

Mineralogy and microbial diversity of the microbialites in a Hungarian thermal well water (Köröm, Hungary)

36. OTDK Biology Section

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Summary

Stromatolites are the oldest known life forms; their presence on Earth can be tracked back to 3.7 Bya in carbonate fossils. Microbialites are the modern analogues of the prehistoric stromatolites, as the organomineralization processes are still active and observable. Several cm thick, colored, multilayered biofilms with unique microbialite deposition patterns were formed near the 79.2 °C outlet of Köröm thermal well (Tokaj Mountains). Light microscopy observations revealed vertical laminar mineral depositions in the red biofilm layers in comparison to the green biofilms containing clotted mesostructure. The precipitated microcrystals formed aggregates reaching up to 250 µm in diameter, which enabled their visibility by naked eye. The electron microscopic images showed the network structures formed by filamentous microbes, different sized cocci and rod bacteria, and mineral particle clusters (needle and spherulite). Based on micro-XRD method, the biofilm layers contained mainly calcite beside aragonite, but aragonite dominated in the bottom layer of the green biofilm samples. The comparison of the bacterial diversity of the samples was performed by 16S rRNA gene amplicon sequencing. The results implied that numerous undescribed thermophilic taxon might contributed to the carbonate mineralization. The biofilms were mainly composed of the phyla Bacteroidota and Cyanobacteria but they differed on low taxonomic levels (e.g. *Geitlerinema* PCC-8501 and *Raineyia* were characteristic to the green biofilm, while unclassified Oxyphotobacteria, unc. Saprospiraceae and unc. Cytophagales were abundant in the red biofilm samples).

Introduction

Habitats with highly elevated temperature values are considered as one of the most extreme environments. Despite this fact, some organisms can thrive in such conditions. These species are the ones called thermophiles (optimal growth temperature above 50 °C). According to their preferred growth temperature ranges, 3 categories can be distinguished: the moderate thermophiles (optimal growth temperature 50-60 °C), the extreme thermophiles (optimal growth temperature 60-80 °C) and the hyperthermophiles (optimal growth temperature over 80 °C) (Gupta et al. 2014). The deeper understanding of these creatures is an important aspect of the environmental and evolutionary microbiology, because according to our current knowledge, the origin of life can be directly linked to habitats with extreme temperature values. Subsurface marine hydrothermal springs were the cradle, where the first living organism formed from the available macromolecules (Corliss et al. 1981, Russell et al. 2010) and supposedly propagated to microbial mats (Chiriac et al. 2017) during adaptation to the hostile environmental conditions. The high organic content of these microbial formations leads to high degree of degradation during fossilization. To obtain relevant evolutionary information from the fossilized structures, it is important to find analogues between them and the actively forming microbial mats. Microbial mats are in general cm-thick structures formed by microbial communities in a stratified arrangement. In some cases, mineral depositions can be observed in these mats (Riding 2011a), which can occur through organomineralisation and leads to the formation of microbialites. Microbialites are microbially produced deposits - in most cases carbonate minerals - located on the benthic surface (Riding 2011b). According to the mesostructure of the deposited minerals inside the microbial mats, microbialites can be classified into 4 different groups: stromatolites (laminated mesostructure of the mineral deposits), dendrolites (dendritic mesostructure of the mineral depositions without lamination), thrombolites (macroscopic size, clot like mineral fabric without lamination) (Riding 1991) and leolites (aphanitic internal carbonate fabric) (Riding 2011b). Microbialites can be found in a great variety of environments including marine and continental hot springs (Kees et al. 2022), alkaline (Chan et al. 2014) and hypersaline lakes (Schneider et al. 2013), underwater caves (Guido et al. 2022) and even marine coastal regions (Morris et al. 2019). Their high adaptation capacity may be the result of the great variability of the microbial communities forming these structures.

The knowledge obtained from these extreme temperature tolerant microorganisms led to a wide range of different technological improvements and breakthroughs. Numerous enzymes with high enzymatic activity at high temperature values were isolated from continental hot springs. Thermophilic amine transaminases originating from Iceland's Grensdalur hot spring had high activity up to 90 °C and additionally showed great resilience against organic solvents as well (Ferrandi et al. 2017). *Bacillus licheniformis* and *Thermononas hydrothermalis* isolated from Jordanian hot springs potentially produce thermotolerant amylase and protease enzymes under moderately thermophilic conditions (43-60 °C) (Mohammad et al. 2017). The possible biotechnological application of thermotolerant enzymes along with the evolutionary data conserved in microbialites from prehistoric hot springs outlines the importance of the study of habitats with elevated temperature.

Aims of the research

The Köröm thermal well is one of Hungary's hot springs, where intensive travertine (carbonate) deposition occurs along the well's flowing water. Red and green slimy biofilms can be observed in significant quantities on certain surface parts of the travertine along the outflowing water (*Fig. 1*). The aim of this research was to assess the prokaryotic diversity of the well water, the carbonate deposition and the multilayered biofilm communities along the outflowing water using next generation amplicon sequencing of the V3-V4 region of the 16S rRNA gene. The objectives of the studies were also to visualize the structure of the biofilms and the association between the identified microbes to the deposited minerals using scanning electron microscopy (SEM), light microscopy and X-ray diffraction (XRD) analysis, and to discuss the putative metabolic diversity in the microbial communities, that may influence the process of carbonate mineralization.

Materials and methods

The karst layers around the Tokaj Mountains are covered by thick sediment layers, which are impermeable for water. The infiltrating rainwater from the Bükk Mountains descends to the depth of nearly 2000 m when it travels towards the plain areas, then it heats up and its pressure increases over 42 bars and becomes thermal karst water (Lénárt and Darabos 2012). The Köröm thermal well (N 47.997046° E 20.987198°) was drilled in 1961 to get access to the karst water reservoir under the Tokaj Mountains. The base depth of the well is situated at 1880

m below the surface level, where the temperature reaches 82 °C. The surfacing thermal karst water has 79.2 °C temperature with approximately neutral 6.8 pH (Kele et al. 2015).

