BIBLIOGRAPHIC INFORMATION SYSTEM

Journal Full Title: Journal of Biomedical Research & Environmental Sciences

Journal NLM Abbreviation: J Biomed Res Environ Sci

Journal Website Link: https://www.jelsciences.com

Journal ISSN: 2766-2276

Category: Multidisciplinary

Subject Areas: Medicine Group, Biology Group, General, Environmental Sciences

Topics Summation: 128

Issue Regularity: Monthly

Review Process type: Double Blind

Time to Publication: 7-14 Days

Indexing catalog: Visit here

Publication fee catalog: Visit here

DOI: 10.37871 (CrossRef)

Plagiarism detection software: iThenticate

Managing entity: USA

Language: English

Research work collecting capability: Worldwide

Organized by: SciRes Literature LLC

Manuscript should be submitted in Word Document (.doc or .docx) through

Online Submission

form or can be mailed to support@jelsciences.com

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JOURNAL OF BIOMEDICAL RESEARCH & ENVIRONMENTAL SCIENCES
issn: 2766-2276
SUBJECT AREA(S): MICROBIOLOGY
DOI: 10.37871

RESEARCH ARTICLE

Microbial Community Associated with Ground waters Discharge in Transylvania (Romania) and Balaton Highland (Hungary)

Marwene Toumi[1]*, István Máthé[2], Ádám Tóth[3], Nóra Sztráda[1], Rózsa Farkas[1], Laura Jurecska[1] and Erika Tóth[1]

[1]Department of Microbiology, Eötvös Loránd University, H-1117, Budapest, Pázmány P. stny 1/C, Hungary
[2]Department of Bioengeneering, Faculty of Economics, Socio-Human Sciences and Engineering, Sapientia Hungarian University of Transylvania, 530104 Miercurea Ciuc, Libertății Sq. nr. 1, Romania
[3]József & Erzsébet Tóth Endowed Hydrogeology Chair, Department of Geology, Institute of Geography and Earth Sciences, ELTE Eötvös Loránd University, H-1117, Budapest, Pázmány P. stny 1/C, Hungary

ABSTRACT

In the present study, water samples from 8 sites known as: Taploca, Nagy-borvíz, Piricske (located in Romania) and Szent Jakab, Kiskút, Kossuth Lajos, Polányi kút and Berzsenyi (located in Balaton highland in Hungary) and characterized with low nutrient content, were studied using cultivation independent methods. Diversity indices and cell counts were determined to assess the species richness in relation to the cell counts within the samples. Next generation sequencing was used to reveal the existing microbial community, and taxon specific PCR was used to detect the presence of some species with hygienic. 18 bacterial phyla above a ratio of 2% were identified in addition to 13 archaeal phyla using amplicon sequencing.

Nagy-borvíz water sample showed a unique presence of *Chloroflexi*, *Bacteroidota*, *Desulfobacterota*, *Acidobacteriota* and *Actinobacteriota*. Piricske water sample showed mostly *Caldisericota* and *Spirochaetota*. Taploca water sample contained a unique presence of *Planctomycetota* and *Myxococcota* at the same time with Szent Jakab water sample which showed an additional presence of *Chloroflexi*, Berzsenyi contained *Campylobacterota* at the same time with *Polányi kút* which had important fractions of *Nitrospirota* and *Desulfobacterota*. Finally, Kossuth Lajos water sample was dominated by *Fusobacteriota,* mainly members of *Hypnocyclicus* genus.

Piricske and especially Nagy-borvíz water samples contained important fractions of *Altiarchaeota*, moreover, *Piricske* contained high fraction of *Euryarchaeota* phylum -most of them belong to Methanobacteriaceae family.

Nitrosopumilaceae dominated Taploca and Kiskút in addition to their important presence in the rest of the samples. Diversity indices and cell count showed in general that cell count values are tending to be lower when the diversity is higher. The results of taxon specific PCR reactions showed that water treatment would be essential in some water samples.

Introduction

Recently, natural mineral water consumption has risen all over the world, becoming an alternative for tap water in many countries [1], Most

***Corresponding author(s)**

Marwene Toumi, Department of Microbiology, Eötvös Loránd University, H-1117, Budapest, Pázmány P. Stny 1/C, Hungary

Tel: +36-702-411-242
Email: marwenetoumi@outlook.com

DOI: 10.37871/jbres1701

Submitted: 05 March 2023

Accepted: 19 March 2023

Published: 22 March 2023

Keywords

- Microbial community
- Diversity
- Nutrient depleted aquatic environments
- NGS
- Total cell counts
- Taxon-specific
- Multitemplate PCR

BIOLOGY GROUP

MICROBIOLOGY

VOLUME: 4 ISSUE: 3 - MARCH, 2023

How to cite this article: Toumi M, Máthé I, Tóth Á, Sztráda N, Farkas R, Jurecska L, Tóth E. Microbial Community Associated with Ground waters Discharge in Transylvania (Romania) and Balaton Highland (Hungary). 2023 Mar 22; 4(3): 485-501. doi: 10.37871/jbres1701, Article ID: JBRES1701, Available at: https://www.jelsciences.com/articles/jbres1701.pdf

of these waters are taken from naturally discharging springs or groundwater wells. These waters represent complex ecosystems with high diversity of microorganisms [2]. Taking into consideration that the European Union directive, restrains any water treatments aiming the removal or destruction of the autochthonous microorganisms, natural mineral waters are distributed containing the original microorganisms of the sources [2].

While distributing natural mineral water with its original microorganisms from the source can have some benefits such like keeping the microbial diversity and the water quality, there are also potential risks that need to be considered.

These risks include:

Microbial contamination: Natural mineral water can contain microorganisms, including bacteria and viruses that may pose a risk to human health.

Unpredictable microbial activity: The microbial activity can be unpredictable, and certain microorganisms may become dominant under other conditions. This can lead to changes in the water quality, which may not be desirable for consumers.

The identification and characterization of the autochthonous microbial community of the ground waters is of great importance for several reasons:

Understanding microbial ecology: Groundwater microbial communities play a crucial role in biogeochemical cycling of nutrients, such as carbon, nitrogen, and sulfur. The identification and characterization of these communities help us understand the ecological roles and functions of different microbial groups and their interactions with each other and the environment.

Monitoring environmental changes: Groundwater microbial communities are highly sensitive to changes in environmental conditions, such as changes in temperature, pH, and nutrient availability. Monitoring changes in these communities can provide valuable information about changes in the groundwater ecosystem and can help us identify potential environmental threats or contamination. The aim of the present study was performed on samples collected from groundwaters.

Three of the analysed springs are issuing from carbonate groundwaters located in the southern part of Harghita County, Romania. This region is characterized with an average temperatures that fall between 20 degrees celsius (68˚F) and 25 degrees celsius (77˚F) during the month of June, July, August and September. The coldest month is January with an average maximum temperature of -2˚C (27˚F). The precipitation amounts to 595 mm/year.

