BABEŞ–BOLYAI UNIVERSITY
FACULTY OF BIOLOGY AND GEOLOGY
DOCTORAL SCHOOL OF INTEGRATIVE BIOLOGY

DOCTORAL THESIS

THERMOPHILIC MICROBIAL COMMUNITIES FROM GEOTHERMAL ENVIRONMENTS OF WESTERN PLAIN, ROMANIA

- Summary -

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List of abbreviations

ABC transporters - ATP-binding casette transporters

ATP - Adenosine triphosphate

BAC – Bacterial artificial chromosome

bp / Mbp – Base pairs / Mega base pairs

cDNA – complementary DNA

cDPG - cyclic-2,3-diphosphoglycerate

CoA – Coenzyme A

CRISPR - Clustered regularly interspaced short palindromic repeats

dNTP/ddNTP – Deoxynucleotide / Dideoxinucleotide

EPS - Extracellular Polymeric Substances

GS-MS – Gas chromatography–mass spectrometry

HGT – Horizontal gene transfer

HSP – Heat shock proteins

MDA – Multiple strand displacement amplification

NAD(P) - Nicotinamide adenine dinucleotide phosphate

NGS – Next generation sequencing

OGT - Optimum growth temperature

OLC – Overlap layout consensus

OUT – Operational taxonomic unit

PCR – Polymerase chain reaction

SEM – Scanning electron microscopy

SOB – Sulfur oxidizing bacteria

SRB – Sulfate reducing bacteria

XRD – X-Ray diffraction

YNP – Yellowstone National Park

Key words

Thermophyles, hyperthermal aquifers, microbial diversity, diversity patterns, microbialites, microbial mats.

I. Why thermophiles matter?

Thermophiles and hyperthermophiles are organisms that grow at more than 55 ºC and 80 ºC, respectively (Urbieta et al., 2015). They attracted the interest of scientists for two main reasons, namely because they provide insights into the origin of life, and because they can have important contributions in the realm of biotechnology (Ferrera and Reysenbach, 2007; Urbieta et al., 2015). In this thesis, a Next – Generation Sequencing (NGS) metabarcoding approach was used for the characterization of microbial communities that inhabit the deep subsurface hyperthermal aquifers, together with the microbial mats and microbialites that were formed in the surroundings of several drillings, some of the abandoned.

II.1.1 The origin and physiological limits of life

Throughout most of the Earth's history, the conditions were very different from what we experience today. It is considered that for the first 200 million years the temperatures on the Earth surface were higher that 100 ºC, making the accumulation of liquid water impossible. Microbial life started to develop when the temperatures dropped, but still in fairly hot conditions (Djokic et al., 2017). Therefore, the first organisms to evolve were most certainly thermophiles or at least thermotolerant (Stan-Lotter, 2017). This hypothesis is validated by the universal phylogenetic tree, constructed on the small subunit of the 16S rRNA gene, which puts the thermophile branches close to the root of the tree (Stetter, 2006). Additionally, the majority of thermophiles are anaerobic organisms that can grow through chemolithoautotrophy, characteristics that make them good candidates for life on a planet with oxygen - free atmosphere and intense volcanic activity (Ferrera and Reysenbach, 2007). On the present Earth, environments with temperatures above 55 ºC are rarely encountered and are generally associated with geothermal activity. Thus, a “thermophile boundary” was formulated at 55 – 60 ºC, above which thermophiles are growing (Brock, 1986). In the past decades life, above 100 ºC was beyond belief. However, when high temperature are accompanied by high pressures, life that is dependent on the liquid water can exist even at 122 ºC, as in the case of *Methanopyrus kandleri* strain 116 (Takai et al., 2008). Even though some isolated thermozymes maintained their activity at 130 ºC, no microbial growth was detected at this temperature (Lévêque et al., 2000). A possible explanation for this may be that at 130 ºC, certain low molecular weight compounds, like ATP and NAD, are quickly hydrolyzed, whereas thermolabile amino acids such as glutamate and cysteine are rapidly decomposed (Stetter, 1999).

II.1.2 Applications of thermophiles in biotechnology

The biotechnological applications of thermophiles can be divided in application that use cell cultures *vs.* those that exploit their macromolecules and metabolites. As many industrial processes take place at elevated temperatures, the utilization of thermophiles and their thermozymes may

provide several advantages, including: 1) their ability to function at enhanced production rates; 2) the elimination of cooling steps; 3) the occurrence of metabolic reactions at the same temperature that substrates solubilize; 4) the elimination / inhibition of contamination with mesophilic species (Ferrera and Reysenbach, 2007; Urbieta et al., 2015). Several areas in which thermophiles have important applications are biofuels production, biomining, bioremediation and the synthesis of various thermozymes (Urbieta et al., 2015).

II.2 Life adaptations to high temperatures

Life is dependent on complex metabolic networks that exist among macromolecules and metabolic intermediates, thus the adaptation to extreme temperatures can not be correlated to only one particular molecule or feature (Urbieta et al., 2015). Considering the genome sizes, it was observed that thermophiles tend to have less intergenic space, and thus more compact genomes (Sabath et al., 2013). Another strategy for adaptation my consist in an increased proportion of GC content in the genome due to the higher stability of the three hydrogen bonds between these nucleotides, compared to the A-T pair that has only two (Urbieta et al., 2015). Although this situation was observed in *Methanopyrus kandleri* (Ferrera and Reysenbach, 2007), it is not generally the case, as *Caldisphera spp.* and *Methanotorris spp*., two thermophilic species, have an average GC content of 30% and 32%, while *Escherichia coli* has a GC proportion of 50.7% (Trivedi et al., 2005). In contrast, the GC content of crucial genes, such as for those encoding the 16S rRNA and tRNA genes, is a better indicator of thermophilicity (Ferrera and Reysenbach, 2007; Urbieta et al., 2015). Additionally, the structure of DNA and proteins can be stabilized through the accumulation of various compounds, including cDPG, trehalose (Martins et al., 1997), HSPs, chaperones and chaperonins, agmatine, spermidines etc. (Urbieta et al., 2015). Regarding the thermostability of proteins, no universal mechanism was observed, but an increased numbers of charged and hydrophobic amino acids, decreased lengths in the superficial loops and higher numbers of disulfide bonds in intracellular proteins were reported (Beeby et al., 2005). Moreover, the increased temperatures affect the fluidity of the membranes, and thermophiles changed the lipid composition of the membrane in order to maintain them in a liquid form and to regulate their membrane potential, permeability and function. They have a higher amount of saturates fatty acids than mesophiles or psychrophiles, which leads to a stronger hydrophobicity that stabilizes the membrane at increased temperatures. Also, the monolayer conformation is common, which increases the membrane stability and protects against hydrolysis at higher temperatures.

II.3 Biodiversity in geothermal environments

II.3.1 Geothermal areas

Geothermal areas are rare on a global scale and can be viewed as islands separated by vast geographical distances. Thermal environments are generally associated with volcanic activity along the margins of tectonic plates, areas where tectonic plates are colliding and transforming (Hreggvidsson et al., 2017). These areas may vary greatly in geochemistry, but are generally classified according to their pH values in two types, i.e. the acidic and the neutral-alkaline environment, with are dependent on the heat source (Hreggvidsson et al., 2017; Kristjanssos and Hreggvidsson, 1995). The areas with low pH values are associated with volcanic activity, where volcanic gases are composed mainly of N_2, CO_2 and H_2S. When H_2S is a major component of the emissions, dissolved sulfate and sulfide are found in the heated water. In the subsurface, the gaseous stream has a neutral pH, but when it gets closer to the surface is chemically/biologically oxidized, resulting in the formation of sulfuric acid. At the surface, as these areas are characterized by elevated temperatures, the water is usually in scarce quantities, resulting in the accumulation of sulfuric acid in mud pool or solfataric humid soils, with a pH around 2 - 2.5 (Hreggvidsson et al., 2017). The high availability of sulfide, sulfur and H_2 sustain the growth of chemoautotrophs that produce organic matter for thermoacidophilic heterotrophs (Stetter, 1986).

Alkaline hot springs and geysers are situated outside the volcanically active regions. They are geologically more stable and the constant water flows contain minerals (SiO_2) and dissolved gases (HCO_3^- /CO_3^{2-}). H_2S is usually in trace amounts, the pH being alkaline, in the range of 7 – 10. Around the hot springs, steep gradients are forming in all directions, generating a diversity of habitable zones for a variety of microorganisms (Hreggvidsson et al., 2017; Kristjanssos and Hreggvidsson, 1995). Dense biofilms of phototrophic bacteria can develop at temperatures less than 74 ^oC (Ward and Castenholz, 2000), their dead biomass providing a rich source of nutrients for other heterotrophic thermophiles. More details on the formation and biodiversity of mineralized and non-mineralized microbial mats developing in neutral to alkaline hot springs will be presented in the following sections.

II.3.2 The biogeography of thermophiles

It is considered that microbial diversity in geothermal areas are influenced by the gradients in temperature and pH, the availability of different electron donors and acceptors, and that the communities from distinct geographical areas are similar if they have comparable physico-chemical parameters (Menzel et al., 2015; Inskeep et al., 2010). Many thermophilic and hyperthermophilic species have a worldwide distribution, like those included in the *Thermus*, *Thermoplasma*, *Bacillus* and *Hydrogenobacter* genera. Nevertheless, endemic patterns of distribution can be detected at the

species level for some thermophiles, thermal habitats being expecting to harbor different ecotypes in distant geographical areas. An example in this case is the sulfur-oxidizing species *Sulfolobus islandicus*, that thrive at pH around 3 and at 80 ºC in solfataric geothermal springs throughout the Northern Hemisphere (Whitaker et al., 2003). By analyzing the sequence of nine chromosomal loci in *S. islandicus* strains isolated from Russia, USA and Iceland, Whitaker et al. (2003) concluded that the geographic isolation is the main factor responsible for the observed global distribution pattern.

II.3.2.1 Dispersal of thermophilic species

Because the distance among geothermal areas are generally large, the number of migration events from one place to another is reduced. The conditions in the environments surrounding the geothermal area are very different and can be harmful for thermophilic species, acting as a barrier for dispersal. Dispersion through air/wind is facilitated by the ability of thermophiles to resist dessication or to form resistance bodies like spores. *Geobacillus*, a group of spore-forming obligate thermophilic bacteria, has a worldwide distribution as opposed to different species of *Synechococcus*, which appear endemic to distinct geographical areas. They are very sensitive to various environmental factors, probably as a trade-off for adaptations to increased temperatures (Hreggvidsson et al., 2017; Miller and Castenholz, 2000). Also, distinct geothermal regions can have particular physico-chemical properties, such as low or high pH, high arsenic concentrations, and migratory species may not have the adaptations required to overpass or compete the local microbiota.

II.4 Biodiversity in the continental deep-subsurface biosphere

The environments found below the continental surface or the ocean floor are called the "subsurface". Based on the geothermal gradient (8 – 30 ºC/km), life should be possible down to the limits of 2 – 12 km below the surface. Many of the studies realized by now were conducted on the hot and localized hydrothermal vents that were dominated by chemolithoautotrophs, or on the cold subseafloor sediments that are driven by photosynthetically synthesized organic carbon (Kieft, 2016). In contrast, less studies investigated the biodiversity in deep continental aquifers, some of them focusing on the Fennoscandian Precambrian shield (Itävaara et al., 2011; Pedersen, 2000; Hubalek et al., 2016), the Outokumpu Deep fracture fluids, Finland (Purkamo et al., 2016), the Witwatersrand Basin, South Africa (Onstott et al., 2006) and the Columbia River basalt aquifers (Stevens and McKinley, 1995).

II.4.1 SLiMEs: Subsurface Lithoautotrophic Microbial Ecosystems

Organisms from the deep, hot subsurface can gain their energy from geochemically produced compounds, like H_2 (Nealson et al., 2005). Some studies proved that chemolithotrophic

prokaryotes are indeed the producers in such habitats, including the groundwater feeding the Liddy Hot Springs, USA (Chapelle et al., 2002) and the Witwatersrand Basin, South Africa (Chivian et al., 2008). The processes that can generate H_2 include the oxidation of silicate minerals (Stevens and McKinley, 2000), the water radiolysis (Lin et al., 2006) and the serpentization of ultramafic rocks (Schrenk et al., 2013; Purkamo et al., 2016). Then, CH_4 can be synthesized starting from H_2 and short-chain hydrocarbons through the Fischer-Tropsch type reactions (Sherwood-Lollar, 2007). Altogether these compounds can create relatively energetic ecosystems that are generally populated by a few number of species, like methanogens and sulfate-reducing bacteria (Kieft, 2016).

II.4.2 Prokaryotic diversity in the continental deep subsurface

Because the continental subsurface is geologically highly varied, so is the microbial abundance and diversity (Kieft, 2016). Active communities with extremely low diversity were described in the groundwater feeding the Liddy Hot Springs, USA, environment where hydrogen-consuming methanogens were dominant (Chapelle et al., 2002), and in the deep fracture-derived groundwater from South Africa, where a thermophilic sulfate reducer affiliated to the Firmicutes phylum was the single organism encountered (Lin et al., 2006). A slightly more diverse microbial community was found in a thermal aquifer associated with the Great Artesian Basin, Australia, which was dominated by *Methanospirillum*, *Thermodesulfovibrio* and *Hydrogenobacter* genera (Kimura et al., 2005). This thermal aquifer had the oldest water component estimated to be around 2 million years old and a temperature of 64°C. Aquifers of similar type, with up to 2 million years old water components and different degrees of connectivity to the surface ecosystem, have been encountered in Romania (Țenu, 1981). In this thesis we investigated the prokaryotic biodiversity in two of the hyperthermal aquifers from Romania and tested if the differences in the levels of water connectivity to the surface is an important factor that drives the biodiversity in the subsurface.

II.5 Microbial mat communities and microbialites

Microbial mats are communities that develop on sediments in various habitats, such as hot spring and hypersaline ponds (Stal, 2012; Bolhuis et al., 2014). Typical for microbial mats it is their laminated structure, made of microorganisms performing different functional roles. Along with this biological stratification, a biomineralogical stratification can also occur, where mats precipitate minerals, usually calcite. Similar laminated formations date back to 3.5 billion years ago, an attractive hypothesis being to consider modern lithifying microbial mats as analogous of Precambrian stromatolites (Stal, 2012; Vasconcelos et al., 2006).