Sampling

The sampling was conducted on the 11th of August 2020. The studied sampling sites were the hydrothermal well and several places along the drainage channel of the well itself. Horizontally 5 sampling sites (K1-5) were selected (*Fig. 1*), where the physicochemical characteristics (temperature, conductivity) of the water was measured *in situ* using WTW ProfiLine pH/Cond 3320 device (*Table 1*). Detailed physicochemical analysis of the water parameters was performed *ex situ* on 1.5 dl water samples (*Table 2*) collected at the first (K1) and the last sampling points (K5) to outline the possible horizontal gradients present in the water body.

Table 1: Water parameters measured in situ at Köröm thermal well.

<i>Sampling site</i>	<i>K1</i>	<i>K2</i>	<i>K3</i>	<i>K4</i>	<i>K5</i>
Distance from the well (m)	0	2	7	18	20
Water temperature (°C)	79.2	-	63.7	62.3	61.4
Conductivity (µS/cm)	1554	-	1524	1517	1514

For molecular investigations 1-1 L water samples were collected (KW1-2 samples) next to the water outflow with sterile glasses. Carbonate deposition samples were taken with a sterilized spatula at the first 4 sampling sites (K1-4 samples), where thick mineral depositions could have been observed. Biofilm samples were taken from the two distinct biofilm types neighboring each other with a sterilized shovel and were placed into sterile glasses (*Fig. 1c*). Both of the biofilms were situated right below the water surface and they propagated at a sheltered area of the drainage channel, behind carbonate protrusions easing the water flow. KR (red) consisted of 5 differently colorized layers (KRA-E samples) and KG (green) contained 2 distinct layers (KGA-B samples) with unique coloration (*Fig. 2, 3*). Both the KR and the K2 sediment samples were extracted from habitats situated close to the air-water interface. All samples were kept under 4 °C until processing. During laboratory processing, both biofilm samples were cut to approximately equal sizes (diameter of 1 cm) with a sterilized scissors and the layers were separated by sterilized needles and tweezers (*Fig. 2, 3*).



Fig. 1: The sampling sites and the collected samples: thermal well and sampling point K1, K2 and K3 (a); the drainage channel of the thermal water and sampling point K4 and K5 (b); sampling point KR, KG, K2 and K3 (c); K1 sample (d); KR biofilm sample (e); KG biofilm sample (f).

DNA extraction

The community DNA isolation from all sample types was conducted by the DNeasy PowerSoil® Kit (Qiagen). The water samples were filtered through nitrocellulose membrane filters (MF-Millipore™) (0.2 µm pore diameter) using a vacuum pump to collect the microbial biomass. The filters were sliced up to smaller pieces and these were loaded onto the PowerBead Tube of the DNA isolation kit. Equal amount of the layers of the biofilm samples and 0.2 g of the mineralized sediments were homogenized in a sterile mortar, then the biomass was loaded. The DNA isolation was done according to the manufacturer's descriptions, except in the 3rd step, when a mixer mill (Mixer Mill MM301, Retsch) was used on 30 Hz for 2 minutes for the cell disruption. 5 µl of the isolated DNA's concentration was measured by Qubit dsDNA HS Assay Kit (Thermo Scientific) and the PCR (polymerase chain reaction) mastermix were adjusted according to the measured DNA concentrations.

Next-generation sequencing (NGS)

During the PCR process the V3-V4 region of bacterial 16S rRNA gene-sequences were amplified using Bacteria-specific Bact-341F and Bact-805NR primer pair (Herlemann et al. 2011). Archaeal 16S rRNA gene-sequences were amplified by Arch-519F and Arch-855R Archaea-specific primer pair (Klindworth et al. 2013). In both cases, the first section of the primers 5' end contained Fluidigm CS1 and CS2 tags. The reaction mixture and the temperature profile of the reactions were the same as in Megyes et al. (2021) with the following modifications. The PCR mixtures contained 0.25 µl from Bacteria-specific and 0.3 µl from the Archaea-specific primer pairs (40 µM), respectively. The temperature protocol for the bacterial V3-V4 amplification was conducted with 3 minutes 98 °C for initial denaturation and 95 °C for 10 s denaturation.

All PCR reactions were done in triplicates for standardization purposes from every sample (3x20 µl). The quality of the amplification step was assessed in all samples by 1% agarose gel electrophoresis. The prepared gel contained the following materials: 0.8 g agarose, 80 ml 1x TBE (Tris-Borate-EDTA) puffer, 2 µl ECO Safe Nucleic Acid Staining Solution (Pacific Image Electronics). GeneRuler 100bp DNA ladder (Thermo Fisher Scientific) was used as molecular marker. 5 µl of the PCR products were loaded onto the gel with 5 µl loading dye [30% (v/v) glycerin, 0.25 mM bromophenol blue], and the gel run was conducted under 100 V for 35 minutes. The separated nucleic acid bands were visualized by trans illuminating the gel

with UV light. After the quantity check, the triplicates were merged together in a single PCR tube. The amplified samples were kept under -20 °C until further processing.

DNA concentration of 1 µl of the amplicons was measured by Qubit dsDNA HS Assay Kit and normalized. The final DNA libraries were cleaned, quantified, barcoded and sequenced on Illumina MiSeq platform by the Genomics Core, Research Technology Support Facility, Michigan State University (USA). The sequencing was conducted on MiSeq v2 flow cell, 500 cycles and 2x250 bp paired end format. The sequencing data can be accessed in the NCBI SRA database under the PRJNA836382 Identification Number.

The evaluation of the sequencing data was done by using the mothur v. 1.44.3 program package (Schloss et al. 2009). During the evaluation process, the guidelines of the MiSeq SOP was followed (http://mothur.org/wiki/miseq_sop/ downloaded at 27.04.2021.) (Kozich et al. 2013). Detailed analysis steps were the same as given in Megyes et al. (2021) but UCHIME software was used for the search of chimeric sequences (Edgar et al. 2011). The Shannon, the Inverse Simpson diversity indexes, the Chao1 species richness and the Good's coverage were also calculated by the mothur program package. Bray-Curtis similarity index based UPGMA (unweighted pair group method using arithmetic averages) dendrogram (500 iterations) of bacterial phyla was constructed using PAST3 (PAleontological STatistics) software (Hammer et al. 2001).