The other five investigated springs are situated in Balaton Highland region, this area is characterized with a humid continental climate, but the effects of the Mediterranean influence can be detected in the sunshine duration and annual mean temperature (11˚C). The amount of precipitation, which falls predominantly in the form of rain is 500 to 600 mm/year. The Balaton Highland is the most extensive carbonate aquifer system in Hungary. The region is abundant in naturally discharging springs of various temperatures, creeks and wetlands.

The aim of this study is to: 1). Check the microbial cell counts and diversity indices in different ground waters. 2). Reveal the microbial community structure in these systems using cultivation independent method (NGS) and 3: determine the risks and possible antibiotic resistance among the detected bacteria.

Materials and Methods

Description of the hydrogeological properties of the sampling sites

Three of the analyzed springs are located in the southern part of Harghita county, Romania: Taploca spring (46.3697˚N, 25.8055˚E) is located in the Csíktaploca (Toplița Ciuc), Nagy-borvíz spring (46.3753˚N, 25.8193˚E) (borvíz ["wine water"] means CO_2 rich mineral water) is located in Csíksomlyó (Şumuleu Ciuc) near the pilgrimage site. Today Csíktaploca and Csíksomlyó villages are part of the Csíkszereda (Miercurea Ciuc) town. Piricske spring (46.3696˚N, 25.7954˚E) is found in the forest close to Csíkszereda. Piricske is a freshwater spring. Taploca and Nagy-borvíz springs are CO_2^- rich mineral waters, their dominant ions are HCO_3^-, Na^+, Ca^{2+}, Mg^{2+} and Fe^{2+} [3]. The hydrochemical character of these discharging groundwater is influenced and determined by the geological structure and groundwater-rock interaction resulting in high mineralization of NaCl, sulfate and dissolved gases (CO_2, H_2S) [4]. Five of the investigated springs are situated in Balaton Highland region: Szent Jakab spring of Vászoly (46.9433˚N, 17.7580˚E) and Kiskút of Szentantalfa (46.9126˚N, 17.6745˚E) are located in the elevated hills (~280 and ~190 m above sea level)

and the Kossuth Lajos (46.9561˚N, 17.8950˚E), Polányi kút (46.9434˚N, 17.8669˚E) (Szekér Ernő outflow) and Berzsenyi spring (46.9465˚N, 17.8739˚E), can be found in Balatonfüred near the shoreline of the Lake Balaton (~105-115 m above the sea level). These springs issue from carbonate aquifers or at the boundary of sandstone, metamorphic and carbonate formations, where a hydraulic barrier forces groundwater discharge [5]. The water temperature in the elevated parts is close to the mean annual temperature of the air (~10-11˚C) because these springs are fed by local and shallow groundwater flow. In turn, the springs of Balatonfüred receives a deeper groundwater flow component which is responsible for the higher water temperature (~14-17˚C) and the natural occurrence of the CO_2 [6]. The figure bellow is showing the location of the different samples.

Collection of water sample

The water sample located in Romania (Taploca, Nagy-borvíz, Piricske) and in Balaton Highland region (Szent Jakab, Kiskút, Kossuth Lajos, Polányi kút and Berzsenyi) were collected from the spring's water outflow. The sampling was carried out during the period from 2018 to 2021. The water samples (2-2 L) were aseptically collected into clean, sterile, glass bottles according to ISO 19458:2006 standard, transferred at 4TFFC in a cooler bag and filtered for cell count determination and molecular studies immediately upon arrival at the laboratory.

Determination of the physical and chemical parameters of the samples

The physical and chemical parameters of the samples were determined by measuring each sample in replicates to ensure accuracy and precision.

The pH and temperature were measured on site using a Hach HQ40D portable multimeter (Hach, Loveland, CO, USA). All other parameters mentioned bellow were determined in the laboratory according to standard methods [7]. Nitrite ion (ASTM 4500-NO_2^--B) was measured following the colorimetric method, using Hach DR2000 spectrophotometer (Loveland, Colorado, USA). Nitrate ion (ASTM 4500-NO^{3-}-B) was measured using the Ultraviolet Spectrophotometric Screening Method, using Perkin Elmer Lambda 35 UV/VIS spectrophotometer (Waltham, Massachusetts, USA). Sulfate ion (ASTM 4500-SO_4^{2-}-E) and iron (3500-Fe-D) were measured with Hach DR2000 spectrophotometer (Loveland, Colorado, USA). The amount of total organic carbon (TOC, ASTM 5310-B) Combustion-Infrared Method was determined by a Multi N/C 2100S analyser (Analytik Jena, Germany). Ortophosphate (ASTM 4500-P-E) was measured following the Ascorbic Acid Method, Hach DR2000 spectrophotometer (Loveland, Colorado, USA).

Determination of the total cell counts of the samples

200 ml from each replicate of each water sample was filtered using a polycarbonate membrane filter (0.2 µm GTTP, Millipore, USA), the obtained filters were treated with 2% paraformaldehyde solution (Sigma-Aldrich, Germany) dissolved in 0.1 m phosphate buffer (NaH_2PO_4 3.2 g, Na_2HPO_4 10.9 g in 1000 ml distilled water, pH 7.2) overnight in order to fix the cells. The obtained filters were stored at -20˚C until further analysis. Microscopic cell counts were determined using Nikon80i epifluorescent microscopy and NisElements program package according to [8].

The images were analyzed using the image analysis software to count the cells in each image, and the mean cell count was calculated across the 20 images.

DNA extraction from the water samples and amplicon sequencing

For DNA extraction, 250 ml water sample from each replicate was filtered using a 0.22 µm pore size sterile mixed cellulose filter (MF-Millipore GSWP04700, Billerica, MA, USA) using DNeasy® PowerSoil® DNA Isolation Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions [9].

A mechanical cell disruption was performed by shaking the filters at 30 Hz for 2 min using a Retsch Mixer Mill MM400 (Retsch, Haan, Germany).

To perform the PCR reactions 3 µl quantity of the template DNA was used. The PCR reaction for amplifying the 16S V4 region was done in triplicate based on the following protocol: 98˚C for 3 min; 25 cycles: 95˚C for 30s, 55˚C for 30s and 72˚C for 30s; and 72˚C for 5 min for bacteria and 98˚C for 3 min; 25 cycles: 95˚C for 30s, 60˚C for 30s and 72˚C for 30s; and 72˚C for 10 min for archaea using the following primers: CS1-TS-B341F and CS2-TS-805NR for bacteria [10] and CS2-TS-Arch-855R and CS1-F-A519F for archaea [11]. Before sequencing, DNA concentration of the PCR products was determined using a Qubit meter (Invitrogen life technologies corporation, Austria) and a minimal concentration of 4 ng/µl and 50µl of PCR product was respected.

Illumina MiSeq platform with MiSeq standard v2 chemistry was used by the Genomics Core Facility RTSF, Michigan State University to perform the amplicon sequencing.

The forward and reverse fastq files obtained from Illumina sequencer, were processed and analyzed using the Mothur v1.41.5 software [12] following the protocol of Benedek [13]. Deltaq was adjusted to 10 with the command "make.contigs", for chimera detection UCHIME [14] was used within the mothur commands, singletons were removed according to Kunin [15].