II.5.1 Microbial mat composition and lithification

Microbialites are organominerals resulted from the complex interaction between the microbial mats and the surrounding environments. These organominerals may be produced by two

processes, i.e. microbially - induced and microbially – influenced mineralization. The microbially-induced mineralization means that the precipitation is the direct result of microbial activity, while the microbially - influenced process is referring to the passive mineralization of the organic matter, mainly due to external, environmental factors (e.g. high pH) rather than the precipitation driven by living organisms (Dupraz et al., 2009). Microbial mats have the ability to alter the equilibrium between the more reduced and oxidized forms of carbon (organic matter vs. CO_2). CO_2 dissolves in water forming carbonic acid (H_2CO_3), which will rapidly dissociate in bicarbonate ion (HCO_3^-). Depending on the water pH, carbonate ions may bind cations, forming carbonate minerals. As cations may vary, so are the minerals; they can take the form of calcite/aragonite ($CaCO_3$), magnesite ($MgCO_3$), dolomite ($CaMg(CO_3)_2$), strontianite ($SrCO_3$) etc. In a typical microbial mat, 5 - 7 functional microbial groups operate together to achieve complete cycling of major elements, such as C, O, N and S. These groups include: 1) photolithoautotrophs (e.g. cyanobacteria); 2) aerobic chemoorganoheterotrophs; 3) fermenters; 4) anaerobic heterotrophs (e.g. SRB); 5) SOB; 6) anoxygenic phototrophs (purple and green (non-) sulfur bacteria) and 7) methanogenic archaea.

Beside the physico - chemical factors that influence the mineralization process, certain microbial activities may promote the precipitation by increasing the environment alkalinity, or they may produce organic acids that trigger the mineral dissolution (Visscher and Stolz, 2005). In hot spring environments, the seasonality fluctuations are lacking, significant changes taking place only in light intensities. In these habitats, the composition and complexity of microbial mats are mainly determined by temperature (Miller et al., 2009). At higher temperatures, the primary production may be attributed to SOB, while at lower temperatures organic matter is a result of oxygenic and anoxygenic photosynthesis (e.g. Cyanobacteria, Chloroflexi, *Chlorobium*), allowing the diversification of the community (Bolhuis et al., 2014).

In mats that form at moderate high temperatures, cyanobacterial photosynthesis can be an important process in favoring the precipitation of $CaCO_3$ (Visscher and Stolz, 2005; Stal, 2012). Oxygenic fixation of CO_2 results in the increase of the environmental pH, sometimes to values higher than 10, and also in the bicarbonate ion formation (Visscher and Stolz, 2005). Anoxygenic photosynthesis performed by purple and green sulfur bacteria uses H_2S instead of water as electron donor. Because during this process HS^- is produced, its oxidation decreases the environmental alkalinity (Visscher and Stolz, 2005). The result of anoxygenic photosynthesis is the production of one mole of $CaCO_3$ for every two moles of fixed CO_2. Similarly, the reduction of sulfate by heterotrophic SRB also facilitates the microbial mats lithification, producing one mole of $CaCO_3$ per one mole of oxidized CH_2O. Some of SRB use H_2 as electron source instead of organic compounds, and are able to fix CO_2 through the carbon monoxide hydrogenase pathway (Visscher

and Stolz, 2005; Schauder et al., 1989). The result of their metabolism consists in one mole of $CaCO_3$ produced per two moles of H_2 oxidized (Visscher and Stolz, 2005). Chemolithoautotrophy is sustained by the oxidation of various compounds, such as H_2, CO, Fe_2^+, NH_4^+ and HS^-. SOB are dominant members in sulfidic hot springs, and their metabolic activity results in the dissolution of $CaCO_3$ (Visscher and Stolz, 2005). Through their metabolism, the aerobic chemoorganoheterotrophs lead to the dissolution of one mole of a $CaCO_3$ for each mole of CH_2O that is oxidized. Among all modes of respiration, methanogenesis is the least energetically favorable. However, hydrogenotrophic methanogenesis is prevalent in freshwater and marine microbial mat environments (Visscher and Stolz, 2005), and also in hot spring microbial mats (Sonne-Hansen and Ahring, 1997). The net result of two moles of HCO_3^- reduction is the production of one mole of $CaCO_3$. Thus, photosynthesis and sulfate reduction lead to the $CaCO_3$ precipitation and possibly the lithification of the microbial mats, while aerobic respiration and sulfide oxidation results in mineral loss.

II.6 Methods for the study of biodiversity in thermal habitats

Culture – dependent techniques were used in early studies for the characterization of microbial communities, but later findings showed that less than 10% of the environmental microbes can be cultivated. As a result, culture-independent methods were developed, which targeted the amplification of the nearly complete 16S rRNA gene from metagenomic DNA. Later, NGS techniques emerged, and the biodiversity started to be investigated through the metabarcoding and the shotgun metagenomic approaches. These methods allow for the characterization of the genomes, transcriptomes and metabolomes of individual thermophilic species and communities. Some future prospects in this study area include the single cell manipulation and NGS, functional metagenomics and metabolomics, the study of viral metagenome etc. (Urbieta et al., 2015).

III. The aim of the thesis

The purpose of the present work was to use of NGS techniques for the study of prokaryotic diversity in three different kind of samples, aiming:

i) to describe the microbial diversity patterns in the two hyperthermal aquifers from the Western Plain of Romania. We also wanted to investigate the impact of physico-chemical parameters and the role of the connectivity to the surface ecosystems in shaping their biodiversity;

ii) to describe the composition of minerals in the carbonate deposits from Ciocaia and to characterize their microbial diversity, which could be involved in the mineralization process;

iii) to explore the relationships between the physico-chemical parameters of the geothermal

waters and the prokaryotic composition of several microbial mat collected from the surroundings of three drilling.

IV. Materials and methods

IV.1. Sampling and sample processing

a) Duplicate hot water samples were collected from 11 drilling in sterile glass bottles of two liters each. Three fractions from each of the collected waters were vacuum filtered using 0.22 μm pore size sterile nitrocellulose filters (Fioroni, France) that were stored at -20°C until DNA extraction.

b) Three sedimentary structures (microbialites), each set in a specific microenvironment, at 32ºC, 49ºC and 65ºC were samples. For DNA extraction approximately 3 cm^3 of mineral deposit were placed into sterile 50 ml Falcon tubes and immediately frozen in liquid nitrogen. For SEM and mineralogical analyses, approximately 5 cm^3 of sample were collected in duplicate using sterile instruments and placed into sterile Petri dishes.

c) For DNA and RNA extraction, approximately 3 g of non-mineralized microbial mats was collected in 15 mL Falcon tubes in triplicate and TRIzol TM Reagent (Invitrogen) was immediately added to fill up the tubes in order to maintain the integrity of the total RNA. All the samples were transported on ice to the laboratory, and processed within the same day.

IV.2. DNA and RNA extraction

a) Three filters were obtained for each water sample, and the total DNA was extracted from the filters using the Chelex ® 100 Resin (Bio-Rad) based protocol (Suenaga and Nakamura, 2005).

b) For DNA extraction from microbialite samples, a DNA extraction protocol was adapted after Reyes-Escogido et al. (2010) that combines the use of a chelating agent (Chelex-100, Bio-Rad, USA) and microwave radiation.

c) From the non-mineralized microbial mat samples, total DNA and RNA were extracted in triplicate for each microbial sample according to the manufacturer instructions for the TRIzol TM Reagent, (Invitrogen).

IV.3. Polarized light microscopy and Scanning Electron Microscopy (SEM)

For microbialite samples, Micro-scale observations were made using a Nikon TE-2000 petrographic microscope equipped with a Nikon D90 digital camera (Nikon Inc., Melville, NY, USA). For SEM, the samples were fractured in liquid nitrogen, fixed on copper holders, covered with a 10 nm gold layer and observed with a JEOL JSM 5510LV electron microscope (JEOL, Tokyo, Japan).

IV.4. Mineralogical analysis using X-ray Diffraction analysis (XRD)

The mineral composition was determined by XRD using a Shimadzu 6000 diffractometer with CuKα radiation and Ni filter. The samples were hand milled in an agate mortar and measured from 2 to 60°2θ, with a scan speed of 2°/min.

IV.5. Quantitative real time PCR (qPCR)

a) The abundance of prokaryotes was determined in the hot water samples and in the non-mineralized microbial mats through qPCR using the universal PRK341F/PRK806R primer pair (Yu et al., 2005). All the details on the reaction mixture and program can be found in thesis.

b) The abundance of bacteria and archaea in the microbialite samples were determined using 16S rRNA gene group-specific primers (931F Archaea AGGAATTGGCGGGGGAGCA (Einen et al., 2008), M1100R Archaea BGGGTCTCGCTCGTTRCC (Einen et al., 2008), 338F Bacteria ACTCCTACGGGAGGCAGCAG (Lane, 1991), 518R Bacteria ATTACCGCGGCTGCTGG (Muyzer et al., 1993). All the details on the reaction mixture and program can be found in thesis.

IV.6. Microbial community analysis

a) Libraries for the V3-V4 regions of the 16S rRNA gene were prepared for each hot water sample and non-mineralized microbial mat sample. For PCR amplification the primer pair PRK341F/PRK806R (Yu et al., 2005) was used, modified by the addition of Illumina-specific adaptors. The concentration of amplicons was measured with the Qubit ® dsDNA HS Assay Kit using the Qubit® Fluorometer and equal amounts of amplicons for each sample (45 ng) were pooled into a normalized library. The library concentration was measured using the PerfeCta ® NGS Quantification Kit for Illumina (Quanta BioSciences, USA) and the pooled library was diluted in Tris pH 8.5 to a final 4 nM concentration. The sequencing step was performed on a MiSeq platform (Illumina, USA) using V3 sequencing chemistry with 300 bp paired-end reads.

b) For the microbialite sample the variable region V4 of the 16S rRNA gene was amplified using the Archaea/Bacteria universal primer pair 515F-806R (Lundberg et al., 2013).

IV.7. Amplicon analysis

Raw sequence data was processed and quality filtered through a combination of Usearch v8 and QIIME pipelines (Caporaso et al., 2010; Edgar, 2010). QIIME was used to extract the barcodes from the sequence data, to join the forward and reverse Illumina reads and to demultiplex the sequence data. Singleton removal and quality control filtering were performed using the Usearch v8 pipeline. Both *de novo* and reference chimera checking were carried out in Usearch v8, using the latest version of the Greengenes database ('13_8') as a reference (DeSantis et al., 2006). Taxonomy was assigned using the default classifier in QIIME against the updated '13_8' version of the Greengenes database, and the mitochondrial/plastidial sequences were filtered out of the OTU table.

IV.8. Statistical analysis

For estimating alpha-diversity, the PD-whole tree, the Shannon and Simpson diversity indices and the Chao1 richness estimator were calculated using QIIME. The Unweighted and Weighted Unifrac distances were calculated to evaluate the diversity among samples. Mantel tests, ANOSIM tests and the tests for the determination of multilevel indicator OTUs were performed in R. All the details on the specific packages used in each case can be found in the thesis/papers.

IV.9. Functional predictions

For functionality prediction with PICRUSt (Langille et al., 2013), the OTUs were picked using an open reference approach, and *de-novo* OTUs were removed, keeping in the final OTU-table only the OTUs that had matching Greengenes IDs ('13_8'). Data in the BIOM OTU-table was normalized using the 16S rRNA gene copy number for each OTU, and the normalized table was used for KEGG functional orthologs predictions. The weighted NSTI was computed in order to evaluate the PICRUSt predictions accuracy for each sample.

V. Results and discussion

V.1 The Pannonian and Triassic deep-subsurface aquifers

(*This section is part of the article: Microbial composition and diversity patterns in deep hyperthermal aquifers from the Western Plain of Romania. Microbial Ecology. DOI: 10.1007/s00248-017-1031-x*).

V.1.1 Abundance of bacterial 16S rRNA genes

In the Pannonian aquifer, the 16S rRNA gene copy number varied between $1.3 \times 10^5 – 1.4 \times 10^6$ mL^{-1}, higher values being observed at 55°C (sample P3), while in the Triassic aquifer the values ranged between 1.05×10^2 - 1.2×10^4 mL^{-1}, with the lowest values being detected at 92 – 104°C (Figure 1).

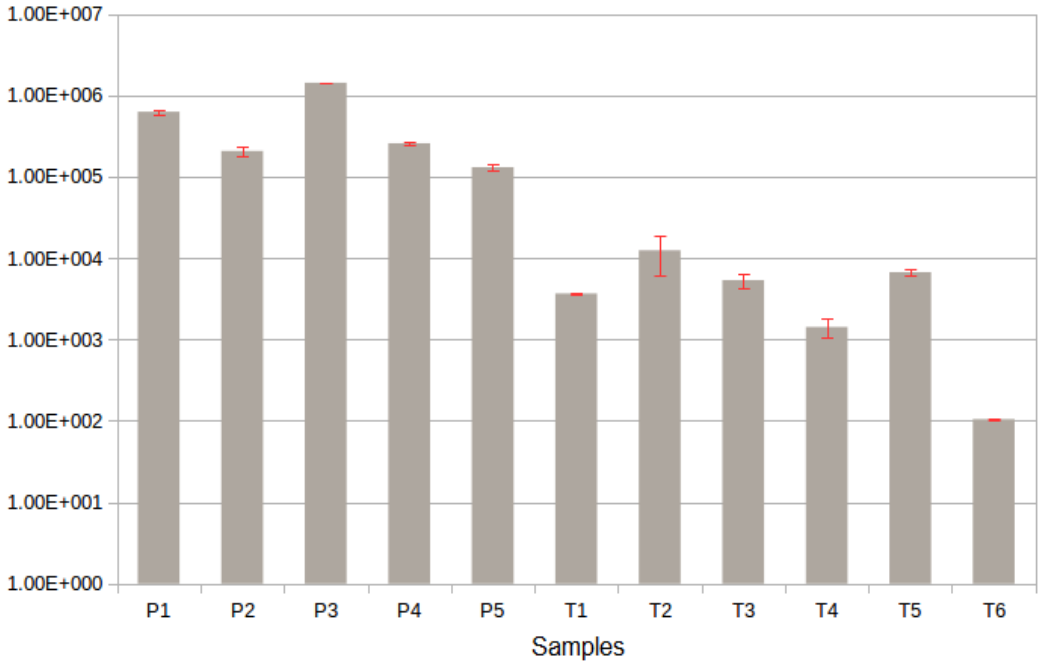

Figure 1. The 16S rRNA gene copy numbers of microbial communities in the 11 upwelling water samples collected from the Pannonian and Triassic aquifers. Values are given in ml^{-1}.