Microscopic observations

Light microscopy images were taken of the multilayered biofilm samples (KRA-E and KGA-B) at 40-1000x magnification using a Canon camera applicated to the microscope and QuickPHOTO CAMERA 3.1 software. SEM imaging was conducted as the same in Anda et al. (2015). The samples were observed using an EVO MA 10 Zeiss scanning electron microscope at an accelerating voltage of 10 kV.

XRD analysis

Mineralogical composition of the KRA-E., KGA-B. and K1-K4 samples was measured using a RIGAKU D/MAX RAPID II diffractometer following the micro-XRD method described in Kovács et al. (2020).

Results and discussion

The measured water parameters indicated some important features according to the spillway of the thermal hot spring. The temperature of the well water decreased moderately but it was above 60 °C at all sample points in the well drainage channel (*Table 1*). The pH shifted towards alkali conditions compared to the neutral pH value measured directly from the tube itself. Another important standpoint is the decreasing Ca^{2+} concentration along the horizontal gradient. The low availability of total organic carbon (TOC) and the total nitrogen (TN) are great limiting factor for the members of the community. Therefore, the inhabitants of the drainage channel might adapt to oligotrophic conditions (*Table 2*).

Microbialite descriptions incorporate a range of scales, from macrometers to micro/nanometers (Dupraz et al. 2011). Macrostructural forms of the two multilayered biofilms were flat shape with several centimeters in diameter (*Fig. 2, 3*). However, the different colored biofilms followed distinct microbialite deposition trends visible with the naked eye or microscopic investigations. The red biofilm (KR) contained laminar mineral depositions, with vertical lamination axis compared to the benthos (*Fig. 2*). Based on Riding (2011b) article the mesostructure of these layers were characterized as stromatolitic, with agglutinated microstructure. In contrast, both layers of the green biofilm (KG) were thrombolytic with dense mesoscopically clotted texture, but the KGB lower layer had mineral inclusions with much higher density. This dense mineral deposition caused the faint green color of this layer (*Fig. 3*). This great extent of the differences between the structure of the deposited minerals in the separate biofilm layers should be the outcome of the microbial community alterations.

Table 2: Detailed water parameters of Köröm thermal well. Total organic carbon: TOC; Total nitrogen: TN.

Parameter	Sampling site	
	K1	K5
pH	8.49	8.69
Conductivity ($\mu\text{S}/\text{cm}$)	1450	1421
Na^+ (mg/L)	330	339
NH_4^+ (mg/L)	<0.1	<0.1
K^+ (mg/L)	31	32
Mg^{2+} (mg/L)	7	7
Ca^{2+} (mg/L)	18	7
Cl^- (mg/L)	70	35
SO_4^{2-} (mg/L)	33	17
NO_3^- (mg/L)	2	2
HCO_3^- (mg/L)	680	725
CO_3^{2-} (mg/L)	44	51
TOC (mg/L)	67	2.1
TN (mg/L)	0.7	0.5

The SEM observations of the laminated biofilm samples visualized several coccus, rod and filamentous structures in the prokaryotic size range embedded into mucilaginous material (Fig. 4a-f). This material (extracellular polymeric substance, EPS) was generated by the biofilm forming prokaryotic organisms. The net like contexture was provided by filamentous bacteria, and it may be responsible for the structural stability of the cm thick biofilms (e. g. Fig. 4b, e, g-h). Different minerals with 20-60 μm diameter were detected in the close proximity of bacterial cells. Many of these mineral precipitates aggregated into grains, which had the diameter up to 250 μm . This size range of the aggregates enabled their visibility by naked eye. A large proportion of microbes were seen in the immediate vicinity of the crystal precipitates, on their surface, or interwoven with them. This was confirmed by the presence of bacterial imprints and bacteria-shaped holes on the surface of the minerals. These were supposedly created, when the crystals formed in the proximity of cells, which lysated afterward (Fig. 4e-f). Distinctive needle-like (acicular) aragonite crystals were observable in the KGB sample (Fig. 4c-d). This characterization was confirmed by the micro-XRD analysis of the depositions (Table 3). Other layers of the two biofilms contained globular (spherulite) mineral depositions (Fig. 4c, e-f). According to the micro-XRD measurement, these were characterized as calcite (Table 3).