The sequences taxonomy were classified using he ARB-SILVA SSU Ref NR 138_1 reference database [16], bootstrap confidence score is more than 80 with 1000 iterations. Based on the taxonomic classification output, both non-archaeal and non-bacterial sequences were removed from the analyses.

The Operational Taxonomic Units (OTUs) were determined using a distance matrix with distances larger than 0.3 [17].

Eventually, the Mothur's commands, sub sample was used to normalize the data, in fact reads were subsampled to the read number of the sample having the lowest sequences count.

The command rarefaction single was used to calculate the rarefaction curve in addition to Shannon-Weaver and inverse Simpson (1/D) diversity indices and Chao-1 and ACE (Abundance-based Coverage Estimator) richness metrics.

Sequence reads were deposited in the NCBI SRA database and are accessible through the Bio Project ID PRJNA628507, under the references: SRS6537389 for Taploca, SRS6537391 for Piricske, SRS6537423 for Nagy-borvíz, SRS9983636 for Berzsenyi, SRS9983634 for Polányi kút, SRS9983633 for Kossuth Lajos, SRS9983477 for Kiskút and SRS9983632 for Szent Jakab.

Taxon specific and multitemplate PCR reactions

The presence of the following bacterial groups was tested using taxon specific/multitemplate PCR reactions, using the same DNA extracted for NGS analysis: coliform bacteria and *E. coli* according to Bej AK, et al. [18]; *Acinetobacter baumannii* based on Huang LY, et al. [19] *Legionella* sp. [20]. *Stenotrophomonas maltophilia* [21]; *Legionella pneumophila* [22]. *Pseudomonas aeruginosa* according to Lavenir R, et al. [23]. The ESBL producing bacteria were tested by multiplex PCR according to Trung NT, et al. [24] and the presence of macrolide resistance genes by simultaneous detection of 5 genes (ermA, ermB, ermC, msrA, mef) according to Zmantar T, et al. [25].

Statistical analysis

Environmental variables (physical and chemical parameters), diversity indices, cell counts and the revealed OTUs (archaea and bacteria) were represented by Principal Components Analysis Ordination (PCA) put together with vector-fitting [26].

In order to fit the variable as vectors, the "envfit" function from the vegan package was applied [27].

Shannon diversity index was calculated in order to assess the population diversity within the samples based on Operational Taxonomic Units (OTUs) [28].

Results

Physical and chemical parameters of the water samples

Physical and chemical parameters of the different water samples are given in table 1.

Microscopic cell counts and diversity indices of the samples

The relationship between the number of bacteria (cell count values) and diversity among the samples is shown on figure 1. The cell count values of Piricske water sample were higher than all the other samples by one order of magnitude (10^6) (Table 1). The majority of the samples had similar Shannon diversity index values between archaea and bacteria. The exception was detected in the case of Kossuth Lajos water sample where it was the most diverse in term of archaea and the least diverse in case of bacteria, the opposite was observed in the case of Nagy-borvíz water sample. A general trend was seen within the majority of the samples (except Kossuth Lajos) showing that the cell count values is tending to be lower when the diversity is higher (Figure 2) (Supplementary Tables 1,2).

Bacterial community composition of the different samples based on amplicon sequencing

The results of amplicon sequencing identified 18 bacterial phyla presented within a ratio higher than 2% in at least one of the 8 samples. The results of the rarefaction curves (Supplementary figure 1) showed that the sequencing depth was sufficient to identify the majority of the bacterial taxa.

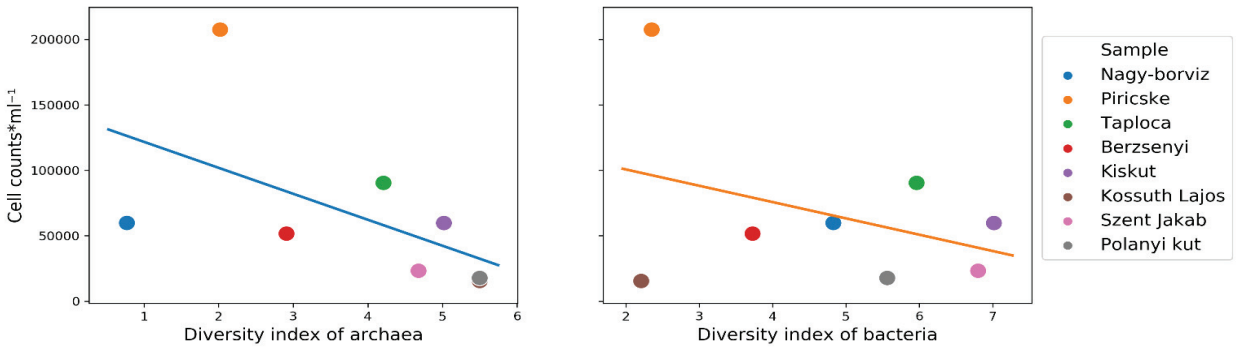

Figure 1 Relationship between the cell count values and Shannon diversity indices of the samples.

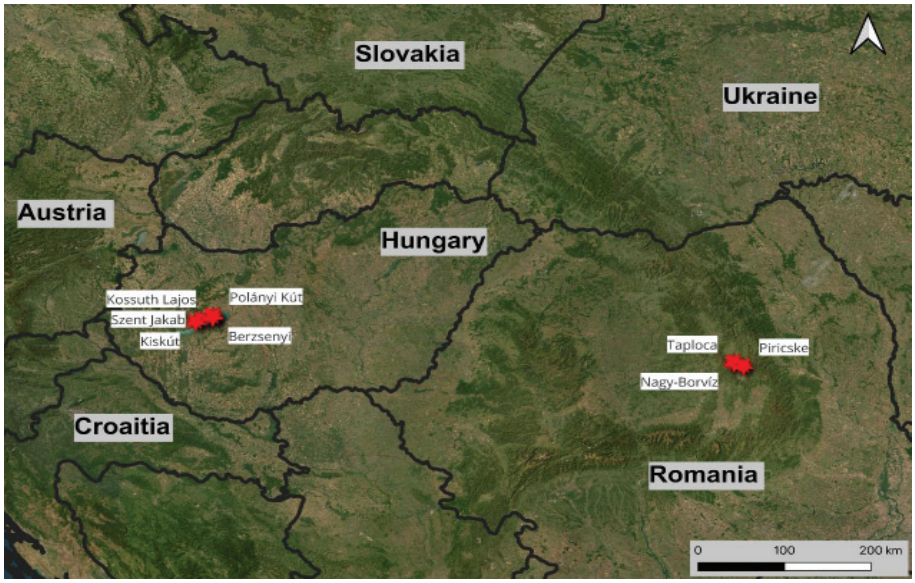

Figure 2 Sampling sites map.

Table 1: Physical and chemical parameters and cell count values of the samples.