The bacterial abundances appear to decline slowly with depth and with the increase in water temperature, an aspect generally reported in similar works (Kieft, 2016; Nyyssönen et al., 2014; Itävaara et al., 2011). Abundances in the range of 10^5 – 10^6 cells/mL, as those observed in the Pannonian basin, were also encountered in the ~ 2 million years old geothermal aquifer associated with the Great Artesian Basin, Australia, with upwelling water temperature of 64°C (Kimura et al., 2005). The low abundances of 16S rRNA genes, especially at the highest depths and temperatures of the Triassic aquifer, may be derived from the reduced energy fluxes in thermal aquifers and the slow lifecycle of subsurface microorganisms, making the characterization of these communities a more difficult process (Kieft, 2016).

V.1.2 Prokaryotic community structure

Following filtering, sequences were clustered using the 97% sequence identity threshold into 43 to 121 OTUs per sample. In the Pannonian aquifer, 95 - 99% of the sequencing libraries were composed of thermophile and hyperthermophile OTUs, which validated this deposit as a pristine environment. On the other hand, the Triassic aquifer contained 28 - 98% indigenous species, the contamination with less mesophiles being observed in T3 and T4 (Figure 2). The presence of contaminant species is not unexpected, as this aquifer receives large quantities of meteoric waters and takes part in the hydrological cycle (Țenu, 1981).

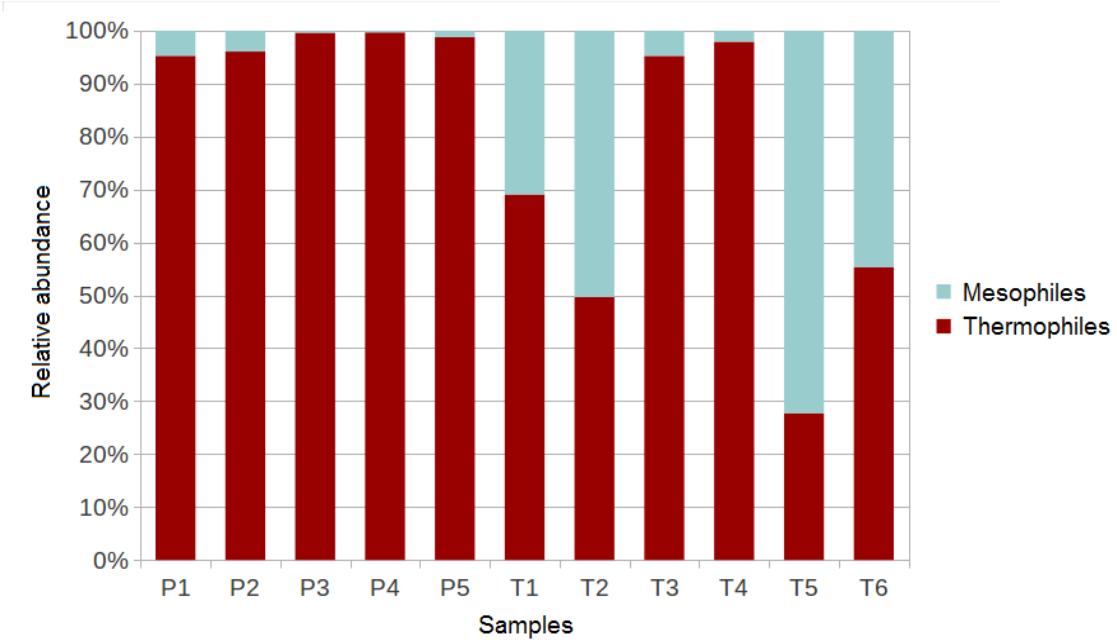

Figure 2. The percentage of indigenous thermophiles and hyperthermophiles together with the mesophilic species in the water samples from the Pannonian and Triassic aquifers.

The taxonomy data revealed that the prokaryotic communities in the investigated aquifers were represented by 24 different phyla, 13 of them with abundance above 1%. The most abundant phylum in the Pannonian aquifer was Proteobacteria (Alpha-, Beta-, Gamma-, Delta- and Epsilonproteobacteria), with 31.9 - 93.9% of the sequencing libraries, while in the Triassic waters both Proteobacteria (6.4 – 95.8%) and Firmicutes (2.8 – 57.5%) were prevalent (Figure 3). Thus, even though these two water deposits are geographically close, they have distinct prokaryotic communities. Moreover, out of the 224 OTUs found in the 11 samples, only 5 of the dominant OTUs were shared among them, i.e. *Rhodocyclaceae*, *Thermoanaerobacteriaceae*, *Thermodesulfovibrio*, *Archaeoglobus* and *Acinetobacter*, highlighting that the Pannonian and Triassic subsurface waters were inhabited by distinct microbial communities.

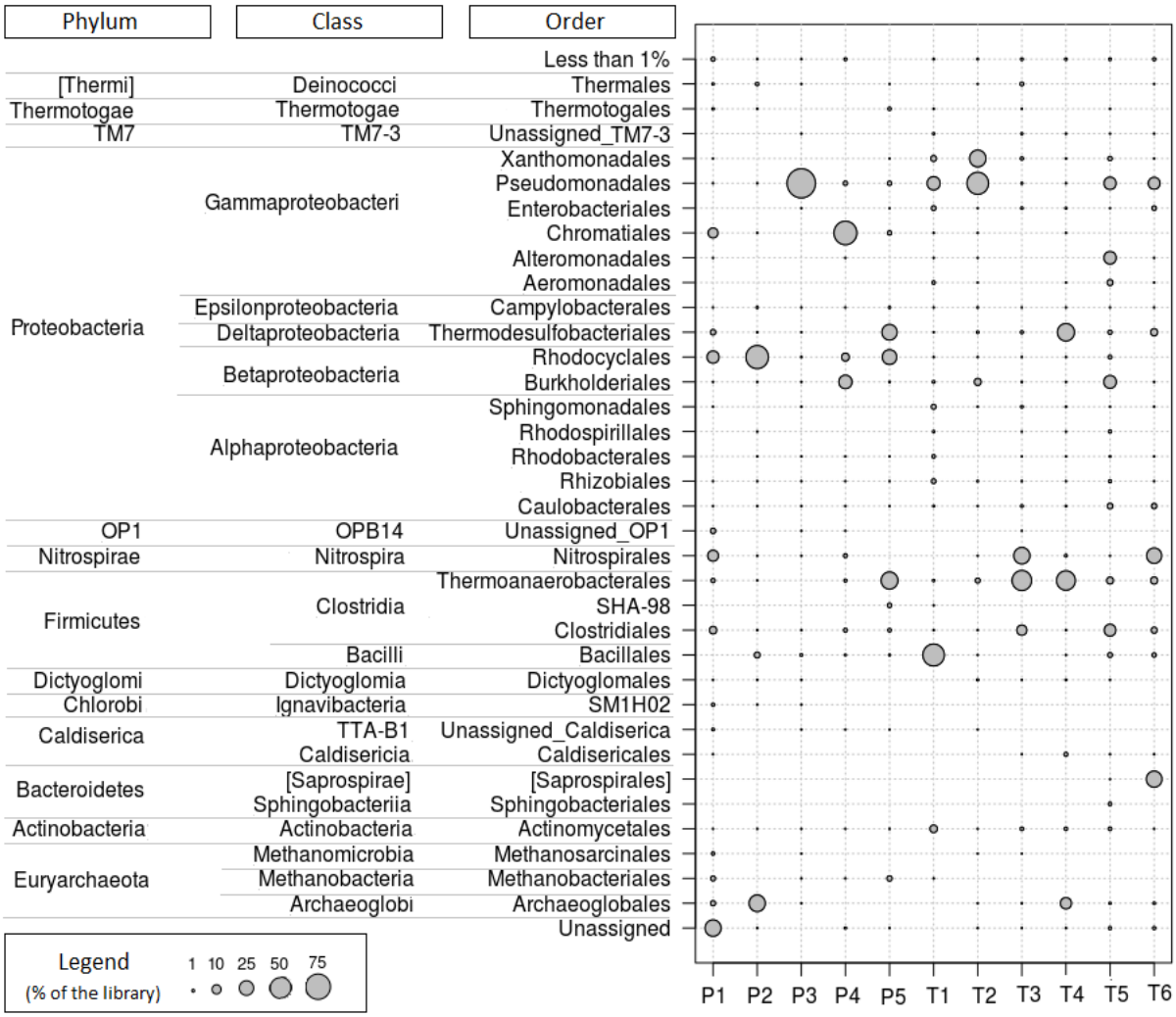

Figure 3. Prokaryotic community composition at the phylum, class and order level. The relative abundances of dominant (> 1% of the sequencing library) bacterial and archaeal taxonomical groups in the 11 geothermal water samples collected from the Pannonian (P) and Triassic (T) aquifers.

V.1.3 Prokaryotic diversity patterns

Unexpectedly, the overall species number increased with temperature, the Pearson correlation coefficient between the OTUs number and temperature being $r = 0.747$ ($p < 0.05$). These results were surprising considering that prokaryotic communities at higher temperatures are usually less complex than those at lower temperatures, hot waters being dominated by only a few genera that are adapted to temperature stress (Kieft, 2016; Inskeep et al., 2010). But the results revealed a different picture when the proportion of thermophiles and mesophiles were analyzed separately. Thus, in the case of thermophiles and hyperthermophiles, their proportion showed a significant negative correlation with temperature ($r = -0.65$, $p < 0.05$) and depth ($r = -0.61$, $p < 0.05$), which is in accordance with the other studies. In our samples, especially in the Triassic aquifer, typical thermophilic and hyperthermophilic species are found together with ubiquitous, aerobic, mesophilic bacteria. A possible explanation may reside in the continuous water refilling of the Triassic aquifer

(Țenu, 1981) that creates a hot, "shallow" environment (Hubalek et al., 2016; Kieft, 2016). In this context, the number of OTUs, as well as the Simpson, Shannon and the PD-whole tree indices (Figure 4) were all strongly correlated with the proportion of mesophiles in our samples (r between 0.58 - 0.66, $p < 0.05$).

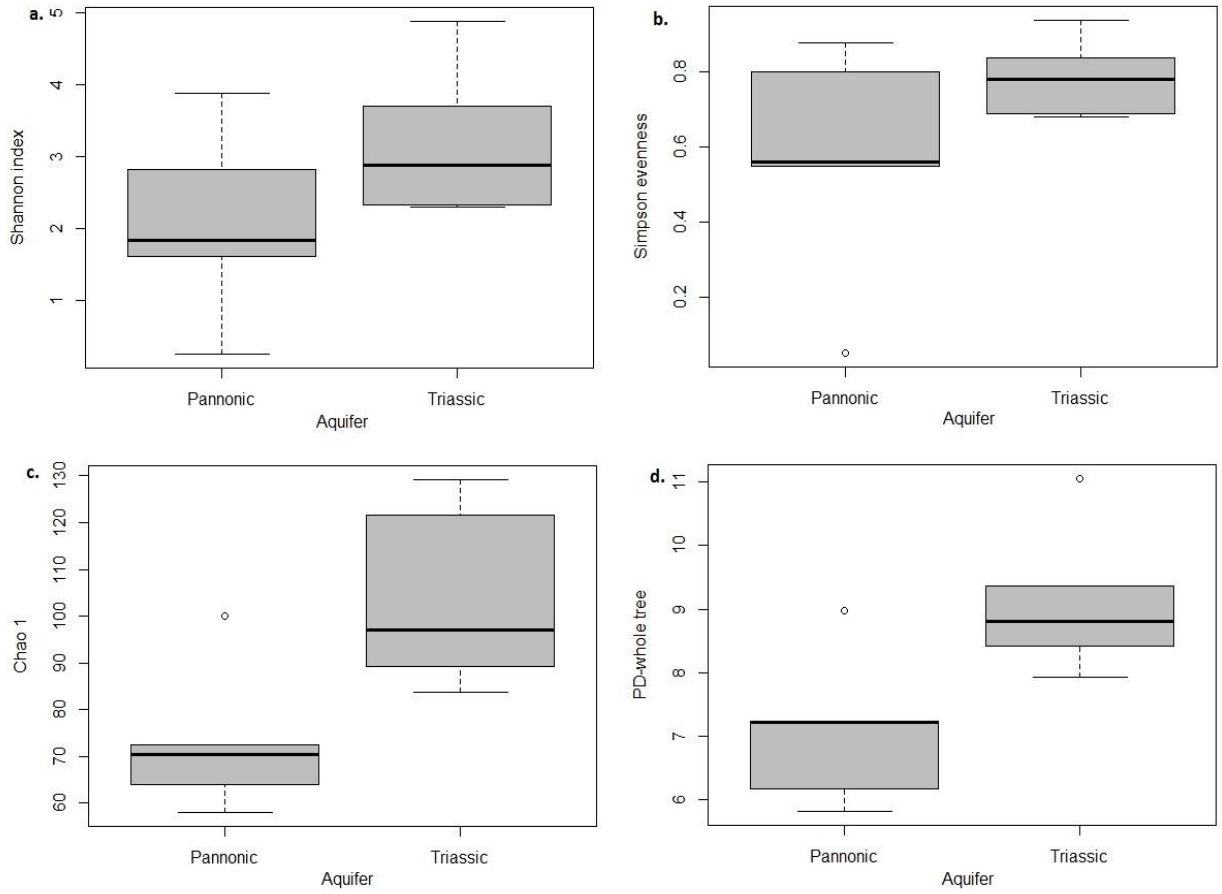

Figure 4. The alpha-diversity indices calculated for the samples within the Pannonian and Triassic aquifers: A) the Shannon index; B) the Simpson diversity index; C) the Chao1 richness estimator and D) the PD-whole tree index.

The presence of mesophiles here probably led to an overall false positive increase in species richness, evenness and phylogenetic diversity along with the temperature and depth (Figure 4). Actually, the communities become more even and more dispersed (low cell abundances) because of temperature stress, nutrients limitation and seclusion (Hubalek et al., 2016).

In order to observe the beta-diversity patterns, PCoA plots were generated using the Unweighted and Weighted Unifrac distance matrices. The clustering pattern generated by the Weighted Unifrac distances was neither strongly correlated with temperature, depth, abundance of mesophiles, nor with any of the physico-chemical parameters (Figure 5a). Interestingly, by using the Unweighted Unifrac distances, a clustering pattern corresponding to distinct aquifers was observed (Figure 5b). Next, the ANOSIM test was run for statistical support, resulting in an r value

of 0.752 ($p = 0.004$), which indicated that the grouping of samples in distinct aquifers is strong and statistically significant. Additionally, environmental parameters, that are specific for each aquifer, like temperature, depth, Na^+ , Ca^{2+} , SO_4^{2-}, pH and electric conductivity were significantly correlated with the diversity pattern ($p < 0.05$).