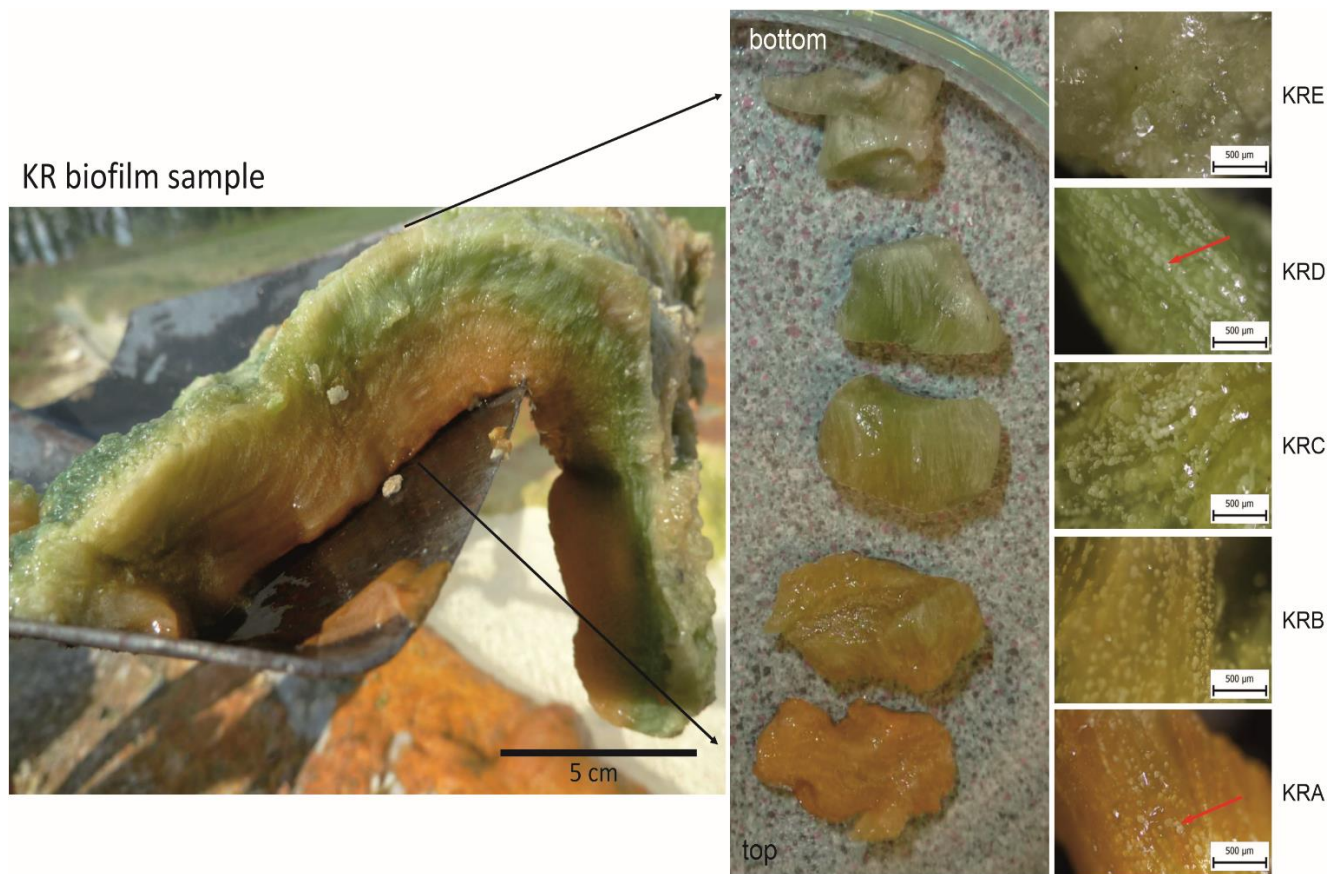


Fig. 2: KR biofilm sample and the separated layers (red arrows: carbonate precipitates). The photo (middle) and stereomicroscopic images (right) show dissected biofilm layers.

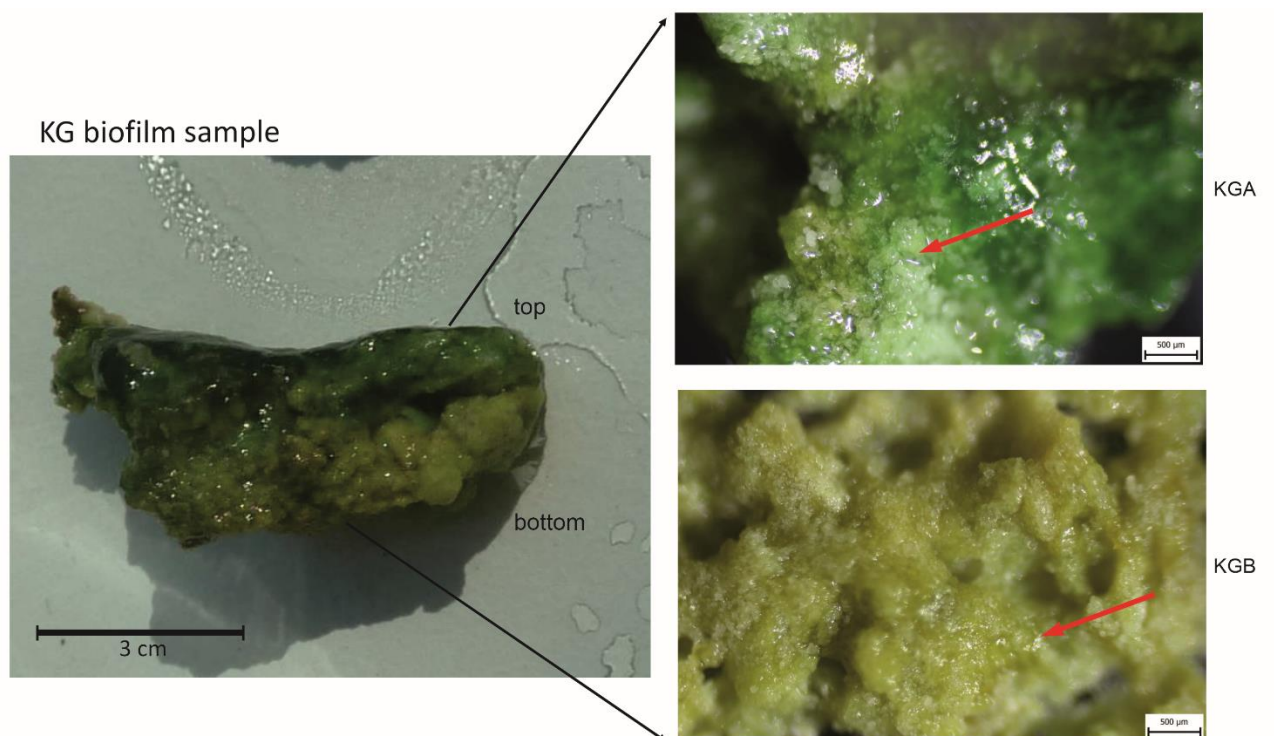


Fig. 3: KG biofilm sample and the separated layers (red arrows: carbonate precipitates).