Parameter	Nagyborvíz	Piricske	Taploca	Berzsenyi	Kiskút	Kossuth Lajos	Szent Jakab	Polányi kút
T (˚C)	14.1	8.5	17.7	17.2	15	17.1	19.7	18.5
pH	6.18	6.74	6.08	6.23	7.19	6.66	7.04	6.23
conductivity (µS/cm)	2785	163	1490	1627	913	1185	581	1526
ORP[1] (mV)	48.1	14.8	27.8	55.9	25.9	49.6	18.6	50.1
Oxygen potential (mgl^{-1})	0.63	5.16	0.39	0.58	5.25	4.01	7.07	0.70
TOC[2] (mgl^{-1})	1.72	2.81	0.85	0.50	1.40	1	13	8.40
NO_3^- (mgl^{-1})	< 0.50	3.10	< 0.50	1.70	22	1.50	19	1.60
NO_2^- (mgl^{-1})	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10
NH_4^+ (mgl^{-1})	1.38	0.06	2.66	0.10	< 0.10	0.10	< 0.10	0.50
SO_4^{2-} (mgl^{-1})	3.9	6	2.7	275	18	73	18	210
Fe (mgl^{-1})	8.63	0.07	5.42	2	< 0.10	1.80	< 0.10	2.40
PO_4^{3-} (mgl^{-1})	0.31	0.14	0.79	0.20	0.50	< 0.10	0.10	0.20
Cell count *ml^{-1}	$5.9^{*}10^4$	$20.7^{*}10^4$	$9.0^{*}10^4$	$5.1^{*}10^4$	$5.9^{*}10^4$	$1.5^{*}10^4$	$2.3^{*}10^4$	$1.7^{*}10^4$

Verrucomicrobia phylum was present in all samples with high abundance (ranging from 3% to 15%) except Piricske water sample. Most of the identified members of this phylum belongs to the Omnitrophaceae family.

All samples had distinct bacterial community. In fact, Nagy-borvíz water sample showed the presence of remarkable fractions of *Chloroflexi*, *Bacteroidota*, *Desulfobacterota*, *Acidobacteriota* and *Actinobacteriota*. Piricske water sample was dominated by *Caldisericota*, also showed the presence of important fraction of *Spirochaetota*. Taploca water sample contained a unique presence of *Planctomycetota* and *Myxococcota* at the same time with Szent Jakab water sample which showed an additional presence of *Chloroflexi*, *Cyanobacteria*, *Bacteroidota* and *Actinobacteriota*. Berzsenyi contained *Campylobacterota* at the same time with *Polányi kút* which had important fractions of *Nitrospirota* and *Desulfobacterota*. Finally, Kossuth Lajos water sample was dominated by *Fusobacteriota* which contained mainly members of *Hypnocyclicus* genus.

All samples were characterized by the abundance of *Proteobacteria* (Figure 3), and *Patescibacteria*. The phylum *Patescibacteria* contained high ratio of *Parcubacteria* and many candidatus genera like *Falkowbacteria*, *Magasanikbacteria*, *Azambacteria*, etc. Within the *Proteobacteria* (Figure 4). Nagy-borvíz water sample showed the presence of the families Comamonadaceae, Hydrogenophilaceae and Nitrosomonadaceae, Piricske water sample contained mainly members of the families Pseudomonadaceae and *Spirochaetota*, Taploca

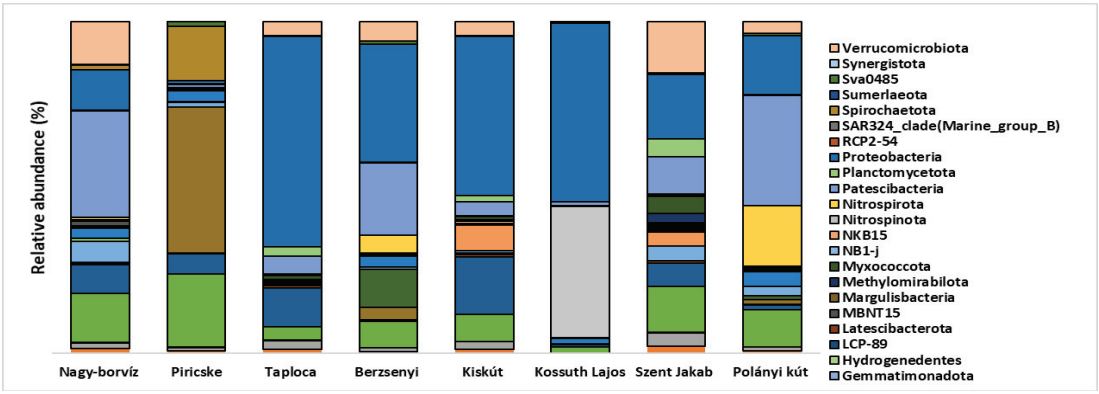

Figure 3 Distribution of the abundant (98%) bacterial phyla based on 16S rRNA gene amplicon sequencing in the water samples.

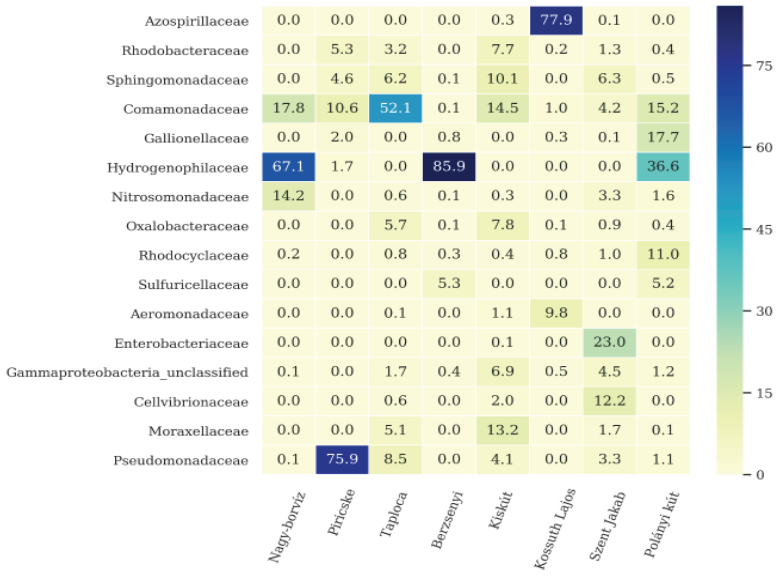

Figure 4 Heat-map of microbial community composition within the Proteobacteria. The colour intensity in each panel shows the percentage ratio of the given taxon in a sample, referring to the colour key at the right.

water sample is characterized by a high presence of the Caulobacteraceae family. Berzsenyi water sample contained mostly Hydrogenophilaceae and Sulfuricellaceae. Kiskút water sample contained unclassified both *Alphaproteobacteria* and *Burkholderiales*, Rhizobiaceae, Sphingomonadaceae, and Moraxellaceae. The *Proteobacteria* community of Kossuth Lajos water sample was split mainly between Aeromonadaceae and Azospirillaceae. Szent Jakab was not dominated by any family - similarly to Kiskút water sample - because of the high diversity within the *Proteobacteria* phylum in these samples, however, important fractions of Cellvibrionaceae and Enterobacteriaceae were identified, the latter family contained members of the genera *Lelliottia* and unclassified Enterobacteriaceae. Polányi kút contained a relatively important fraction of the three families Comamonadaceae, Gallionellaceae and Hydrogenophilaceae.