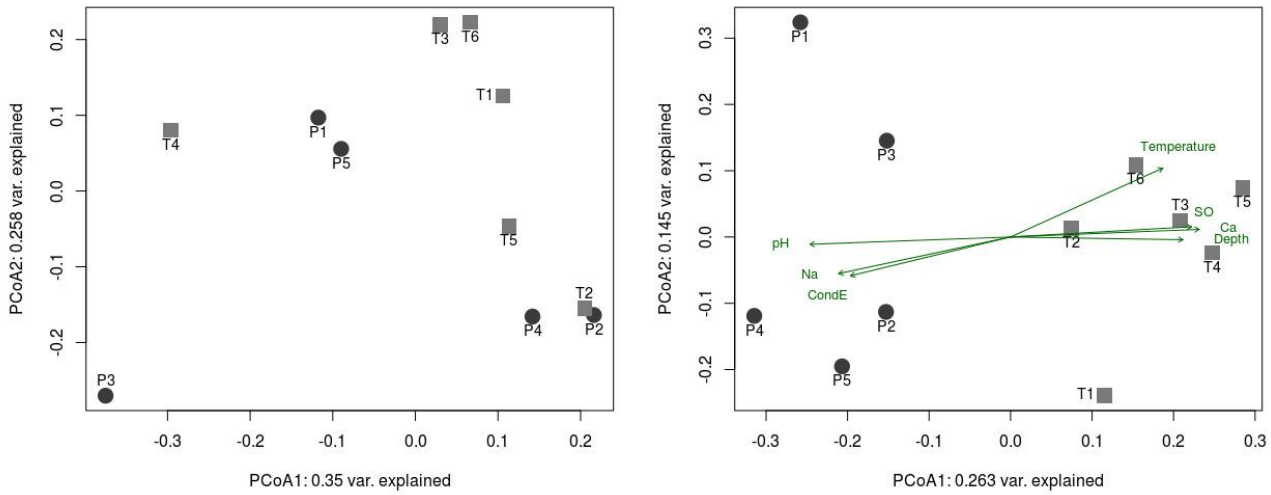

Figure 5. a) Plot of the first two principal coordinate axes for PCoA using Weighted UniFrac distance matrix. b) The same plot using Unweighted UniFrac distances. The environmental factors that exert a significant influence ($p < 0.05$) on the beta-diversity were fitted onto the PCoA graphic.

Thus, the particular physico-chemical variables and the water refilling, factors that are specific to each aquifer, most probably have a cumulative effect in shaping the community structure, especially in the rare taxa distribution.

V.1.4 Functionality prediction in the microbial communities

Methanogenesis appeared more common in the Pannonian aquifer, where *Methanosaeta* species are able to utilize acetate as electron donor (Kamagata et al., 1992) and those that belong to *Methanothermobacter* genus can reduce carbon dioxide to methane using molecular hydrogen as electron donor (Figure 6) (Wasserfallen et al., 2000).

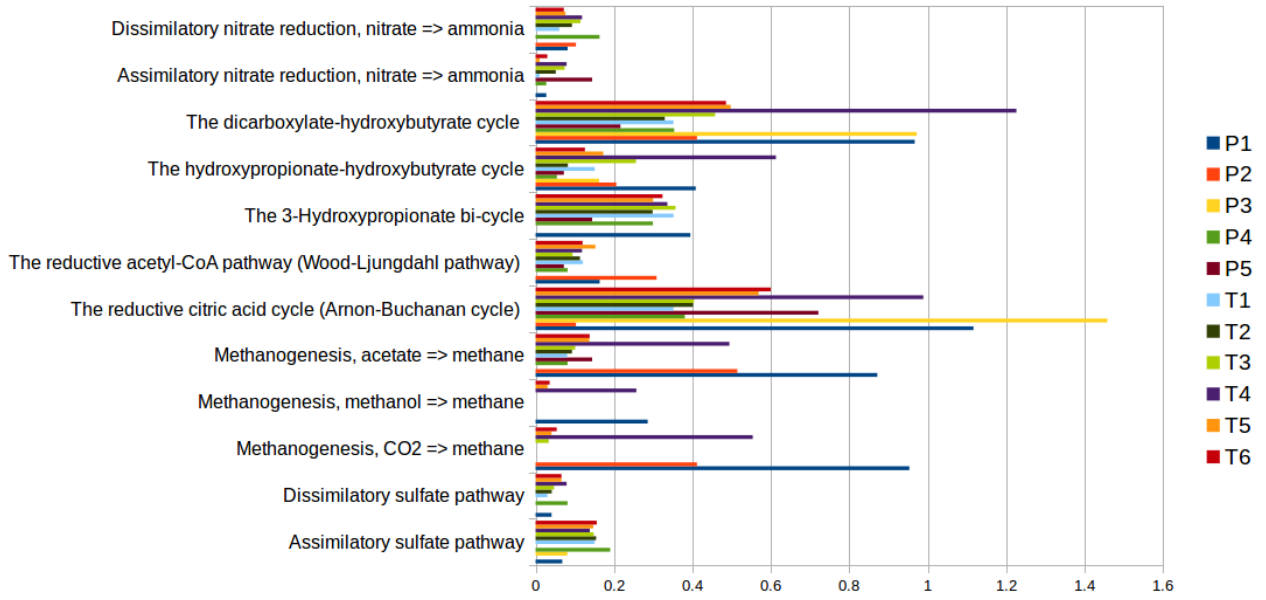

Figure 6. The predicted percentage of genes involved in the nitrogen, methane and sulfur metabolism, as well as the carbon fixation pathways in prokaryotes, in the Pannonian and Triassic geothermal water samples, generated using PICRUSt.

As methanogens and sulfate-reducers normally compete for resources, the former group is dominant in anaerobic habitats where sulfate is limited, which is the case of the Pannonian deposit, and vice versa (Oremland and Polcin, 1982). The organisms responsible for dissimilatory sulfate-reduction are most likely archaea from the *Archaeoglobus* genus in the Pannonian aquifer (Widdel, 2006), while the high sulfate ion concentrations in the Triassic groundwaters favor the presence of clostridial and non-clostridial sulfate-reducers. Sulfate is reduced by these organisms to hydrogen sulfide that can be further assimilated by other prokaryotes as a substrate for growth (Hartzell and Reed, 2006). Although different pathways for carbon fixation were predicted in our samples, the reductive citric acid together with the dicarboxylate - hydroxybutyrate cycles seem to dominate in almost all communities, being characteristic for the microaerophiles and anaerobes, or to the strictly anaerobic hyperthermophilic archaea (Berg, 2011).

V.2 Structure, mineralogy and microbial diversity of geothermal spring microbialites

(*This section is part of the article: Structure, mineralogy, and microbial diversity of geothermal spring microbialites associated with a deep oil drilling in Romania, Frontiers in Microbiology 6:253. DOI: 10.3389/fmicb.2015.00253*)

V.2.1 Mineralogy of the carbonate deposits

Macroscopically, the carbonate crusts show an overall light cream color, with a visible fibrous structure. Dark thin layers (from <1 mm up to few mm thickness), parallel with the substrate (base of the deposit) gives a banded, rhythmical appearance in the case of C32 and C49. The X-ray diffraction data are similar for all the samples investigated and indicate the presence of mostly calcite, accompanied by some clay minerals (probably illite and smectite). Very weak lines, which may be tentatively assigned to aragonite, are present. Polarized light microscopy reveals that the carbonate samples from Ciocaia consist of bundles of elongated scalenohedral crystals displaying slight radial orientation with a more or less regular lamination of darker and lighter layers. In C65, the crystal growth is more irregular and no dark layers were observed. Dark layers consist in mainly organic material, and the lamination pattern was observed at 32^{o}C, but it starts to fade away with the increase in temperature, the precipitation of minerals being random at 65°C.

V.2.2 Microbial mat-carbonate crystal interactions

Several types of microbial mat-carbonate crystals interactions were observed using SEM: i) trapping and binding of crystals (Figure 7A), a key interaction involved in the genesis of lithified communities (Dupraz et al., 2009); ii) microbial mats forming connecting bridges between crystals (Figure 7A, B); iii) microbial mat and individual bacterial filaments or rods colonizing the mineral mass (Figure 7C, D). The bacterial filaments observed are 0.5 to 2.5 μm in diameter and tens of μm in length (Figure 7C, D). The individual filaments (Figure 7E), as well as the entire microbial biofilms (Figure 7F) are included in what appears to be a thick mass of EPS, which may have an important role in carbonate precipitation (Dupraz et al., 2009).

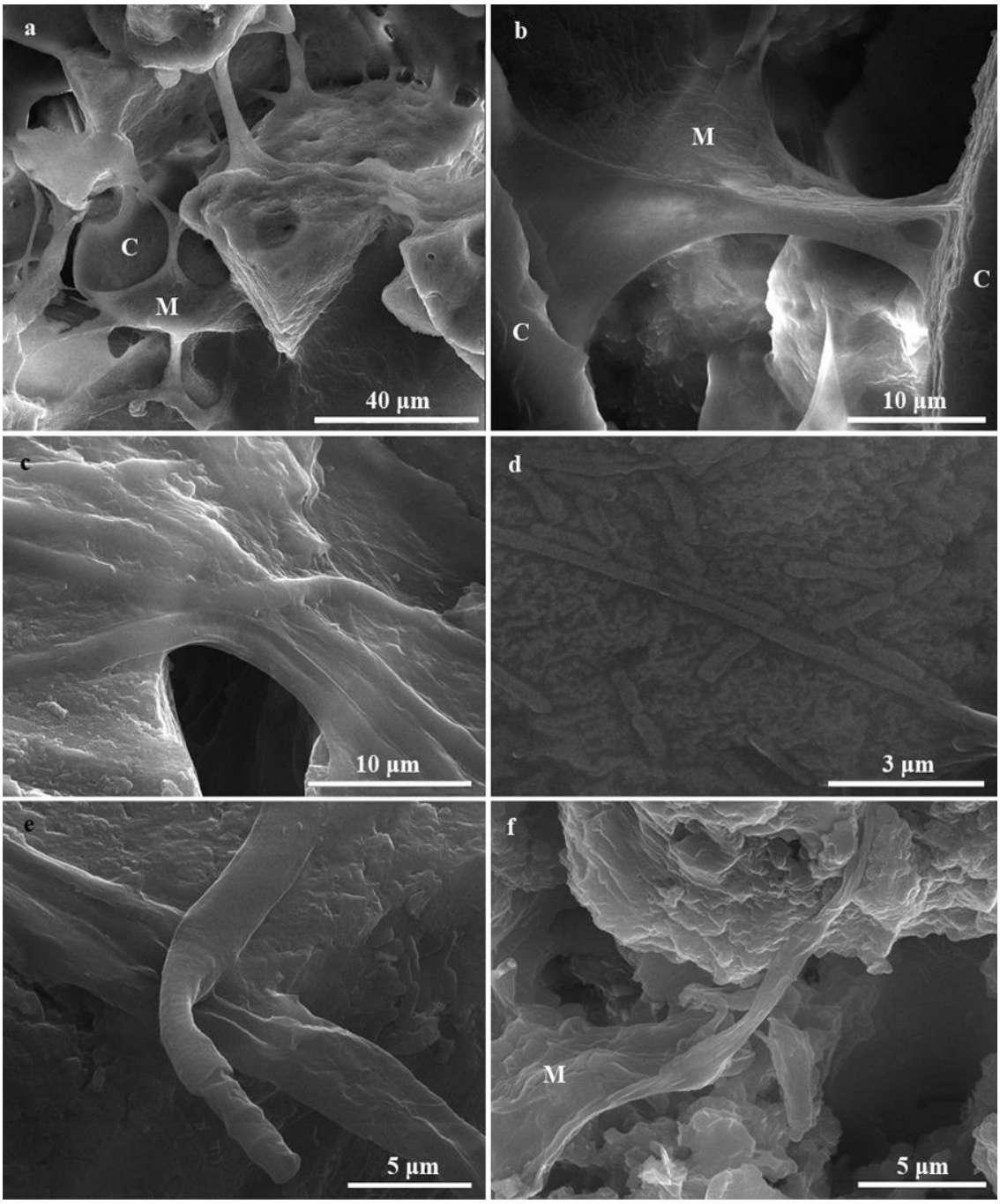

Figure 7. Scanning electron microscopy images presenting the microbial mat-crystals interaction in the carbonate deposits from Ciocaia. A) The microbial mat traps and binds the crystals (C32). B) The microbial mats form connecting bridges between crystals (observed in C49 and rarely in C65). C) and D) The microbial mat or the individual bacterial filaments or rods colonizing the mineral deposit (C – C32; D – C65). E) and F) Individual filaments (E – C32), as well as the entire microbial biofilms (F – C49) are included in what appears to be a thick mass of extracellular polymeric substances. M: microbial mat. C: carbonate crystal.

V.2.3 Microbial abundance and diversity

Bacteria dominated the microbial community in C32 ($2.1\pm0.01\times10^8$ cells/g wet weight; 98.6% of total cell numbers) and C49 ($7.2\pm0.01\times10^8$ prokaryotic cells/g of carbonate; 99.8% of total prokaryotic cell numbers). In C65, bacterial cells accounted for 54.6% of total prokaryotic cell

numbers ($5.7 \pm 0.02 \times 10^7$ prokaryotic cells/g of carbonate). Archaea represented less than 2% in the C32 and C49 samples ($2.9 \pm 0.01 \times 10^6$ prokaryotic cells/g of carbonate - 1.34% and $1.0 \pm 0.01 \times 10^6$ prokaryotic cells/g of carbonate - 0.2%, respectively) and were in almost equal amounts with Bacteria in C65 ($4.7 \pm 0.03 \times 10^7$ prokaryotic cells/g of carbonate – 45.4%).

V.2.4 Microbial taxon richness and diversity coverage

The Chao1 index was calculated to be 531 for the C32 sample, while the corresponding values for the C49 and C65 samples were 156 and 104, respectively. Shannon`s index was similar for C49 and C65 samples (3.0 and 2.7), but doubled in value for the C32 sample (5.94). As the three rarefaction curves reached saturation, it was considered that the sequencing data is reliable for an accurate characterization of prokaryotic diversity in the investigated samples.