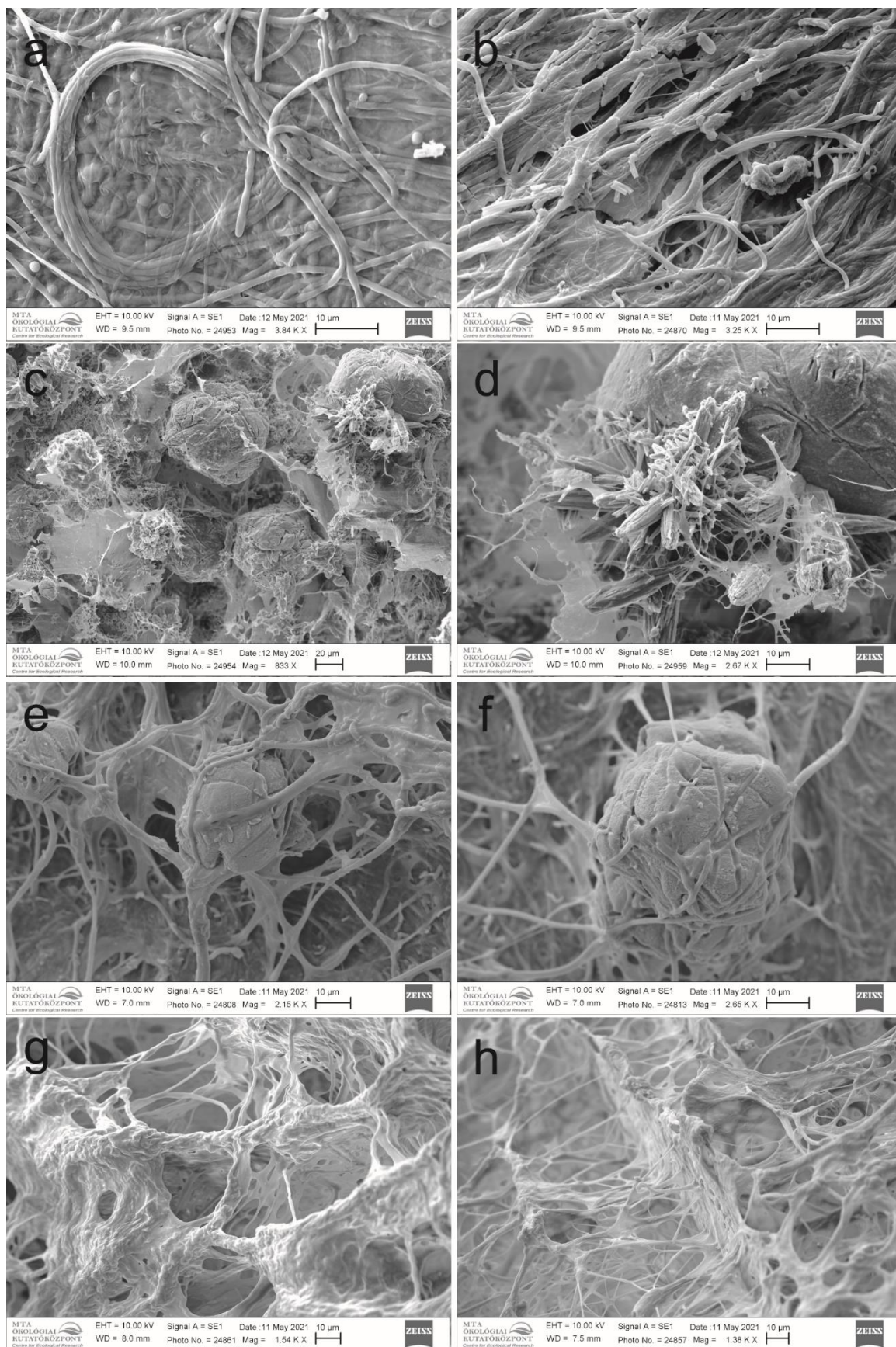


Fig. 4: Comparison of SEM micrographs of multilayered biofilm samples from Köröm thermal well (KGA [a-b]; KGB [c-d]; KRA [e-f]; KRC [g]; KRE [h] samples).

The micro-XRD characterization of the observed carbonate depositions (K1-4 samples) (Table 3) indicated that aragonite formation was dominant in every observed sample except the K2 deposition. It was calcite and it formed outside the water canal (Fig. 1a, c). The structural difference between the 2 minerals were even observable visually. The calcite deposit was much sturdier and was harder to extract from the sediment layer. The red biofilm (KR) contained calcite with higher proportions; in contrast, the green biofilm (KG) produced aragonite over calcite. This phenomenon might be the outcome of the fundamentally different community composition in the 2 distinct biofilm types (Table 3).

Table 3: Mineral content of the samples from Köröm thermal well. K1-4: carbonate depositions; KRA-E: red biofilm layers; KGA-B: green biofilm layers.

Sample	Result of micro-XRD analysis
K1	Aragonite
K2	Calcite
K3	Aragonite
K4	Aragonite
KGA	Calcite ~ Aragonite
KGB	Calcite < Aragonite
KRA	Calcite
KRB	Calcite > Aragonite
KRC	Calcite >> Aragonite
KRD	Calcite
KRE	Calcite > Aragonite

After the bioinformatical analysis, 432314 bacterial and 1023 archaeal sequences remained. The very low obtained archaeal sequence number only gave a brief overview on the archaeal community structure, so the diversity indexes were disregarded in this case. In case of the bacterial sequences, the Good's coverage implied, that the sequencing of the bacterial community was representative and acceptable because the coverage value exceeded 99% in all samples. The diversity indices (Table 4) showed that the KGA (deep green) and the KRE (grey) layers, with the K4 sediments sample had the most diverse bacterial community among the observed regions in the canal.

Table 4: Diversity indices and Good's coverage calculated by the *mothur* program package based on the subsample of 23525 sequences.

Sample	Number of reads	Number of OTUs	Good's coverage (%)	Chao1	Shannon	Inverse Simpson's (1/D)
K1	37356	81	99.95%	87.0	1.83	4.77
K2	23525	161	99.99%	161	2.45	6.83
K3	42202	483	99.49%	542	2.68	5.53
K4	31605	228	99.84%	238	2.77	7.65
KGA	43909	565	99.39%	638	3.26	9.75
KGB	30704	392	99.81%	403	2.91	7.58
KRA	29691	411	99.80%	421	3.13	8.65
KRB	35405	515	99.62%	545	3.15	8.55
KRC	32619	332	99.79%	347	2.83	7.24
KRD	24228	307	99.97%	307	2.46	4.74
KRE	29899	480	99.76%	493	3.54	10.0
KW1	34292	269	99.79%	287	1.39	1.73
KW2	36879	68	99.96%	72.2	0.57	1.22

The phylum and the genus level identification of the community members unfolded serious compositional differences between the distinct sampling sites (Fig. 5, 6). In the two water samples (KW1-2), the phylum Aquificota showed clear dominance by exceeding 70% relative abundance in both samples (Fig. 5, 6) distinctly to the other samples. From this phylum, the *Hydrogenobacter* genus was identified as the most abundant taxon. This genus contains extremophile, obligate chemolithotroph aerobic organisms, which utilizes H₂ as electron donor (Pitulle et al. 1994, Igarasi et al. 1990). Other aerobic H₂ oxidizers from the genus *Hydrogenophilus* (phylum Proteobacteria) were also detected in the carbonate depositions with small abundance (K1-4) (Manaia et al. 2019). The reason behind the differing relative abundance of the two groups may be due to the competition for the limited H₂ substrate.