Archaeal community composition of the different samples based on amplicon sequencing

Altogether, 13 archaeal phyla were detected in the water samples (Figure 5). The rarefaction curves of the samples (Supplementary figure 2) showed that the sequencing depth was sufficient to recover the majority of the archaeal taxa.

Unclassified Archaea, *Nanoarchaeota*-belonging to the *Woesearchaeales* order -, *Thermoplasmatota*, *Halobacterota* - low fraction within Taploca water sample - and *Crenarchaeota* phyla were present in all the samples.

Thermoplasmatota phylum contained mainly unclassified families characterizing both Nagy-borvíz and Piricske water samples, the rest of the samples contained unclassified *Thermoplasmata*. Concerning *Crenarchaeota* phylum Nagy-borvíz and Piricske water samples contained mostly unclassified *Crenarchaeota* in addition to *Bathyarchaeia* taxa in case of Nagy-borvíz. The rest of the samples contained variable fractions of taxa belonging to the *Nitrososphaeria* class.

Piricske and especially Nagy-borvíz water samples contained important fractions of *Altiarchaeota*, moreover, *Piricske* contained higher fraction of *Euryarchaeota* phylum-most of them belong to Methanobacteriaceae family. With the exception of Piricske water sample, all the rest of the samples contained variable fractions of *Micrarchaeota.*

Among the families in the *Crenarchaeota* phylum, all samples contained the following ammonia oxidizing archaea (figure 6) except Nagy-borvíz and Piricske water samples:

Nitrosopumilaceae: dominated Taploca and Kiskút with important presence in the rest of the samples, Nitrososphaeraceae is mainly present in Kiskút, unclassified *Nitrososphaeria* is present with important fraction in Szent Jakab. Lastly, Nitrosotaleaceae is present with important fractions in Taploca, Kiskút and Szent Jakab.

Results of taxon-specific and multitemplate PCR reactions

The genus *Legionella* was present in all the samples except Piricske water sample, but not in any case *L.*

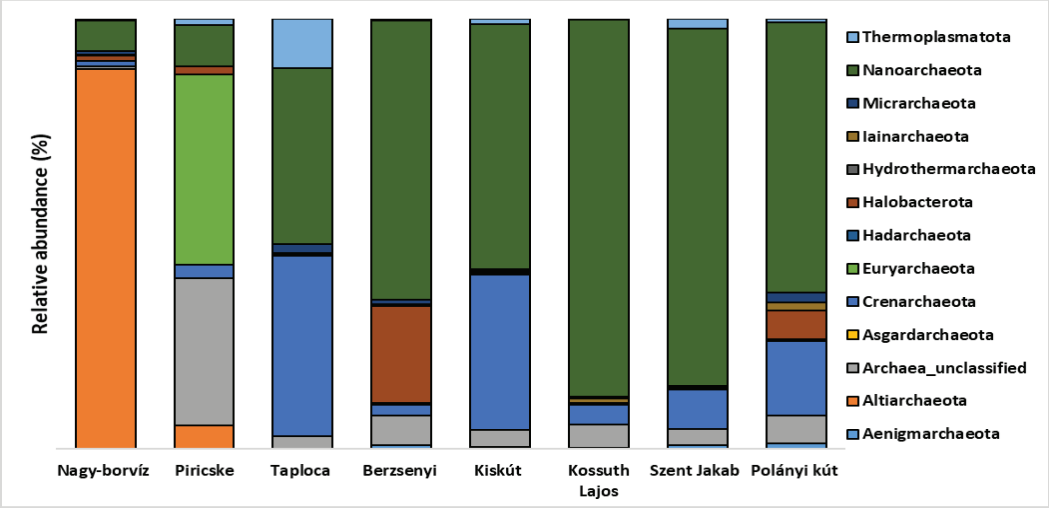

Figure 5 Distribution of the abundant (98%) archaeal phyla based on 16S rRNA gene amplicon sequencing in the water samples.

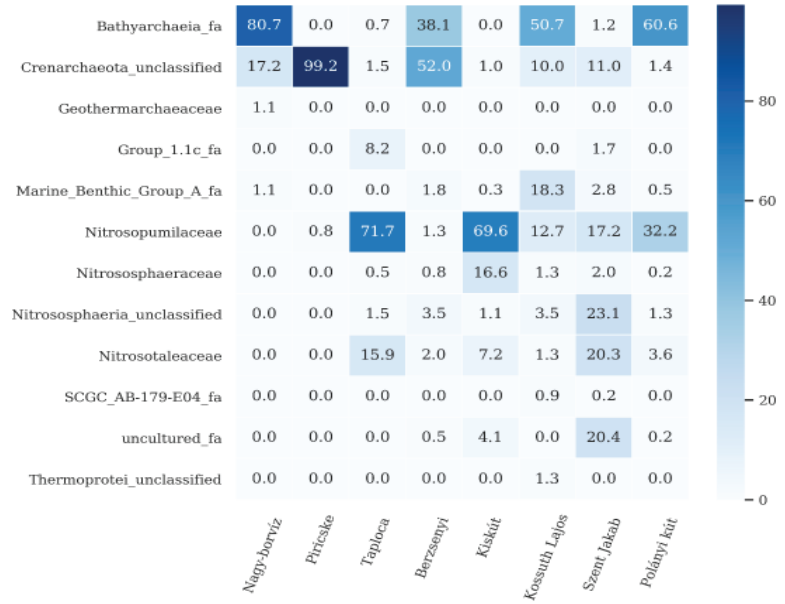

Figure 6 Heat-map of microbial community composition within the *Crenarchaeota phylum*. The colour intensity in each panel shows the percentage in a sample, referring to the colour key at the right.

pneumophila was detected. *Pseudomonas aeruginosa*, *E. coli* or coliform bacteria were not present in the samples. The presence of *Stenotrophomonas maltophilia* (being an emerging facultative pathogen bacterium) which is a potential cause of concern, was present in six of the samples. On the other hand, neither ESBL producer, nor macrolide resistant bacteria were detected (Table 2).

Discussion

Microbial communities of the different samples based on amplicon sequencing

According to the results of Kuznetsov SI, et al. [29], oligotrophic environments are characterized by less than 1.36 mg/L suspended organic substance and 15.24 mg/L for dissolved organic material. More recent studies such like the one obtained by Ho A, et al. [30] confirmed that oligotrophs are characterized by their ability to grow under low substrate concentrations (e.g. carbon in the nano and picomolar range).

TOC values of our samples are lower in each case therefore all samples can be stated oligotrophic.

All samples were characterized by a high percentage of unclassified and uncultured OTUs, table 3 and table 4 further demonstrate this large fraction, in fact over 27% in case both Archaea and Bacteria were not classified with any specific taxa and were therefore determined as Unclassified organisms.

Previous studies show that low nutrient content environments and groundwaters discharge data sets are usually dominated by unknown taxa. Previous studies of Lopez-Fernandez M, et al. [31] and Gayner NJ [32] showed that almost 50% of the identified phyla in groundwater samples were archaeal or bacterial candidates, moreover, the percentage of unknown and candidate phyla increased with depth, which highlights the importance of further studies to characterize deep biosphere microbial communities.