V.2.5 Archaeal diversity and community structure

After quality filtering, 35,916 archaeal 16S rRNA partial gene sequences were obtained. Their abundances in the C32 and C49 libraries are low, representing only 0.17% and 0.31%, respectively. The percentage of archaeal sequences increased dramatically in the C65, counting for 35.88% of the entire library. Clustering at 97% cut-off shows the existence of 11 OTUs in C32 library, 18 OTUs in C49 and 14 OTUs in C65. The diversity at the class level can be observed in Figure 8.

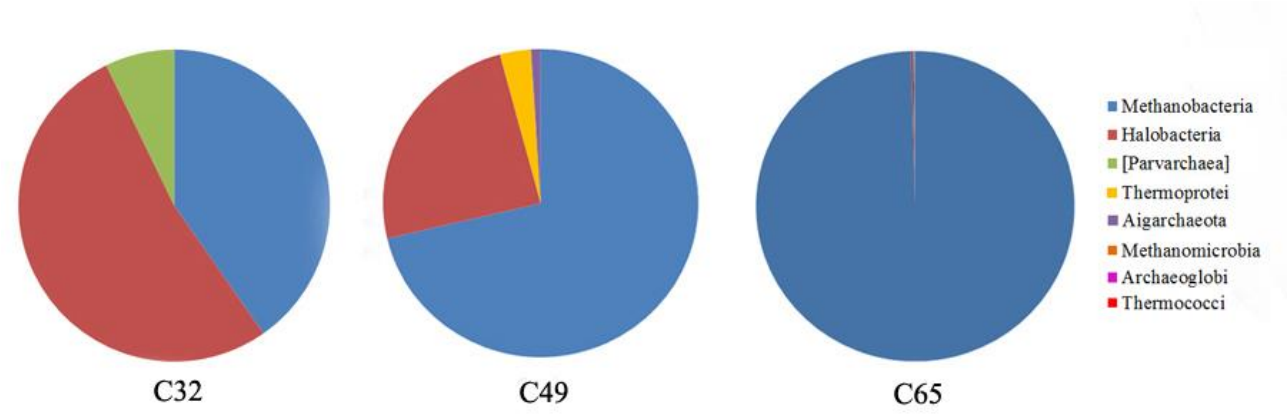

Figure 8. Comparison of archaeal taxonomic diversity in the carbonate deposits from Ciocaia based on the percentage of sequencing reads attributed to OTUs. The C32 sample is dominated by halophiles, followed by methanogens and the proposed lineage [Parvarchaea]. As the temperatures increases, the dominance shifts towards methanogens (sample C49 and C65).

V.2.6 Bacterial diversity and community structure

Among the sequences representing the bacterial community, 66,367 were retrieved for C32, 123,284 for C49 and 63,255 for C65, respectively. The number of observed OTUs decreased from 518 in C32 to 84 in C65. The dominant phylum in C32 was Cyanobacteria (~34%), followed by Proteobacteria (~29%), Firmicutes (~9%), Bacteroidetes (~7%), Chloroflexi (~6%), Thermi (~6%),

Actinobacteria (~3%), Planctomycetes (~3%), and Defferibacteres (~1%). Within C49, there was a striking dominance of the phylum Proteobacteria (~91%), followed by Firmicutes and Bacteroidetes (each with 2%) and Armantimonadetes (~1%) as major groups. In C65, the dominant phyla were Proteobacteria (~50%), Firmicutes (~20%), [OP1] (~10%), Defferibacteres, Thermi and Thermotogae (each with ~5%), [EM3] and Nitrospirae (each with less than 2%) (Figure 9).

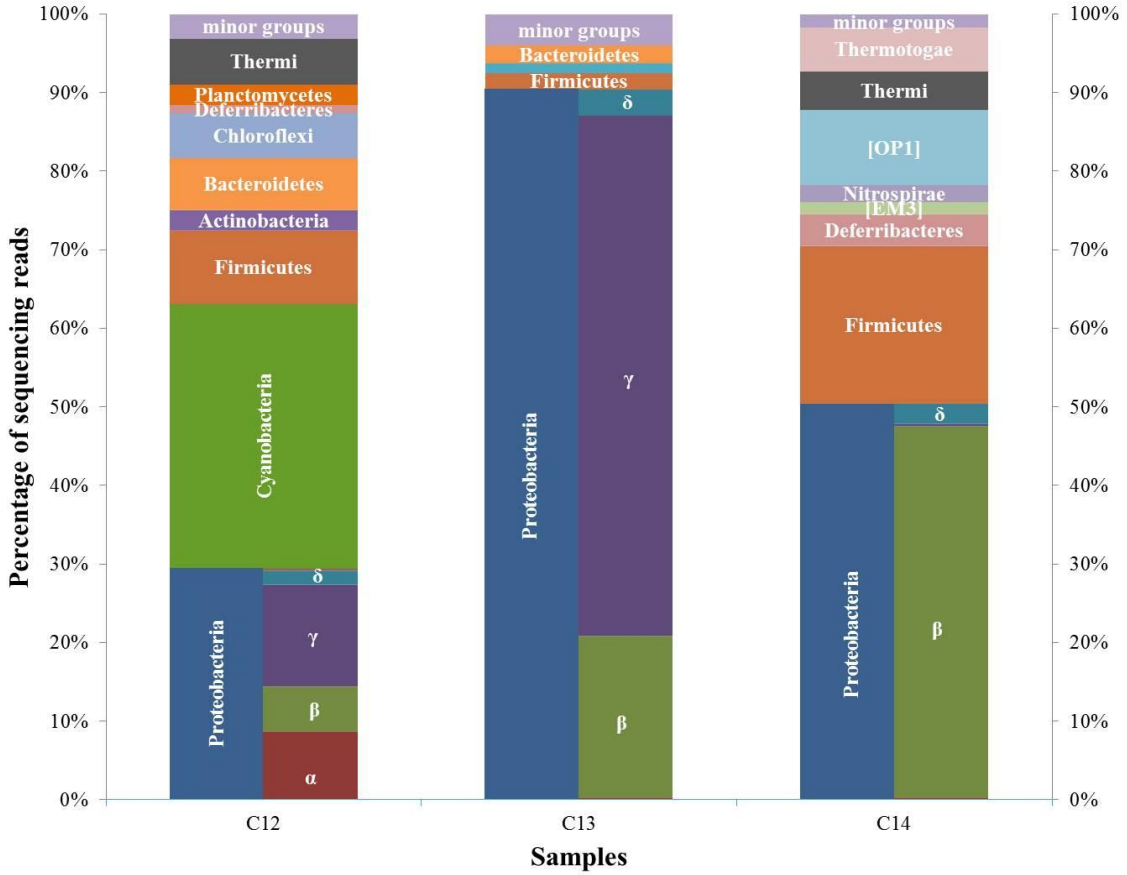

Figure 9. Distribution of major bacterial phyla and classes in the carbonate deposits from Ciocaia based on the percent of sequencing reads that could be attributed to Operational Taxonomic Units (OTUs) within specific bacterial groups.

There are noticeable differences in microbial communities between the three types of microenvironments (microbialites). The C49 and C65 diversities are more similar to each other than to other microbial mats and microbialites, while the microbial diversity in C32 resembles to that of Alchichica crater lake (Figure 10) (Centeno et al., 2012). The bacterial diversity at 49°C and 65°C (samples C49 and C65) has unique characteristics when compared to other modern carbonate deposits worldwide, while the bacterial diversity of the mat collected at 32°C presents a high degree of similarity with other moder stromatolites described in literature (Figure 10).

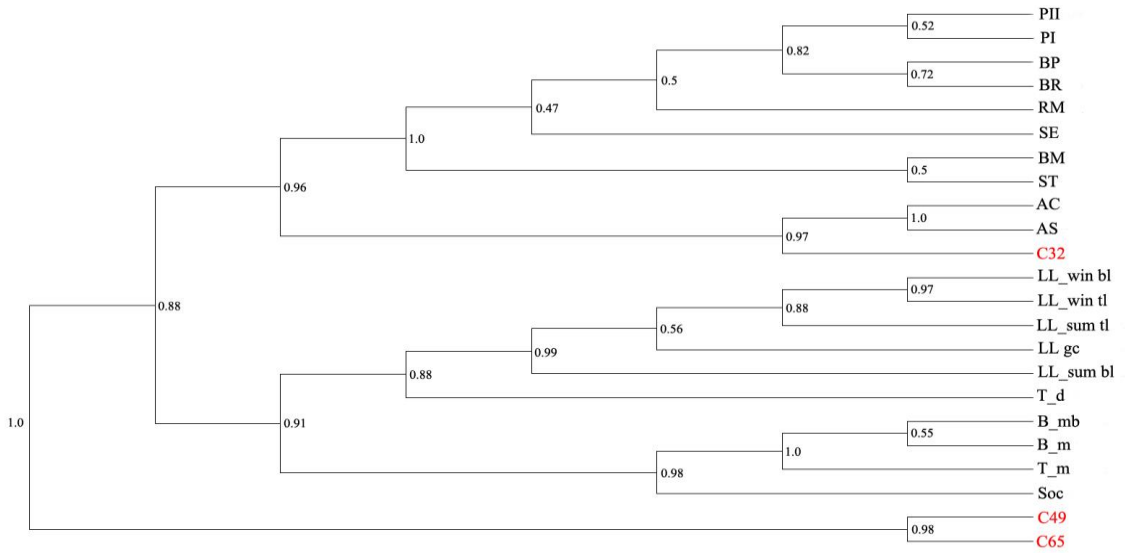

Figure 10. Comparative analysis (UPGMA similarity tree) of microbial diversity in the Ciocaia samples with existing microbial mat/microbialite studies. A closed-reference OTU picking was performed via QIIME (Caporaso et al. 2010) at 97% sequence similarity, after quality filtering and trimming via UPARSE (Edgar, 2013). Sample codes: PI and PII – Pozas Azules I and II; BP – Pirate Channel; BR – Los Rapidos; RM – Rio Mesquites; SE – lagoon microbialite; BM – soft microbialite; ST – microbial mat; AC – columnar microbialite Alchichica lake; AS – spongy microbialite Alchichica lake (SRR350006; Centeno et al 2012); LL_win bl – Salar de Llamara winter sample, bottom layer; LL_win tl – Salar de Llamara winter sample, top layer; LL_sum bl - Salar de Llamara summer sample, bottom layer; LL_sum tl – Salar de Llamara summer sample, top layer (SRR961678, SRR952918, SRR952917, SRR952915, SRR952913; Rasuk et al., 2014); T_d – Tebenquiche dome; B_mb – Brava microbialite; B_m – Brava mat; T_m – Tebenquiche mat (SRR627689, SRR627690, SRR627691, SRR627395; Farias et al., 2014); S – Socompa stromatolite (SRR329490; Farias et al., 2013).

V.2.7 Carbonate-specific and carbonate-unspecific bacterial groups observed in the Ciocaia samples

Among the Bacteria, we identified several other taxa that have not been previously described in association with modern carbonates (Table 1).

Table 1. Bacterial groups described for the first time in association with carbonate deposits.

OTU/Group	Taxonomic rank	Phylum/class	Sample
4C0d-2/YS2	Class/Order	*Cyanobacteria* or [Melainabacteria]	C32
Azoarcus	Genus	*Betaproteobacteria*	C49
Hydrogenophilus	Genus	*Betaproteobacteria*	C49, C65
Thermacetogenium	Genus	*Betaproteobacteria*	C65
Thermanaerobacterales	Order	*Firmicutes*	C65
Bacillus saliphilus *Bacillus aurantiacus*	Species	*Firmicutes*	C32

At 32°C (sample C32), four cyanobacterial genera were identified (*Oscillatoria*, *Pseudoanabaena*, *Leptolyngbya* and *Gloeobacter*), all common inhabitants of carbonate microbialites (Foster et al., 2009; Goh et al., 2009; Schulze-Makuch et al., 2013). At 49ºC, dominant genera are *Idiomarina* (~ 34%) and *Halomonas* (~ 24%) from Gammaproteobacteria,

together with *Hydrogenophilus* (16%) from Betaproteobacteria. The potential for carbonate precipitation by the halophilic members of the *Idiomarina* and *Halomonas* genera was previously documented (Heijs et al., 2006; González- Muñoz et al., 2008). The 65°C carbonate deposit was dominated by *Hydrogenophilus*, with 94% of the entire Proteobacterial sequences and about 47% of the entire library of partial 16S rRNA gene sequences. *Thermacetogenium* spp. is well represented, with about 17% of total. As far as we know, this taxon was never described as being a major group in other carbonate microbial mats.

V.2.8 Functional diversity and possible mechanisms for genesis of carbonate crusts

According to Kim et al. (2012), the abiogenic precipitation prevails at the exit area of the geothermal water, while the level of biogenic influence on precipitation is in direct relationship with the increasing distance of the microbial mat from the geothermal water source and with the decrease of temperature. Based on that, in the sample closest to the exit hole of the drilling (C65), the mineral precipitation could be abiogenic. This possibility is supported by the SEM investigations that did not reveal a well-developed microbial mat in this sample. Also, the microbial diversity is that of a subsurface environment rather than of a terrestrial community. As we move farther apart from the exit point of the geothermal water, both the temperature and water flow decrease and the microbial mats start to thrive in those environments. Thus, the carbonate mineralization could be influenced by both water and microbial mat (C49) or mainly by the microbial mat (C32), as the community is hydrated there only by sprinkling.

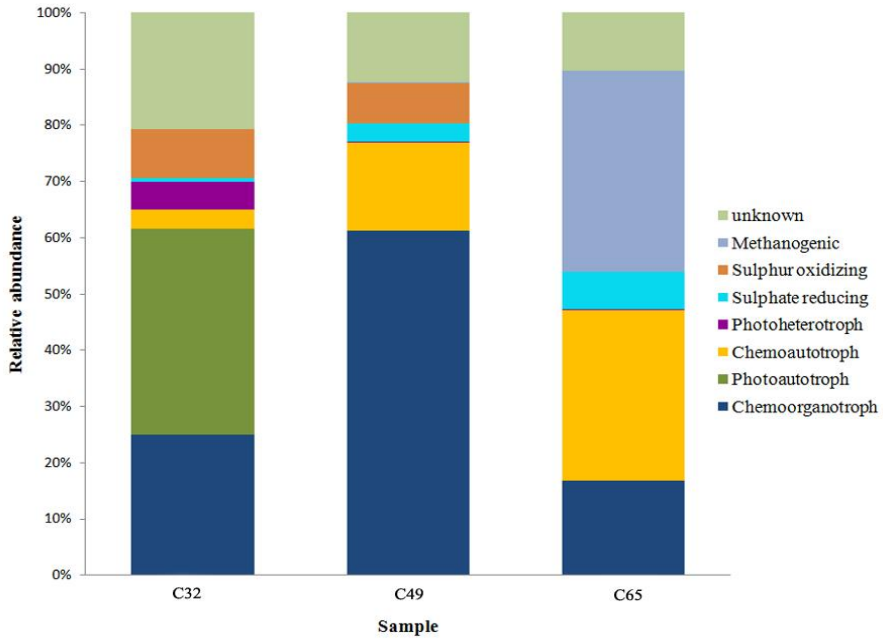

Figure 11. Relative abundance distribution of putative bacterial functional groups in the carbonate deposits from Ciocaia. The histograms are constructed based on the taxonomic affiliations inferred from the 16S rRNA genes.