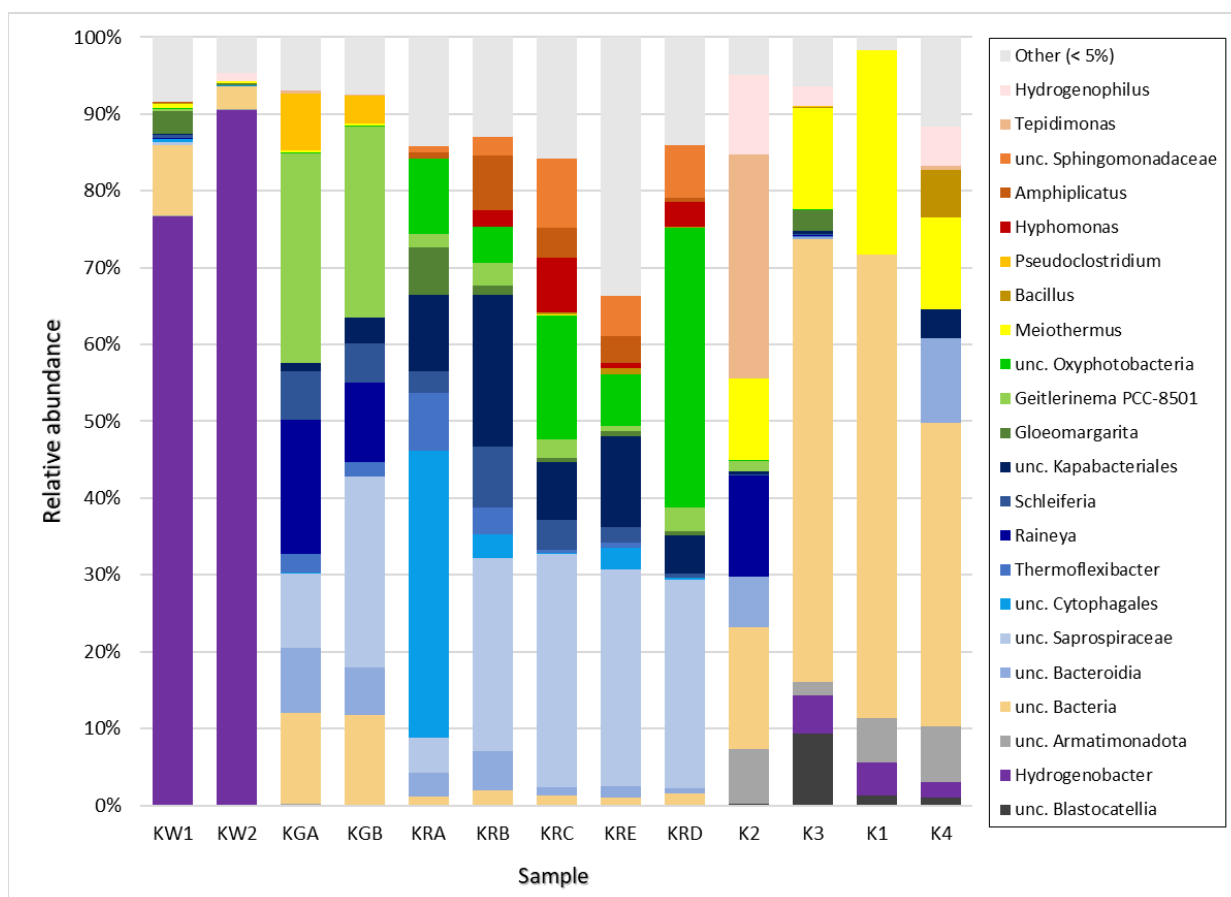


Fig. 6: Assessment of the bacterial diversity in Köröm thermal well samples by NGS technique. K1-4: carbonate depositions; KRA-E: red biofilm layers; KGA-B: green biofilm layers; KW1-2: water samples.

The phyla Proteobacteria, Cyanobacteria and Bacteroidota had outstanding relative abundance in all 5 layers dissected from the red biofilm (KR). The Bacteroidota phylum was represented by the following 6 taxa in the observed red biofilm samples with varying abundance profiles: class *Kapabacteriales*, genera *Schleiferia* and *Thermoflexibacter*, uncultured members of order *Cytophagales*, family *Saprospiraceae* and class Bacteroidia. Every red biofilm layer except the KRA contained the *Saprospiraceae* family with considerably high relative abundance. The representatives from the type genus of this taxon, *Saprospira* can form long multicellular filaments with coloration caused by the great variability of carotenoid pigments (pink, yellow, orange or red) (Lewin, 2015). The *Kapabacteriales* (formerly *Ignavibacteriales*) class had outstanding abundance in the KRA-B samples. Previously, this taxon was mostly found during metagenome analysis of toxic algal blooms (Al-Saud et al. 2020). Large portion of the community in the KRA biofilm layer originated from the *Cytophagales* order. The organisms from this order are strictly aerobic (in most cases) chemoorganotrophs. Colonies formed by them are often colored yellow, orange, pink or red by the abundant pigment material

in the cells (Nakagawa, 2015). The outstanding presence of this taxon in the KRA layer might be the reason behind the bright red coloration of this layer. The members of the *Schleiferia* genus appeared in every layer of the red biofilm but had outstanding abundance in the KRB sampling point. This taxon contains strictly aerobic, thermophilic organisms. The type species of this genus, *Schleiferia thermophila* forms colonies with orange coloration (Albuquerque et al. 2011). The unusual mixed (orange/green) color of the KRB biofilm layer was supposedly result of the coexistence of the pigmented colonies of the *Schleiferia* taxon and the Cyanobacterial filaments with green coloration. The *Thermoflexibacter* genus (formerly *Flexibacter* genus) appeared only in two sections of the red biofilm layers with mentionable abundances (KRA-B). The type strain of this genus was also described from hydrothermal area in Iceland by Hankle and co-workers (2016). Some of the detected threads in the biofilm layers could be formed by the members of this taxon, which is known for the formation of unusually long filaments (Lewin, 1969), which were also observable on the SEM images (Fig. 4).