Even thought, a high percentage of OTUs affiliation are unclassified taxa, many of the classified ones are associated with previously known groundwater and poor nutrient environments microbes.

Many taxa are recently discovered as CPR and DPANN organisms.

Woesarchaeota, presented in a relatively large proportion of this microbial dataset (10%), especially in Szent Jakab and Kossuth Lajos water samples with a percentage of 16.92 % and 17.55%, respectively. They are part of DPANN superphylum, previous studies based on genome analyses and metabolic reconstructions propose that they are anaerobic heterotrophic organisms with a potential of symbiotic relationships especially with methanogens [33,34]. This feature can remove the thermodynamic bottlenecks and enables several metabolic reactions under nutrient-depleted conditions [35]. Some

Table2: Results of taxon-specific and multitemplate PCR reactions (positive: +, negative: -).

Sample name	Stenotrophomonas maltophilia	Pseudomonas aeruginosa	Legionella spp	Legionella pneumophila	E.coli/coliform	Acinetobacter baumannii	NOB (nitrite-oxidizing bacteria)	ESBL (Extended Spectrum Beta-Lactamase) producer bacteria	Macrolide resistance
Nagy-borvíz	+	-	+	-	-	-	+	+	-
Piricske	-	-	-	-	-	-	-	-	-
Taploca	+	-	+	-	-	-	+	-	-
Berzsenyi	-	-	+	-	-	-	-	-	-
Kiskút	+	-	+	-	-	-	-	-	-
Kossuth Lajos	+	-	+	-	-	-	-	-	-
Szent Jakab	+	-	+	-	-	-	-	-	-
Polányi kút	+	-	+	-	-	-	-	-	-

Table 3: Top 2 archaea taxa affiliations. Known taxa affiliations from OTU taxon classification were condensed to display the percentage of that taxon in the dataset.

Taxonomy	% Across Dataset
Archaea; Nanoarchaeota; Nanoarchaeia; Woesearchaeales; Woesearchaeales_unclassified; Woesearchaeales_unclassified	15.91
Archaea; Archaea_unclassified; Archaea_unclassified; Archaea_unclassified; Archaea_unclassified; Archaea_unclassified	7.91

Table 4: Top 2 bacteria taxa affiliations. Known taxa affiliations from OTU taxon classification were condensed to display the percentage of that taxon in the dataset.

Taxonomy	% Across Dataset
Bacteria; Bacteria_unclassified; Bacteria_unclassified; Bacteria_unclassified; Bacteria_unclassified; Bacteria_unclassified	10.4
Bacteria; Proteobacteria; Gammaproteobacteria; Burkholderiales; Comamonadaceae; Comamonadaceae_unclassified	1.84

studies present Woesarchaeota as fermenters with iron or methane metabolism [36].

Amplicon sequencing showed the presence of members of Thermoplasmatota phylum, known previously of inhabiting not only extreme environments but also a wide range of environments [37]. Taxa existing within this phylum could be classified only in case of Nagy-borvíz and Piricske water samples where mainly they belonged to Marine_Benthic_Group_D (Thermoprofundales) and DHVEG-1 (Thermoplasmata).

Based on few available cultures and genomes of Thermoplasmata, researchers could have an insight on their myxotrophic lifestyle [38] which is crucial for their sustainability in oligotrophic ecosystems in order to compensate the lack of organic nutrients [39].

Shuming MCJ, et al. [40] demonstrated that Crenarchaeota and Halobacterota had relatively complete gene families involved in dissimilatory sulfate reduction, these findings are in accordance with the chemistry analyses as Taploca water sample (characterized with the lowest fraction of Halobacterota) contained the lowest amount of SO_4^{2-}. Sulfate-Reducing Bacteria (SRB) and Sulfate-Reducing Archaea (SRA) can use sulfate as a terminal

electron acceptor in anaerobic respiration. During this process, sulfate is reduced to sulfide, which can lead to the accumulation of hydrogen sulfide gas in the water. Hydrogen sulfide gas can be toxic to other aquatic microorganisms.

Among the families under the *Crenarchaeota* phylum, all the samples except Nagy-borvíz and Piricske water samples contained the following ammonia oxidizing archaea: Nitrosopumilaceae [41], Nitrososphaeraceae [42] and Nitrosotaleaceae [43] (Figure 6). These Archaea are responsible for the first step of nitrification, which is the oxidation of ammonia to nitrite. The produced nitrite is then oxidized by other microbes to nitrate, which can accumulate in the spring water.

Most of these samples contained higher values of NO^{3-} (Table 1), this can be explained by the high rates of nitrification in which certain bacteria oxidize ammonia to nitrite, and then further oxidize nitrite to nitrate. This process can increase the nitrate concentration in spring water [44].

Nagy-borvíz water sample which is characterized by high ammonia content, contained members of Nitrosomonadaceae family which are lithoautotrophic ammonia oxidizing bacteria [45]. In addition, species from the phyla candidate division Zixibacteria-found especially in Piricske water sample and Chloroflexi-present in all the samples also known to contain nitrification genes [46].

Candidatus Parcubacteria, considered as a part of CPR superphylum, members of this phylum are known to harbour a variety of metabolisms with the possibility of acquiring fermentative processes able to produce acetate, ethanol, lactate, and hydrogen [32]. Previous genomic analyses of Parcubacteria revealed the existence of nitrite reductases which can transform nitrite to produce nitric oxide (gene nirK) and ammonium (gene nirB) [36].

The predominance of the superphylum Patescibacteria in groundwaters is often related to their mobilization from soils and their good survival under oligotrophic conditions [47]. Co-occurrence network analysis pointed to potential associations of Patescibacteria with specific organisms involved in nitrogen, sulfur and iron cycling [47]. Other capability of Candidatus Patescibacteria members in oligotrophic habitats is their abundance under ultra-small cells and acquirement of reduced genome size (pass through 0.2 µm pore size filter) [48,49]. These features are thought to be evolutionarily advantageous, as the increased surface-to-volume ratio optimizes the uptake of the sparse nutrients [50] and the loss of expendable genes leads to a lower metabolic cost of reproduction [51], these characteristic are also seen in Caldisericota genome [52] which was revealed in Piricske water sample.

Chloroflexi phylum known to harbour many phototrophic microorganisms - was present in all water samples. Members of this phylum are correlated with the fraction of TOC level within the environment, this explain their abundance in all the samples as they are characterized with TOC values below the oligotrophy level [53].

The rest of the OTUs were specific to one or many water samples, among them:

Altiarchaeaceae: High presence in Nagy-borvíz and Piricske water samples, literature shows that they predominate anaerobic groundwater due to evolved metabolic and structural features where they may represent an important carbon dioxide sink [54], cultivated member of this family has autotrophic metabolism [54].

Methanogenic archaea: They are present in many samples in different families, Methanobacteriaceae and Methanoregulaceae in Piricske, Methanoperedenaceae in Nagy-borvíz, Berzsenyi and Polányi kút water samples. They are able to thrive in nutrient-poor, low ionic-strength environments [55].