Phototrophs are dominant in C32 (Figure 11), being represented by both oxygenic and anoxygenic groups. SRB, identified in all samples (Figure 11), can take part in increasing the environment's alkalinity by generating carbonate ions in the process of sulfate reduction. Heterotrophs, dominant in C49 and well represented in C32 and C65, depending on the buffering conditions, can also increase the environment pH towards alkalinity by decomposing organic matter (Konhauser, 2007).

V.3 Drivers of microbial diversity in hot spring microbial mats

(*This section is part of the article: Differences in temperature and water chemistry shape distinct diversity patterns in thermophilic microbial communities, Applied and Environmental Microbiology 83(21): e01363-17. DOI: 10.1128/AEM.01363-17*)

V.3.1 Abundance of prokaryotic 16S rRNA genes and transcripts

Generally, the copy numbers in the cDNA transcripts were found to be two to three orders of magnitude lower than the numbers observed in the DNA sample. This difference may suggest that evaluating the abundance of microorganisms should not be based solely on the 16S rRNA genes and that the total extracted DNA most probably reflects not only the living microbes present in the environment, but also the extracellular DNAs (Valentini et al., 2009). In the CH (Chiraleu) samples, the number of 16S rDNA copies decreased with temperature from $1.1 \pm 0.01 \times 10^8$ at 40ºC to $3.4 \pm 0.6 \times 10^6$ copies/mg at 53ºC, whereas in the other sampling sites no clear pattern was observed. In the CI (Ciocaia) DNA samples, the abundance of 16S rDNA genes ($1.2 \pm 0.05 \times 10^7$ - $2.3 \pm 0.27 \times 10^8$ copies/mg) were comparable with those reported in a previous study focused on the microbialites described at temperatures between 32 and 65ºC in the proximity of the currently investigated microbial mats (Coman et al., 2015). The copy number of 16S rRNA in the MB (Mihai Bravu) samples ($2.89 \pm 0.1 \times 10^7$ – $9.9 \pm 0.2 \times 10^8$ copies/mg) were similar with the other two locations, and no direct relationship with temperature was observed.

V.3.2 Microbial communities revealed by 16S rRNA gene sequencing

Following raw sequences quality filtration, a number of 498,539 sequences were kept in the final dataset that included both the 16S rDNA and 16S cDNA libraries. Each sample was represented by 3,499 to 78,280 high quality sequences that were clustered at a 97% identity threshold in 639 OTUs. Archaea is present in all samples except MB65r, but prevails in the CI65 and CI35r (Figure 12), being mainly represented by aceticlastic methanogens from the *Methanosaeta* (31.6%) genus in the former case, while *Methanoculleus* (5.3%) and *Methanothermobacter* (3.2%) dominated in the latter case. Regarding the bacterial taxonomic composition, the groups with the highest abundances were Proteobacteria (13.2 – 66.2%),

Cyanobacteria (up to 40.3%), Chloroflexi (up to 24%), Bacteroidetes (up to12.8%), and Firmicutes (up to 11.3%). The remaining sequences were widespread affiliated across the Bacteria domain, in the Deinococcus-Thermus (or [Thermi]) (5.7 – 11.2%), Thermatogae (up to 9.03%), Nitrospirae (2.6 – 15.75%), Spirochaetes (up to 5.94%), Verrucomicrobia (up to 4.32%) and Aquificae (up to 10%) phyla.

The CH samples were mainly dominated by Proteobacteria and Cyanobacteria (Figure 12). The abundance of the Proteobactaria phylum and the *Chloroflexus* genus increased with temperature in CH samples, an inverse relationship being observed for the oxygenic phototrophic group. Microbial mats developed at lower temperatures (20 and 35^{o}C) near the CI hot spring were represented by almost equal populations of Cyanobacteria (34.2 – 40.4%) and Proteobacteria (32.2 – 40.7%). Bacteroidetes was the next most abundant group (5.6 – 6.5%) in both samples. The CI65 sample was dominated by Betaproteobacteria, especially by sequences affiliated to *Hydrogenophilus* genus, a group of thermophilic and aerobic organisms, that can have a facultatively chemolithoautotrophic metabolism, using H_2 as electron donor and CO_2 as a carbon source (Hayashi et al., 1999).

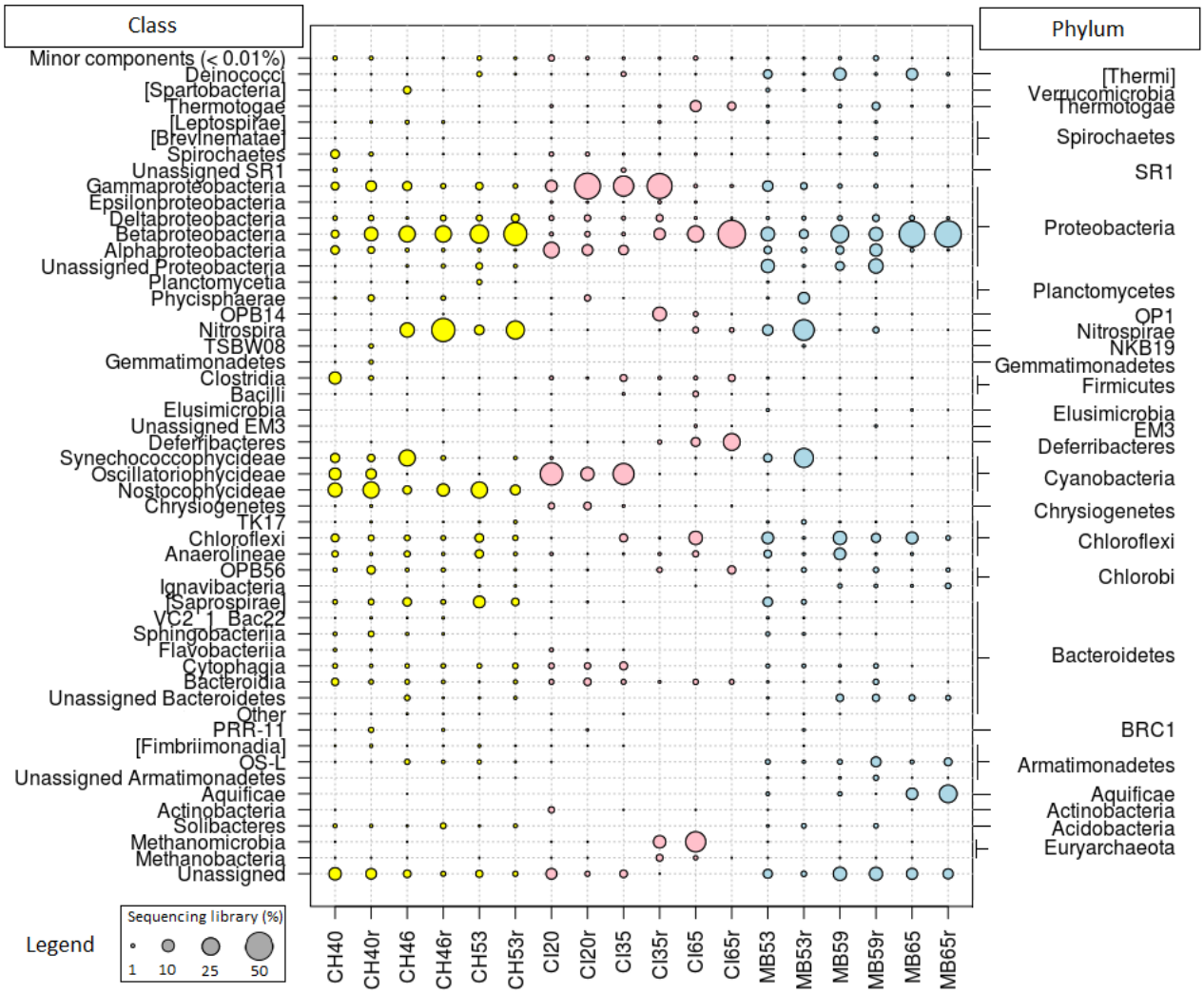

Figure 12. The relative abundance of prokaryotic classes and phyla encountered in the hot spring microbial mats. The sequencing results from both the DNA- and cDNA - based approachs are shown side by side. Different colors were used to represent the three sampling sites: yellow – Chiraleu, pink – Ciocaia, blue – Mihai-Bravu.

The microbial mats from MB were encountered at the highest temperature range, from 53 to 65^{o}C. In these microbial mats, Cyanobacteria is present as a major group only at 53^{o}C (5.4%) (Figure 12). It seems that the environmental conditions favor the Chloroflexi phylum that rises from 15% in MB53 and MB65, to 24% of the library at 59^{o}C. In contrast, the proportion of Betaproteobacteria increased with temperature, being mostly represented by *Hydrogenophilus* members.

V.3.3 Comparative analysis of DNA and cDNA libraries and putative functional traits of microbial mats

For the samples analyzed in this study the DNA - cDNA comparison showed important changes in relative abundance of several groups within the Cyanobacteria, Proteobacteria, Nitrospira, Aquificae and Methanomicrobia phyla (Figure 13).

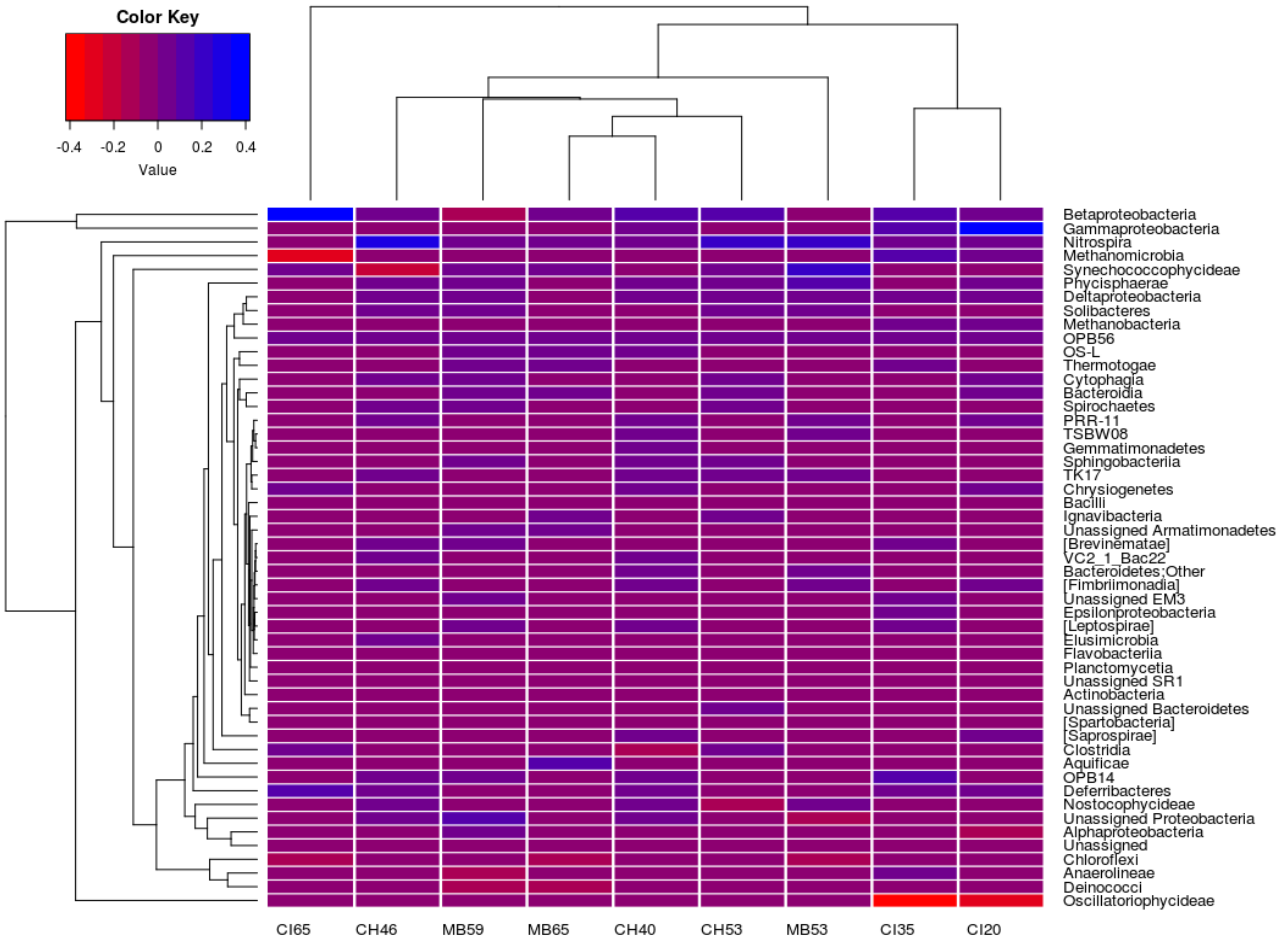

Figure 13. Heatmap of the proportion of change observed in the relative abundance of major taxonomic classes in the DNA versus cDNA libraries. A value of 1 corresponds to 100% of the library.

By analyzing the microbial mats from CH and CI, a decrease in the abundances of *Synechococcophycideae* (-18.5%), *Nostocophycideae* (-12.6%) and *Oscillatoriophycideae* (- 34.2%) sequences were detected in the cDNA libraries. This aspect does not necessarily imply a reduction in the population size, but rather in the protein synthesis activity in Cyanobacteria (Blazewicz et al., 2013). Betaproteobacteria and Gammaproteobacteria had high rRNA/rDNA ratios in the CI microbial mats, being mainly represented by the increase of *Ectothiorhodospira* (CI20r) and *Hydrogenophilus* (CI65r) genera. For this reason, it may be assumed that both genera are potentially important contributors to the primary production in these microbial ecosystems. Surprisingly, *Metanosaeta* genus is the most abundant OTU in sample CI65 (31.6%), but is absent in CI65r. Either this is the result of the RNA extraction bias or these microorganisms are carried by the water flow from the subsurface, but are not able to establish populations in the microbial mats.

