiotic lifestyles (Chaudhari et al., 2021), was present in all samples at low abundance (Fig. 3B). Most representatives of the superphylum, commonly detected in groundwater microbiomes (Gios et al., 2023; Pratama et al., 2025), are estimated to have ultra-small diameters ranging from 0.1 μm to 0.3 μm (Beam et al., 2020), therefore the use of a filter with a pore diameter of 0.22 μm likely resulted in underrepresentation in our samples.

4.3. Fungal and protozoan communities in Karst waters reflect surface inputs and anthropogenic impact

Fungal presence in cave waters reflects transfer from surface via air and water pathways—rain, streams, and soil infiltration carry fungal spores and other microorganisms underground (Zhang and Xu, 2018; Retter and Nawaz, 2021). Cross-comparison of fungal taxa showed that the core ASVs present in all waters belonged to *Cladosporium* and *Penicillium.* Both genera are ubiquitous in groundwater, soils, plants, and air (Retter et al., 2024; Babič and Gunde-Cimerman, 2021). Interestingly, all samples analysed in this study contained large numbers of ASVs identified as ectomycorrhizal Basidiomycetes (*Russula* and Agaricomycetes). One of the possible explanations would be presence of their spores and residues in humus and other plant debris washed from the nearby environment through porous karst terrain, as these fungi are unlikely to grow individually in the caves (Wang et al., 2015; Summers Engel and Northup, 2008). Apart from a few abundant core ASVs mentioned above, the composition of fungal biota varies between the samples. Jelševniščica (CW5) and Kompoljska jama (CW6) hosted many soil- and plant-associated fungi (Fig. 4B), which is in line with their environmental characteristics. Kompoljska jama with its spring has a wide entrance colonized by mosses and other vegetation, where water encounters plant and soil material, like the groundwater of Jelševniščica, when it reaches the surface.

Anthropogenic impacts on cave waters can be observed indirectly through certain fungal taxa associated with human activities. For instance, urban wastewater, occasionally carried by rivers, can be a source of human and animal pathogens (e.g. *Candida, Malassezia, Pseudallescheria*) *(*Assress et al., 2019*)*. The highest numbers of fungi associated with anthropogenic activities were found in samples from Krka River – Krška jama (CW3) and Kompoljska jama (CW6). The possible reason is the location of the caves with large recharge areas influenced by human activities, particularly agriculture.

Besides tracking the presence of fungi related to urban waste, presence of certain fungal taxa, such as melanised *Exophiala* (order Chaetothyriales), previously detected in groundwaters (Retter et al., 2024), can indicate the pollution with aromatic hydrocarbons or heavy metals. Melanised fungi were detected in Pivka River – Planinska jama (CW4) and Jelševniščica (CW5). While Pivka River is occasionally affected by urban waste, Jelševniščica, however, was polluted with phenols and heavy metals caused by improper storage of industrial wastes at the end of previous century (Hudoklin, 2004). Despite elimination of the pollution sources, the presence of melanised fungi, which association with remaining pollutants cannot be excluded, should be monitored on a long-term, due to their potential of disseminated infections in fish and, amphibians (Hudoklin, 2004; Seyedmousavi et al., 2013).

The description of protozoan communities was hampered by the unusually low abundance of protozoan sequences at some sampling sites. Since the lowest number of protozoan sequences was observed in the sample Krka River – Krška jama (CW3) and Pivka River – Planinska jama (CW4), the latter cave system being recognized as one of the highest biodiversity hotspots in the world (Zagmajster et al., 2021), it seems unlikely that the observed low abundance truly reflects the absence of protozoa. Their low abundance or absence in the samples is likely to be temporary. Due to their predatory nature, protozoa may follow the cycles of their primary food sources or be affected by seasonal fluctuations. Additional sampling is needed to address this issue. At the same time, the observed abundance of other microorganisms in the same water samples limits the possibility of DNA degradation or inconsistencies in the methodological approaches used in the study. Despite the obvious advantages of amplicon-based metagenomic approaches for microbial community analysis, the efficient description of protozoa and other eukaryotes in natural microbial communities remains a challenge. The selection of an appropriate primer set for protozoa usually represents a trade-off between minimizing the amplification of bacterial sequences and efficiently covering the target group of eukaryotes (Moreno et al., 2018; Kounosu et al., 2019). With the goal of comprehensive coverage of protozoan diversity in cave waters, the trade-off of the selected primer set used in our study (Hugerth et al., 2014) is reflected in the detection of other eukaryote groups besides protozoa, including fungi, sponges, algae, plants, and selected taxa of metazoans. At the same time, most of the animal groups contributing to the recognized biodiversity of the studied cave waters, including crustaceans (especially amphipods, isopods, copepods, and decapods), annelids, molluscs, and finally olms (Zagmajster et al., 2021), were not detected by the primers used in our study.

5. Conclusions

We used HTS to characterize the groundwater microbiomes in several presumed-pristine subterranean environments of the Slovenian Dinaric Karst. Our analysis assessed the abundance and diversity of viruses, archaea, bacteria, fungi, and protozoa, identifying core taxa and providing a baseline catalogue of natural microbial communities in cave waters.

The observed diversity in microbial composition and species richness across sampling sites indicates notable ecological variation, despite similar abiotic conditions. The sampling sites in the study were selected based on the presence of olm populations. In this regard, the observed differences in the complexity and composition of microbial communities between sampling sites provide an essential information for assessing potential microbial threats (viruses, bacteria, fungi) to individual olm lineages and for developing targeted conservation strategies for this highly endangered amphibian and its natural environment. Future strategies for the sustainable conservation and monitoring of groundwater should prioritize site-specific over generalized approaches.

Some of the microorganisms identified in the study are closely associated with certain anthropogenic activities. These include the bacterial species from the phylum *Bacillota* (genera *Romboutsia* and *Turicibacter*), fungi from the genera *Candida*, *Pseudallescheria* and *Malassezia*, as well as crAss-like phages, which are recognized to be part of the mammalian gut microbiota. Additionally, archaea from the phylum Nitrososphaerota were detected, commonly found in ammonia-enriched environments. The study shows the potential of microorganisms as source-specific indicators of groundwater pollution in karst systems, particularly from fecal contamination, agricultural runoff, and industrial discharge. Combined with chemical monitoring, they can serve as an invaluable tool for long-term assessment of groundwater quality.

CRediT authorship contribution statement

Bacnik Katarina: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Denis Kutnjak:** Writing – review & editing, Visualization, Supervision. **Janez Mulec:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Monika Novak Babič:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Cene Gostinčar:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Rok Kostanjšek:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Martina Turk:** Writing – review & editing, Writing – original draft, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Ion Gutierrez-Aguirre:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Gunde Cimerman Nina:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2026.119890.

Data availability

Supplementary material is available at https://doi.org/10.5281/zenodo.18390617. All raw sequencing data generated in this study are available in NCBI SRA database - BioProject number PRJNA1010541.

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