Sulfate-Reducing Bacteria (SRB): The phylum Desulfobacterota-found in most samples - contains sulfate-reducing and sulfate related fermentative and syntrophic taxa [56]. Cultured Desulfobacterota showed preference for anoxic conditions, and many utilize sulfate, sulfite, thiosulfate, elemental sulfur, or iron and amino acids) [60].

Members of the Sphingomonadaceae as terminal electron acceptor in respiratory and/or disproportionation processes [57].

The phylum Nitrospirota - found in many samples - currently includes a limited number of bacterial species with validly published names, among them Sulfate-Reducing Bacteria (SRB) [58], these taxa contain gene set for dissimilatory sulfate reduction [59].

Among proteobacteria phylum: Azospirillum

genus dominated Kossuth Lajos water sample, it can use NH^{4+}, NO_3^-, amino acids and N_2 as nitrogen sources for growth. They can grow under anaerobic conditions using nitrate as electron acceptor, microaerobic (N_2 or NH_3 as nitrogen sources) and fully aerobic conditions with combined nitrogen only (NH_3, NO^{3-} family were detected in all the samples except Nagy-borvíz and Kossuth Lajos water samples (Table 5).

Iron oxidisers: Polányi kút water sample contained important ratio of the family Gallionellaceae where its members belong to Gallionella and Sideroxydans genera. Both are adapted to chemolithoautotropy [67]. Chemistry measurements showed important fraction of iron within that sample.

Hydrogenophilaceae: Most members of this family are chemolithotrophic using various inorganic electron donors such as reduced sulfuric compounds or hydrogen [32]. Sequences at the genus level could not be classified however, their high abundance in Nagy-borvíz, Polányi kút and Berzsenyi water samples - characterized with medium to high SO_4^{2-} content comparing to the other samples can give an insight about their potential role as sulfate reducers. This is endorsed also by the presence of members of sulphur oxizing bacteria "sulfuricellaceae" in the last two mentioned samples.

Certain orders of Proteobacteria such as Burkholderiales, have been associated with nutrient poor conditions [68].

Influence of the environmental factors on the diversity of archaeal and bacterial communities.

It is known that hydrogeological and other ecological factors influence the microbiological processes [69]. On the other hand, the metabolism of microorganisms usually affects the water quality of groundwater systems [69]. In addition, the fate and transport of microorganisms in groundwaters are the result of their physicochemical characteristics (flow velocity, gain, size, porosity, solid organic carbon content, temperature, pH, and other chemical characteristics of the water) [70].

In order to assess the effect of environmental factors on the prokaryotic communities, the PCA ordination was performed (Figures 7,8). The analysis revealed the impact of chemical characteristics ($p < 0.1$) on archaea and bacteria OTUs distribution.

Microorganisms have an important role in mediating reactions in aquifers that affect geological processes, at the same time physical and chemical variations result in changes in microbial communities [71]. Nagy-borvíz and Piricske water samples were grouped together, they contained common presence of Altiarchaeum and Methanobacterium taxa (Figure 7). In previous studies, these two taxa appeared together in many other environments [72] where CO_2 fixation could be carried out by members of the class Altiarchaeia meanwhile Methanobacterium utilize H_2/CO_2 as sole carbon and energy source. These two water samples are located in the Transylvanian region characterized with the high presence of dissolved gases (CO_2, H_2S).

Taploca, Szent Jakab and Kiskút water samples were grouped together due to the presence of OTUs related to the nitrogen cycle (as an example: Nitrosopumilaceae and Nitrosoarchaeum). These water samples contained the highest fractions of either NO_3^- or ammonia among the samples.

Sulfate had an important impact on the archaeal community structure of Berzsenyi, Kossuth Lajos and Polányi kút (Figure 7). The most abundant taxa in

Table 5: Most abundant members of the family Sphingomonadaceae within the samples and their potential ecological role.

Genus	Sample name	Description
Blastomonas	Berzsenyi Kiskút	Can be isolated from oligotrophic environments [61], characterized with slow growing, this change in growth dynamics can increase the cell volume [62].
Novosphingobium	Taploca Kiskút	Known with high genomic and functional plasticity allowing it to rearrange its genome according to environment variations and adapt their metabolic capabilities to better cope with severe conditions (degrade a wide range of aromatic hydrocarbons) [63].
Sphingomonas	Taploca Kiskút	Has the ability to degrade the copper pipes in drinking water distribution systems (White, et al. 1996).
Sphingopyxis	Kiskút	Has the capacity to thrive in harsh environments [65], commonly isolated also from freshwater and marine habitats and many are facultatively chemolithotrophs, often producing H_2 during their metabolism [33].
Sphingorhabdus	Kiskút Szent Jakab	Isolated from diverse environments [66]

these samples is Methanoperedens (known previously to perform anaerobic oxidation of methane coupled to nitrate reduction [73]). The study of Timmers [74] showed that this taxon was found to be abundant in aquifers where methane and sulfate were present, moreover the absence of nitrate-dependent anaerobic oxidation of methane activity in the studied samples presented Methanoperedens as an anaerobic methane oxidizer coupled to Sulfate reduction.

Polány Kút, Berzseny and Nagy-borvíz water samples were related with the presence of Gallionellaceae and Desulfobulbales (Figure 8), they are known as ferrous iron-oxidizing and sulfate-reducing bacteria. These samples contained important fractions of iron (Table 1) allowing the abundancy and activity of these microbes in oxic/anoxic interfaces of sediments in groundwaters [75].

Taploca, Szent Jakab and Kiskút were grouped together based in the abundance of both archaeal and bacterial OTUs, they had a common presence of the families Comamonadaceae, Sphingomonadaceae and Rhodobacteraceae. They were mainly chemoheterotrophs with fresh water is their primary habitat. They were deeply involved in carbon biogeochemical cycling with many species possess oligotrophy characters [76,77].

Hygienic of the existing microbial communities in the water samples

The results of taxon specific PCR reactions showed that in some cases water treatment would be essential before use. Legionella species, known to be widespread in natural aquatic systems, and also in aquifers and pipelines pump stations [78-80], were present in all the samples except Piricske water sample, however the samples did not contain the pathogenic *L. pneumophila* (Table 2).

Previous studies showed that *Acinetobacter baumannii* was detected in 38% of the groundwater supplies highlighting the possible significance of Acinetobacter spp. in groundwaters [81]. A. baumannii, though commonly associated with aquatic environments, is an opportunistic pathogen of humans therefore, this bacterium is not favoured in artificial waters (e.g. baths, drinking water, etc.). This bacterium was not present in any tested samples.

On the other hand, *Stenotrophomonas maltophilia*, which is an aerobic, Gram staining negative bacterium, was detected in all the water samples except Piricske and Berzsenyi by taxon specific PCR. This bacterium is ubiquitous in aquatic habitats and soils but considered as an emerging, multidrug-resistant, opportunistic pathogen bacterium [82].