V.3.4 Alpha- and beta-diversity patterns

The microbial richness and diversity indices (i.e., Shannon, PD-whole tree, Chao1 and the total number of observed OTUs) were all negatively correlated to the increase in temperature (Pearson r between -0.708 and -0.468, $p < 0.05$). The Simpson diversity index was an exception as

the correlation with temperature (Pearson r = -0.196) was not significant at a $p < 0.05$. This is not surprising as fewer species manage to adapt when the environments become more extreme (Sharp et al., 2014). By comparing our alpha-diversity results to other hot spring microbial mats reported in literature, we observed that the number of OTUs in the majority of the DNA samples was higher than those from the cDNA library, which may reflect the environmental accumulation of free DNA (Valentini et al., 2009) (Figure 14).

The beta-diversity was found to be mainly driven by the combination of physico-chemical parameters at each sampling site, a standard Mantel test performed for the physico-chemical data and the weighted Unifrac dissimilarities (Mantel r = 0.4313, p = 0.001), revealing that the beta-diversity was directly correlated with the environmental factors. For a better understanding on the effects of specific physico-chemical parameters, and to quantify the relative contribution of each variable and combinations of variables on the microbial diversity, a rank correlation analysis was performed on the weighted Unifrac matrix. The best model that correlated the environmental and community data consisted in a subset of six environmental parameters, including Na^+, K^+, HCO_3^-, PO_4^{3-}, temperature and electric conductivity (Spearman's rho 0.456).

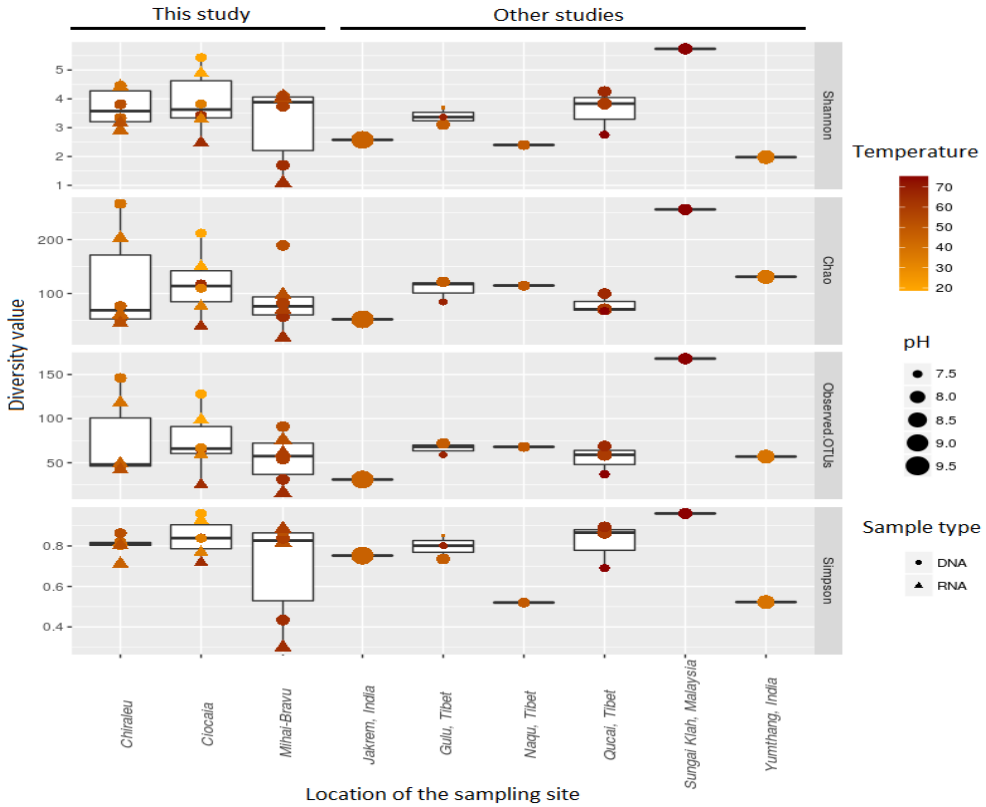

Figure 14. The values of Shannon and Simpson diversity indices, the Chao1 richness estimator and the number of observed OTUs in the samples from Chiraleu, Ciocaia and Mihai Bravu, together with other microbial mats from similar sites (China: SRX206469, SRX206467, SRX206466, SRX206468, SRX206459, SRX206460, SRX206456, Wang et al., 2013; India: SRS932137, SRS932073, Panda et al., 2016; Malaysia: PRJEB7059, Chan et al., 2015).

In order to visualize the beta-diversity among our samples, and also in relationship to other hot spring microbial mats described in literature, we constructed an UPGMA tree based on the Bray-Curtis distances (Figure 15). The UPGMA tree supported the clustering according to the sampling site, rather than the temperature or to the methodological approach (DNA vs. cDNA). We can see that samples from CH form a closed, distinct cluster probably as a result of being dominated by OTUs included in the *Nostocophycideae*, Chloroflexi and Betaproteobacteria groups. The samples with the lowest temperature from Ciocaia (20 and 35ºC) are also grouped together, being mainly composed of methanogenic Archaea, Gamaproteobacteria and *Oscillatoriophycideae* affiliated sequences. The samples from 65ºC had increased abundances of Betaproteobacteria, and particularly *Hydrogenophilus* members, this being probably the factor that supports their clustering with the Mihai Bravu samples. In this analysis, the Tibetan Plateau spring samples described by Wang et al. (2013) formed two clusters based on the temperature, as stated in their original research, showing that the temperature is the main driver of the diversity in that area. GL3.4 and NQ4 have similar thermal regimes (48 and 49ºC) and are dominated by Chloroflxi, Cyanobacteria and *Deinococcus-Thermus* phyla, while the other samples collected from 60 – 75ºC form a distinct group, the majority of OTUs being affiliated to Chloroflexi and/or Aquificeae (Wang et al., 2013).

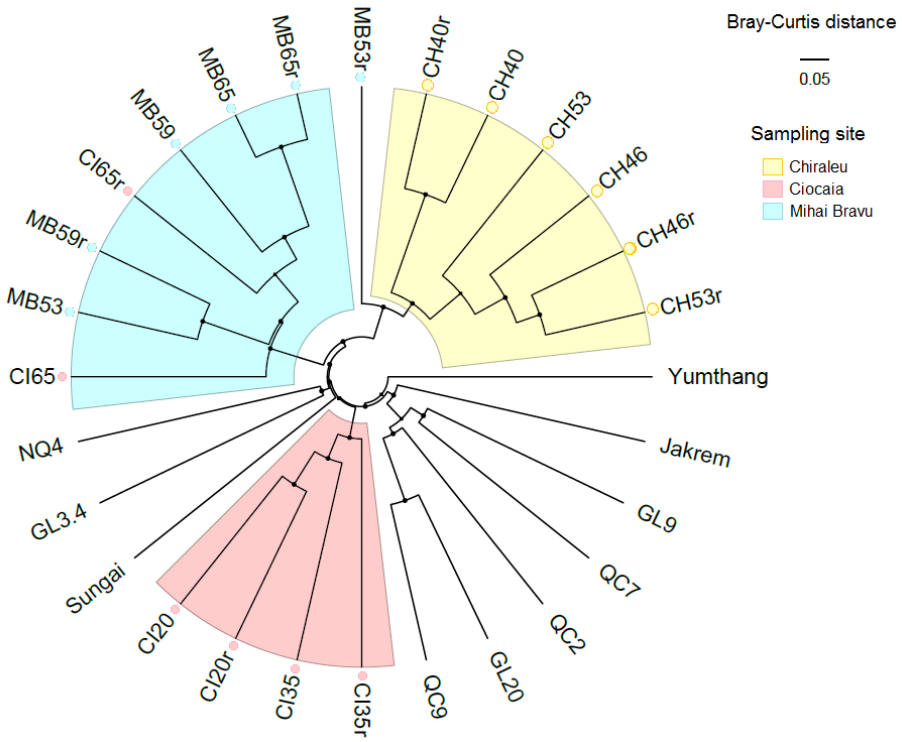

Figure 15. UPGMA tree constructed based on Bray-Curtis distances for a comparative analysis of beta-diversity of the Romanian samples and other hot spring microbial mats reported in literature. Sample codes correspond to: Qucai, Tibetan Plateau, China, samples QC9 - SRX206469, QC7 - SRX206468, QC2 - SRX206467; Naqu, Tibetan Plateau, China, sample NQ4 - SRX206466; Gulu, Tibetan Plateau, China, samples GL3.4 – SRX206459, GL9 – SRX206460, GL20 - SRX206456 (Wang et al., 2013); Jakrem, India – SRS932137, Yumthang, India - SRS932073 (Panda et al., 2016); and Sungai Klah, Malaysia - PRJEB7059 (Chan et al., 2015).

VI. Conclusions

The use of NGS techniques has made possible the comprehensive description of prokaryotic diversity in several types of geothermal environments from the Western Plain of Romania. From the first study it was understood that even though the Pannonian and the Triassic aquifers are in geographical proximity, they are dominated by particular microbial communities. These differences may be caused by the specific physico-chemical characteristics and levels of connectivity to the surface environments. Additionally, mesophilic species were found in high abundances in the Triassic aquifer, probably as a result of meteoric water infiltration in the subsurface, but their origin needs to be confirmed in future studies.

The second study presented the biodiversity of carbonate deposits (microbialites) formed in the surroundings of the Ciocaia abandoned drill hole, at temperatures of 32, 49 and 65^{o}C. The microbialites at lower temperatures presented a laminated structure, while at 65^{o}C the distribution of the carbonate crystals was irregular. Most probably the sample closest to the exit point of the thermal water formed most likely through abiogenic precipitation, a conclusion supported by both the SEM and microbial diversity results, no microbial mat being visible and the prokaryotic diversity was rather similar to that described in subsurface waters. The carbonate precipitation was probably influenced by both water chemistry and microbial mat at 49^{o}C, whereas at the lowest temperature (32^{o}C) it seems mainly determined by the metabolism of the microbial mat.

The last study showed that the prokaryotic biodiversity in the investigated hot spring non-mineralized microbial mats was determined by the combined effect of six parameters, the Na^{+}, K^{+}, HCO_3^{-}, PO_4^{3-} ion concentrations, together with temperature and electrical conductivity. Even though the biodiversity of hot spring microbial mats from other geographic areas seemed to be influenced primarily by temperature as previously reported in literature, our microbial mats have clustered according to their sampling locations.

VII. References

Awramik SM. 1992. The history and significance of stromatolites. In: Schidlowski M, Kimberley MM, McKirdy DM, Trudinger PA (Eds.), Early Organic Evolution. Implications for Energy and Mineral Resources. Springer, Berlin, pp. 435–449.

Beeby M, O'Connor BD, Ryttersgaard C et al. 2005. The genomics of disulfide binding and protein stabilization in thermophiles. *PLOS Biol* 3: e309.

Berg IA. 2011. Ecological aspects of the distribution of different autotrophic CO_2 fixation pathways. *Appl Environ Microbiol* 77: 1925-1936.

Blazewicz SJ, Barnard RL, Daly RA, Firestone MK. 2013. Evaluating rRNA as an indicator of microbial activity in environmental communities: limitations and uses. *ISME J* 7: 2061–2068.

Bolhuis H, Cretoiu MS, Stal LJ. 2014. Molecular ecology of microbial mats. *FEMS Microbiol Ecol* 90: 335-350.

Brock TD. 1986. Introduction: An overview of the thermophiles, pp. 1-6. In: Brock TD (ed) Thermophiles. General, molecular and applied microbiology. Wiley, New-York.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK et al. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7: 335–336.

Centeno CM, Legendre P, Beltran Y, Alcantara-Hernandez RJ, Lidstrom UE, Ashby MN, Falcon LI. 2012. Microbialite genetic diversity and composition related to environmental variables. *FEMS Microbiol Ecol* 82: 724–735.

Chan CS, Chan K-G, Tay Y-L, Chua Y-H, Goh KM. 2015. Diversity of thermophiles in a Malaysian hot spring determined using 16S rRNA and shotgun metagenome sequencing. *Front Microbiol* 6: 177.

Chapelle FH, O'Neill K, Bradley PM, Methé BA, Ciufo SA et al. 2002. A hydrogen-based subsurface microbial community dominated by methanogens. *Nature* 415: 312–315.

Chivian D, Alm E, Brodie E, Culley D, Dehal P, DeSantis T et al. 2008. Environmental genomics reveals a single species ecosystem deep within the Earth. *Science* 322: 275-278.

Coman C, Chiriac CM, Robeson MS, Ionescu C, Dragos N, Barbu-Tudoran L, Andrei AS, Banciu HL, Sicora C, Podar M. 2015. Structure, mineralogy, and microbial diversity of geothermal spring microbialites associated with a deep oil drilling in Romania. *Front Microbial* 6: 253.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie LE, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. 2006. Greengenes, a chimera-checked 16S rRNA gene database and worlbench compatible with ARB. *Appl Environ Microbiol* 72: 5069-5072.

Djokic T, Van Kranendonk MJ, Campbell KA, Walter MR, Ward CR. 2017. Earliest signs of life on land preserved in ca. 3.5 Ga hot spring deposits. Nat Commun 8: 15263.

Dupraz C, Reid RP, Braissant O, Decho AW, Norman RS, Visscher PT. 2009. Processes of carbonate precipitation in modern microbial mats. *Earth Sci R* 96: 141–162.

Edgar R. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* 10: 996–998.

Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26: 2460-2461.

Einen J, Thorseth IH, Ovreås L. 2008. Enumeration of Archaea and Bacteria in seafloor basalt using real-time quantitative PCR and fluorescence microscopy. *FEMS Microbiol Lett* 282: 182-187.

Farias ME, Contreras M, Rasuk MC, Kurth D, Flores MR, Poire DG et al. 2014. Characterization of bacterial diversity associated with microbial mats, gypsum evaporites and carbonate microbialites in thalassic wetlands: Tebenquiche and La Brava, Salar de Atacama, Chile. *Extremophiles* 18: 311–329.