The detected members of the phylum Cyanobacteria originated from 2 different genera and 1 class: the class *Oxyphotobacteria*, the genera *Geitlerinema* and *Gloeomargarita*. The class *Oxyphotobacteria* contains microorganisms, which are capable of oxygenic photosynthesis (Soo et al. 2017). Cyanobacteria are known to contribute to organomineralization through their photosynthetic activity (Shiraishi, 2012). The prominent green coloration of the KRC-D samples was due to the elevated relative abundance of the Cyanobacterial taxa in those layers of the biofilm. The genus *Gloeomargarita* includes unicellular organisms with oxygenic photoautotrophic metabolism capable of precipitating intracellular amorphous calcium carbonate. The cells show green coloration because they contain chlorophyll a and phycocyanin used as photosynthetic pigments (Moreira et al. 2017). Thermophilic representatives of this genus were detected during metagenomic sequencing performed on thermal wells located at the Yellowstone National Park (YNP). The temperature range (73 °C) and the observed laminated biofilms at YNP (Kees et al. 2022) shows high degree of similarity to the ones detected at the Köröm well (79.2 °C).

From the phylum Proteobacteria, the genera *Hyphomonas* and *Amphiplicatus*, with the uncultured members of the family *Sphingomonadaceae* had the greatest relative abundance with some additional groups. The *Hyphomonas* genus consists of aerobic, chemoorganotrophic organisms that are known for the formation of benthic biofilms in marine ecosystems (Abraham 2020). Previously, some representatives were isolated from oxygen rich deep sea hydrothermal effluents (Jannasch and Wirsén 1981, Weiner et al. 1985). Members of the genus *Amphiplicatus*

are obligate aerobic thermophilic organisms. The first described species of this genus was also discovered in a hot spring located in northwest China (Zhen-Li et al. 2014.). The *Sphingomonadaceae* family contains mostly strictly aerobic organisms with phototrophic or chemoheterotrophic metabolism (Yabuuchi and Kosako 2015). A thermophilic member of this taxon was isolated from a Japanese radioactive hot spring and it was capable of producing zeaxanthin (natural yellow pigment) and other carotenoids. (Asker et al. 2012). This family may have played a role in the altering coloration in some of the red biofilm layers (KRC-D and -E.).

Apart from some mentionable changes, the community composition on phylum level remained the same in the red and the green biofilms. The phylum Proteobacteria was almost completely substituted by Firmicutes in the green microbial mat, which was only present in trace amounts in the red biofilm. The phylum uncultured Bacteria had increased abundance in case of the green biofilm. The genus *Pseudoclostridium* were present in both layers from the green microbial mat. The organisms belonging to this genus has their growth optimum in the thermophilic range and can degrade a wide range of sugars (Zhang et al. 2018). With the appearance of this genus, other aerobic heterotrophs vanished from the community. Other abundant participants like Cyanobacteria and Bacteroidota remained permanent members in both biofilm variants. Regarding the green biofilm, the genus *Raineyia* appeared in great quantities. This taxon contains organotrophic organisms. The type species *Raineyia orbicola* is known to develop colonies with orange pigmentation (Albuquerque et al. 2018). Other Bacteroidota taxa, which were detected in the red biofilm, were also identified in the green microbial mat. The sole representative of the Cyanobacterial phylum was the genus *Geitlerinema* as the most abundant taxon in both green layers. This genus was most likely responsible for the deep green coloration of the KGA-B stratum. The filamentous arrangement of cells and extracellular mucus typically produced by this genus (Castenholz et al. 2015) contributed to the structural integrity of both the red and green biofilms.

The bacterial community of the carbonate deposition showed huge variety between samples. The community defining phyla in the K1 sediment sample directly extracted under the well were the unclassified Bacteria and the Deinococcota. In the K3 sediment sample, phylum Acidobacteriota was also represented by the genus uncultured *Blastocatellia* (> 9%) with mentionable relative abundance. The representatives of this genus are known to inhabit hydrothermal hot springs or geothermal soils (Huber et al. 2017). Some filamentous members of the *Meiothermus* taxon were described from hydrothermal areas before (Nobre and da Costa, 2015). The proteobacterial genus *Tepidimonas* was only present in the K2 region of the

carbonate deposition with the highest relative abundance (> 29%) of any genera. Some members are known to adsorb Ca^{2+} ions to their extracellular cell surfaces, which can promote CaCO_3 precipitation. A member of this taxa, *Tepidimonas fonticaldi* AT-A2 even has adsorption maximum of Ca^{2+} ions to surface proteins under extremophilic conditions (150 °C) (Han et al. 2016). The only unexpected element of the community was the appearance of the phylum Firmicutes, more precisely *Bacillus* spp. (> 6%) in the K4 sample. Many representatives from the genus *Bacillus* are known for their involvement in carbonate mineral precipitation through strong urease activity (Kim et al. 2016). One aerobic *Bacillus* strain, isolated from an Italian shallow hydrothermal vent, was even capable of firm EPS production near 70 °C (Nicolaus et al. 2000). Some close relatives of this *Bacillus* strain might have contributed to the biofilm production at the warmer segments of the Köröm thermal well.

Table 5: Assessment of the archaeal diversity at genus level in Köröm thermal well samples with NGS technique. K1-4: carbonate depositions; KRA-E: red biofilm layers; KGA-B: green biofilm layers; KW1-2: water samples.