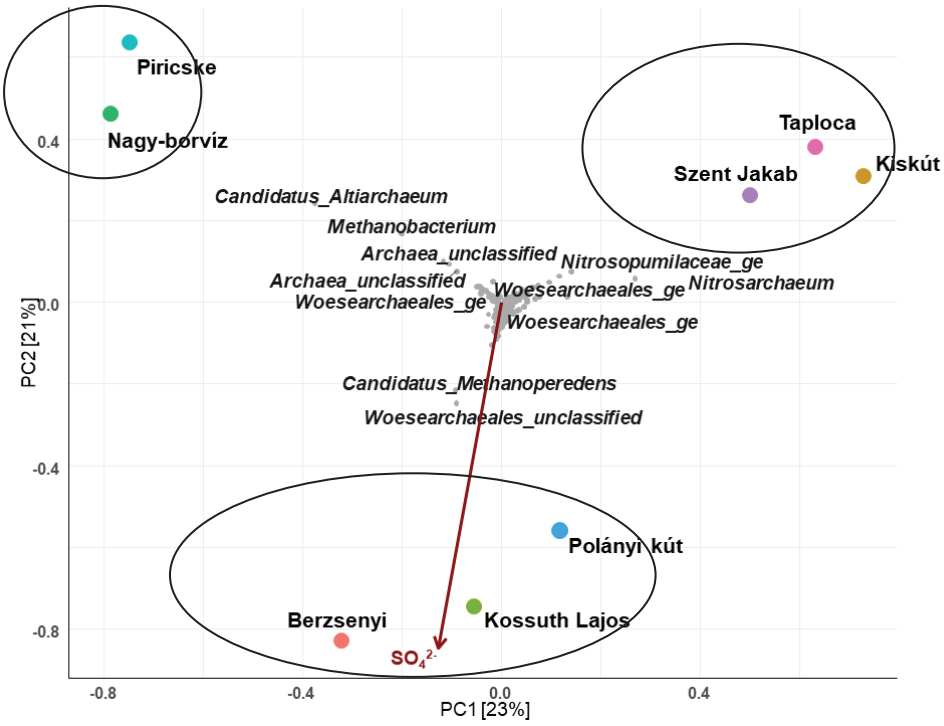

Figure 7 PCA ordination of the water samples based on archaeal community structure and environmental parameters.

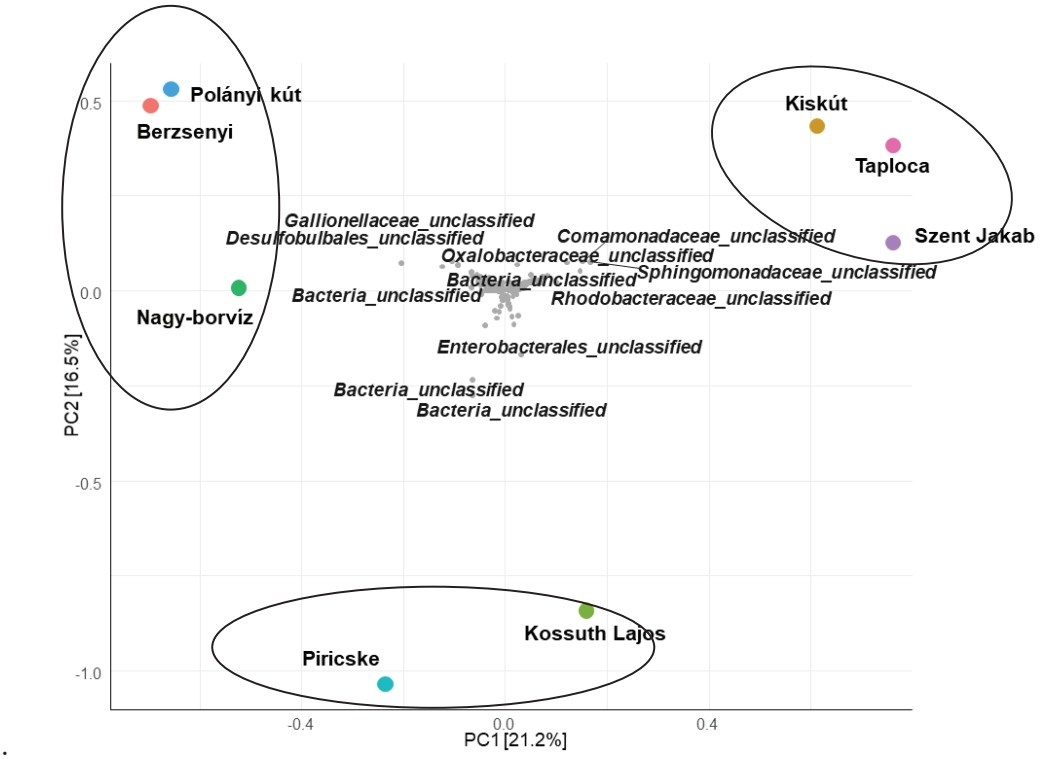

Figure 8 PCA ordination of the water samples based on bacterial community structure and environmental parameters.

NGS results revealed that Szent Jakab was the only sample that showed the presence of Enterobacteriaceae, precisely unclassified and Lelliottia members, this family was also detected in several contaminated groundwater samples in previous researches [83]. These results are of a high concern as this water is used for drinking without any treatment.

The area where Szent Jakab groundwater is located, was characterized with high vulnerability to human contamination due to the absence of confining layer, high hydraulic conductivity of the aquifer and shallow and intense groundwater flow [6].

Conclusion

Microbial cultivation independent methods confirmed the large variations in the microbial community structure among the low nutrient content water samples by presenting a comprehensive profile of the existing microbial community. In fact, nutrient-depleted aquatic environments are highly colonized by microorganisms which are key participants in the different biogeochemical cycles. Among them, methanogenic archaea: Methanobacteriaceae, Methanoperedenaceae. Sulfate reducers: *Desulfobacterota, Nitrospirota.* Iron oxidiser: Gallionellaceae. Nitrate reducers: *Azospirilla*. Ammonia oxidizers: Nitrosomonadaceae. Moreover, the existing microorganism were involved in many different metabolic types. Many are chemotrophic, chemoautotrophic and facultative phototrophic microorganisms. The high percentage of unknown sequences suggests further that many low nutrient content aquatic environments are still undiscovered and further studies are needed.

Declarations

Availability of data and materials

All data generated or analysed during this study are included in this published article or mentioned in the section: DNA extraction from the water samples and amplicon sequencing.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was funded by ELTE Thematic Excellence Programme 2020 Supported by the National Research – Development and Innovation Office – TKP2020-IKA-05.

Authors contributions

MT: Wrote the main Manuscript Text. IM: Sampling and elaboration of the first 24 hours' experiments. AT: Hydrogeological research on the samples. NS: Performed the taxon specific and multitemplate PCR experiments. RF: Material and methods plan. LJ: Performed the physiochemical experiments. ET: Supervision and research plan. All Authors read and approved the final manuscript.

Acknowledgment

The authors are thankful to Gergely Krett, Sára Szuróczki, Balázs Vajna and Attila Szabó and for their technical advice.

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