Farias ME, Rascovan N, Toneatti DM, Albarracín VH, Flores MR, Poiré DG et al. 2013. The discovery of stromatolites developing at 3570 m above sea level in a high-altitude volcanic lake Socompa, Argentinean Andes. *PloS ONE* 8: e53497.

Ferrera I, Reysenbach AL. 2007. Thermophiles. eLS doi: 10.1002/9780470015902.a0000406.

Foster JS, Green SJ, Ahrendt SR, Golubic S, Reid RP, Hetherington KL, Bebout L. 2009. Molecular and morphological characterization of cyanobacterial diversity in the stromatolites of Highborne Cay, Bahamas. *ISME J* 3: 573–587.

Goh F, Allen MA, Leuko S, Kawaguchi T, Decho AW, Burns BP, Neilan BA. 2009. Determining the specific microbial populations and their spatial distribution within the stromatolite ecosystem of Shark Bay. *ISME J* 3: 383–396.

González-Muñoz MT, De Linares C, Martínez-Ruiz F, Morcillo F, Martín-Ramos D, Arias, JM. 2008. Ca-Mg kutnahorite and struvite production by *Idiomarina* strains at modern seawater salinities. *Chemosphere* 72: 465–472.

Hartzell P, Reed DW (2006) The Genus *Archaeoglobus.* In: Dworkin M, Falkow S, Rosenberg E (eds). The Prokaryotes. Springer, USA, New York, pp. 82–100.

Hayashi NR, Ishida T, Yokota A, Kodama T, Igarashi Y. 1999. *Hydrogenophilus thermoluteolus* gen. nov., sp. nov., a thermophilic, facultatively chemolithoautotrophic, hydrogen – oxidizing bacterium. *Int J Syst Evol Microbiol* 49: 83-786.

Heijs SK, Aloisi G, Bouloubassi I, Pancost RD, Pierre C, Sinninghe-Damsté JS et al. 2006. Microbial community structure in three deep-sea carbonate crusts. *Microb Ecol* 52: 451–462.

Hreggvidsson GO, Petursdottir SK, Stefansson SK, Björnsdottir SH, Fridjonsson OH. 2017. Microbial speciation in the geothermal ecosystem. In: Stan-Lotter H, Fendrihan S (eds). Adaption of microbial life to environmental extremes. Springer International Publishing, Cham, pp. 37-67.

Hubalek V, Wu X, Eiler A, Buck M, Heim C, Dopson M et al. 2016. Connectivity to the surface determines diversity patterns in subsurface aquifers of the Fennoscandian Shield. *ISME J* 10: 2556.

Inskeep WP, Rusch DB, Jay ZJ, Herrgard MJ, Kozubal MA, Richardson TH et al. 2010. Metagenomes from high-temperature chemotrophic systems reveal geochemical controls on microbial community structure and function. *PLoS ONE* 5: e9773.

Itävaara M, Nyyssönen M, Kapanen A, Nousiainen A, Ahonen L, Kukkonen I. 2011. Characterization of bacterial diversity to a depth of 1500 m in the Outokumpu Deep Borehole, Fennoscandian Shield: Deep Terrestrial Biodiversity. *FEMS Microbiol Ecol* 77: 295–309.

Kamagata Y, Kawasaki H, Oyaizu H, Nakamura K, Mikami E, Endo G, Koga Y, Yamasato K. 1992. Characterization of three thermophilic strains of *Methanothrix ("Methanosaeta") thermophila* sp. nov. and rejection of *Methanothrix ("Methanosaeta") thermoacetophila*. *Int J Syst Bacteriol* 42: 463–468.

Kieft TL. 2016. Microbiology of the deep continental biosphere. In: Hurst CJ (ed). Their world: A diversity of microbial environments, Springer, Cham, pp. 225–249.

Kim JW, Kogure T, Yang K, Kim ST, Jang YN, Baik HS, Geesey G. 2012. The characterization of $CaCO_3$ in a geothermal environment: A SEM/TEM-EELS study. *Clay Clay Miner* 60: 484–495.

Kimura H, Sugihara M, Yamamoto H, Patel BK, Kato K, Hanada S. 2005. Microbial community in a geothermal aquifer associated with the subsurface of the Great Artesian Basin, Australia. *Extremophiles* 9: 407– 414.

Konhauser K. 2007. Introduction of geomicrobiology. Blackwell Publishing Ltd. Maldon, Oxford, Carlton.

Kristjanssos JK, Hreggvidsson GO. 1995. Ecology and habitats of extremophiles. *World J Microbiol Biotechnol* 11: 17-25.

Lane DJ. 1991. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds). Nucleic acid techniques in bacterial systematics, John Wiley & Sons, United Kingdom, Chichester, pp 115–175.

Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Thurber RLV, Knight R, Beiko RG, Huttenhower C. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol* 8: 1-10.

Lévêque E, Janecek S, Haye B, Belarbi A. 2000. Thermophilic archaeal amylolytic enzymes. *Enzyme Microb Technol* 26: 3–14.

Lin LH, Wang PL, Rumble D, Lippmann-Pipke J, Boice E, Pratt LM et al. 2006. Long term biosustainability in a high energy, low diversity crustal biome. *Science* 314: 479.

Lundberg DS, Yourstone S, Mieczkowski P, Jones CD, Dangl JL. 2013. Practical innovations for high-throughput amplicon sequencing. *Nat Methods* 10: 999–1002.

Martins LO, Huber R, Huber H et al. 1997. Organic solutes in hyperthermophilic Archaea. *Appl Environ Microbiol* 63: 896-902.

Menzel P, Gudbergsdottir SR, Rike AG, Lin L, Zhang Q, Contursi P, Moracci M, Kristjansson JK, Bolduc B, Gavrilov S, Ravin N, Mardanov A, Bonch-Osmolovskaya E, Young M, Krogh A, Peng X. 2015. Comparative metagenomes of eight geographically remote terrestrial hot springs. *Microb Ecol* 70: 411–424.

Miller SR, Castenholz RW. 2000. Evolution of thermotolerance in hot spring cyanobacteria of the genus *Synechococcus. Appl Environ Microbiol* 66: 4222–4229.

Miller SR, Williams C, Strong AL, Carvey D. 2009. Ecological specialization in a spatially structured population of the thermophilic cyanobacterium *Mastigocladus laminosus. Appl Environ Microbiol* 75: 729-734.

Muyzer G, Waal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59: 695-700.

Nealson KH, Inagaki F, Takai K. 2005. Hydrogen driven subsurface lithoautotrophic microbial ecosystems (SliMEs): do they exist and why should we care? *Trends Microbiol* 13: 405-410.

Nyyssönen M, Hultman J, Ahonen L, Kukkonen I, Paulin L, Laine P et al. 2014. Taxonomically and functionally diverse microbial communities in deep crystalline rocks of the Fennoscandian shield. *ISME J* 8: 126–138.

Onstott TC, Lin LH, Davidson M, Mislowack B, Borcsik M, Hall J et al. 2006. The origin and age of biogeochemical trends in deep fracture water of the Witwatersrand Basin, South Africa. *Geomicrobiol J* 23: 369–414.

Oremland RS, Polcin S. 1982. Methanogenesis and sulfate reduction: competitive and noncompetitive substrates in estuarine sediments. *Appl Environ Microbiol* 44: 1270–1276.

Panda AK, Bisht SS, De Mantal S, Kumar NS. 2016. Bacterial and archeal community composition in hot springs from Indo-Burma region, North-east India. *AMB Expr* 6: 111.

Pedersen K. 2000. Exploration of deep intraterrestrial microbial life: current perspectives. *FEMS Microbiol Lett* 185: 9–16.

Purkamo L, Bomberg M, Kietäväinen R, Salavirta H, Nyyssönen M, Nuppunen-Puputti M et al. 2016. Microbial co-occurrence patterns in deep Precambrian bedrock fracture fluids. *Biogeosciences* 13: 3091–3108.

Rasuk CR, Kurth D, Flores MR, Contreras M, Novoa F, Poire D, Farias ME. 2014. Microbial characterization of microbial ecosystems associated to evaporites domes of gypsum in Salar de Llamara in Atacama desert. *Microb Ecol* 68: 483-494.

Reyes-Escogido L, Balam-Chi M, Rodríguez-Buenfil I, Valdés J, Kameyama L, Martínez-Pérez F. 2010. Purification of bacterial genomic DNA in less than 20 min using Chelex-100 microwave: examples from strains of lactic acid bacteria isolated from soil samples. *Antonie van Leeuwenhoek* 98: 465–474.

Riding R. 2006. Microbial carbonate abundance compared with fluctuations in metazoan diversity over geological time. *Sediment Geol* 185: 229-238.

Sabath N, Ferrada E, Barve A, Wagner A. 2013. Growth temperature and genome size in bacteria are negatively correlated, suggesting genomic streamlining during thermal adaptation. *Genome Biol Evol* 5: 966–977.

Schauder R, Preug A, Jetten M, Fuchs G. 1989. Oxidative and reductive acetyl CoA/carbon monoxide dehydrogenase pathway in *Desulfobacterium autotrophicum*: 2. Demonstration of the enzymes of the pathway and comparison of CO dehydrogenase. *Arch Microbiol* 151: 84–89.

Schrenk MO, Brazelton WJ, Lang SQ. 2013. Serpentization, carbon and deep life. *Rev Miner Geochem* 75: 575-606.

Schulze-Makuch D, Lim DSS, Laval B, Turse C, Antonio MRS, Chan O et al. 2013. Pavilion Lake microbialites: morphological, molecular, and biochemical evidence for a cold-water transition to colonial aggregates. *Life* 3: 21–37.

Sharp CE, Brady AL, Sharp GH, Grasby SE, Stott MB, Dunfield PF. 2014. Humboldt's Spa: Microbial diversity is controlled by temperature in geothermal environments. *ISME J* 8: 1166–1174.

Sherwood-Lollar B, Voglesonger K, Lin LH, LaCrampe-Couloume G, Telling J, Abrajano TA et al. 2007. Hydrologic controls on episodic H_2 release from Precambrian fractured rocks – energy from deep subsurface life on Earth and Mars. *Astrobiology* 7: 971-986.

Sonne-Hansen J, Ahring BA. 1997. Anaerobic microbiology of an alkaline Icelandic hot spring. *FEMS Microbial Ecol* 23: 31 – 38.

Stal LJ. 2012. Cyanobacterial mats and stromatolites. In: Whitton BA (ed). The Ecology of Cyanobacteria II. Their Diversity in Space and Time, Springer, The Netherlands, pp. 65 – 125.

Stan-Lotter H. 2017. Physiological boundaries of life. In: Stan-Lotter H, Fendrihan S (eds). Adaptation of microbial life to environmental extremes. Springer International Publishing, pp. 1-21.

Stetter KO. 1986. Diversity of extremely thermophilic archaebacteria. In: Brock TD (ed). Thermophiles: General, molecular and applied microbiology. John Wiley & Sons, New York, pp. 39-45.

Stetter KO. 2006. Hyperthermophiles in the history of life. *Philos Trans R Soc Lond B Biol Sci* 361: 1837-1843.

Stetter, K.O. 1999. Extremophiles and their adaptation to hot environments. *FEBS Lett* 452: 22–25.

Stevens TO, McKinley JP. 1995. Lithoautotrophic microbial ecosystems in deep basalt aquifers. *Science* 270: 450–454.

Suenaga E, Nakamura H. 2005. Evaluation of three methods for effective extraction of DNA from human hair. *J Chromatogr B Analyt Technol Biomed Life Sci* 820: 137–141.

Takai K, Nakamura K, Toki T, Tsunogai U, Miyazaki M, Miyazaki J, Hirayama H, Nakagawa S, Nunoura T, Horikoshi K. 2008. Cell proliferation at 122 °C and isotopically heavy CH_4 production by a hyperthermophilic methanogen under high-pressure cultivation. *Proc Natl Acad Sci USA* 105: 10949–10954.

Țenu A. 1981. Zăcăminte de ape hipertermale din Nord-Vestul Romaniei București, Editura Academiei Republicii Socialiste România, București.

Trivedi S, Rao SR, Gehlot HS. 2005. Nucleic acid stability in thermophilic prokaryotes: a review. *J Mol Cell Biol* 4: 61–69.

Urbieta MS, Donati ER, Chan KG, Shahar S, Sin LL, Goh KM. 2015. Thermophiles in the genomic era: biodiversity, science and applications. Biotechnil Adv 33: 633-647.

Valentini A, Pompanon F, Taberlet P. 2009. DNA barcoding for ecologists. *Trends Ecol Evol* 24: 110–117.

Vasconcelos C, Warthmann R, McKenzie JA, Visscher PT, Bittermann AG, van Lith Y. 2006. Lithifying microbial mats in Lagoa Vermelha, Brazil: Modern Precambrian relics? *Sediment Geol* 185: 175-183.

Visscher PT, Stolz JF. 2005. Microbial mats as bioreactors: populations, processes, and products. *Palaeogeogr Palaeoclimatol Palaeoecol* 215: 87-100.

Wang S, Hou W, Dong H, Jiang H, Huang L, Wu G, Zhang C, Song Z, Ren H, Zhang J, Zhang L. 2013. Control of temperature on microbial community structure in hot springs of the Tibetan Plateau. PLoS ONE 8: e62901.

Ward DM, Castenholz RW. 2000. Cyanobacteria in geothermal habitats. In: Whitton BA, Potts M (eds). The Ecology of Cyanobacteria. Kluwer Academic Publishers, Dordrecht, pp. 37–59.

Wasserfallen A, Nölling J, Pfister P, Reeve J, Conway de Macario E. 2000. Phylogenetic analysis of 18 thermophilic *Methanobacterium* isolates supports the proposals to create a new genus, *Methanothermobacter* gen. nov., and to reclassify several isolates in three species, *Methanothermobacter thermautotrophicus* comb. nov., *Methanothermobacter wolfeii* comb. nov., and *Methanothermobacter marburgensis* sp. nov. *Int J Syst Evol Microbiol* 50: 43–53.

Whitaker RJ, Grogan DW, Taylor JW. 2003. Geographic barriers isolate endemic populations of hyperthermophilic archaea. *Science* 301: 976–978.

Widdel F. 2006. The Genus *Desulfotomaculum.* In: Dworkin M, Falkow S, Rosenberg E (eds). The Prokaryotes. Springer, New York, pp. 787–794.

Yu Y, Lee C, Kim J, Hwang S. 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng* 89: 670-679.