Taxon	Relative abundance (%)												
	K1	K2	K3	K4	KGA	KGB	KRA	KRB	KRC	KRD	KRE	KW1	KW2
Crenarchaeota													
<i>Candidatus Nitrosocaldus</i>		24%	98%	70%									1%
<i>Candidatus Nitrosotenuis</i>							77%	63%	86%	77%	88%	2%	
<i>Nitrosarchaeum</i>	100%	29%					5%		14%		1%		1%
unc. Nitrososphaeraceae				3%	100%		18%			23%			
<i>Candidatus Nitrosotalea</i>						100%		38%					
<i>Thermofilum</i>												21%	25%
<i>Pyrobaculum</i>		48%	2%									73%	74%
Halobacterota													
unc. Haloferacaceae				27%									
Nanoarchaeota													
unc. Woesearchaeales GW2011_GWC1_47_15											7%		
other (< 5%)											4%	5%	

The observed archaeal community consisted of 3 phyla: Crenarchaeota, Halobacterota, Nanoarchaeota (Table 5). Almost half of the archaeal sequences originated from the K3 sediment sample. The most abundant genera were the *Nitrosarchaeum*, *Pyrobaculum*, *Candidatus Nitrosocaldus* and *Thermofilum* in the water and carbonate deposition samples. *Candidatus Nitrosotalea* and uncultured *Nitrososphaeraceae* dominated the KGA layer of the green biofilm. *Candidatus Nitrosotenuis* dominated every layer of the red biofilm. Some of the detected taxa were also observed in thermophilic biofilms discovered in volcanic beds (Devlin

et al. 2022). Huge portion of the archaeal community was from thermophilic, ammonia oxidizer groups that can adapt to the low NH_3 content of the thermal water.

Overall, this was the first time that living/extant microbialites were discovered in Hungary. The microbial community of Köröm thermal well consist of mostly aerobic, chemolithotroph and heterotroph thermophilic taxa, which vary greatly along the horizontal axis. This variation is caused by horizontally altering temperature and TOC values. Moreover, the exceptionally low TN (total nitrogen) and TOC (total organic carbon) concentrations produced an oligotrophic environment presenting another selective pressure for the inhabiting microorganisms. Continuous water flow might permit the mixing of the well water and sustain an oxygenated environment. The detected red and green biofilms contained diverse thermophilic microorganisms in contrast to the extremity of the environment; most of them are capable of producing distinct pigments, which was supposedly the reason behind the altering coloration observed vertically in the extensively forming multilayered biofilms. The water samples were dominated by *Hydrogenobacter* taxon (from the Aquificota phylum) with only trace abundance of other groups. The uncultured Bacteria phyla had exceptionally high abundance in the examined sediment (carbonate deposition) samples extracted from Köröm. The resilience of this taxa against the high temperature values contributed to its prevalent presence in almost every sediment section except K2. The K2 sediment sample had very similar community composition to the red biofilm. The reason behind this phenomenon could be, that both the K2 sampling site and the red biofilm were situated close to the water surface, thus their community was adapted to resembling oxygen and temperature levels as well as to stronger UV radiation. Many different taxa from the phyla Bacteroidota, Cyanobacteria and Proteobacteria appeared in each biofilm layer with varying relative abundance profiles. Great portion of the identified groups were previously reported to be capable of carbonate precipitation to some extent in thermophilic conditions. The detected biofilms contained aragonite and calcite precipitates with either vertical laminated or clotted mesostructures, which characterized them as stromatolite and thrombolite.

Rezümé

A sztromatolitok a Földön fellelhető legősibb szoros együttélésben élő életformák. Jelenlétük egészen 3,7 milliárd évvel ezelőttig követhető vissza a karbonátos fossziliák vizsgálata által. Ezen ősi életközösségek modern analógiái a mikrobialitok, melyek esetén az organomineralizációs folyamatok aktívak és ez által napjainkban is megfigyelhetők. A Tokaji-hegység közelében elhelyezkedő Köröm termálkút 79,2 °C fokos vizet emel a felszínre közel 2 km mélységből. A kút vizének elvezető medre mentén a karbonát kiválások több cm vastagságú, színátmenetes, rétegzett zöld és vörös biofilmekkel tarkítottak, melyek belsejében szabad szemmel is látható apró ásványi szemcsés lerakódások jelentek meg. A biofilm minták fénymikroszkópos vizsgálata során a lerakódások mezoszerkeztúrája a vörös biofilm esetén sztromatolitokra jellemzően vertikálisan rétegzettnek, míg a zöld biofilm esetén trombolitokra jellemzően szabálytalan foltos szerkeztúnek bizonyult. A biofilmekről készült pásztázó elektronmikroszkópos felvételek alapján eltérő kokusz és pálcika morfológiájú baktériumok sokasága volt megfigyelhető fonalas mikrobák hálózatába ágyazva. A mikrobák nagy hányada a (szférikus és tűszerű) kristálykiválások közvetlen közelében, vagy azok felszínén, illetve azokat átszőve jelent meg. A minták ásványos összetételének vizsgálata mikroröntgendiffrakció (mikro-XRD) segítségével valósult meg. A különböző biofilm rétegek aragonitot és kalcitot tartalmaztak változó arányban. Kivételt képez ez alól a zöld biofilm alsó rétege, melyben nagyobb részt aragonit kiválás volt megfigyelhető. Mivel a termálkút térségében ezen hőmérsékleti értékek mellett általában aragonit kiválás dominál, ezért a kalcit jelenléte feltehetőleg a mikroba közösségek aktivitásának köszönhető. A minták Archaea és Bacteria közösségeinek összehasonlítása 16S rRNA gén amplikon szekvenálás segítségével történt. Az eredmények arra utaltak, hogy számos még ismeretlen termofil taxon járulhatott hozzá a karbonát kiválások létrejöttéhez. A biofilmekben legnagyobb arányban a Bacteroidota és Cyanobacteria törzsek voltak jelen, azonban ezen törzsek képviselői mintánként nagy mértékben eltértek alacsonyabb rendszertani kategória szinten (például a Geitlerinema PCC-8501 és *Raineyia* csoportok a zöld biofilmben jelentek meg, míg az Oxyphotobacteria, azonosítatlan Saprospiraceae és azonosítatlan Cytophagales csoportok a vörös biofilmre voltak jellemzőek).

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