Cultivation and Molecular Studies to Reveal the Microbial Communities of Oligotrophic Aquatic Environments

Marwene Toumi

Department of Microbiology, Institute of Biology, Eötvös Loránd University, Budapest

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Ph.D. Program of Environmental Biology, Doctoral School of Environmental Sciences at the Eötvös Loránd University, Budapest

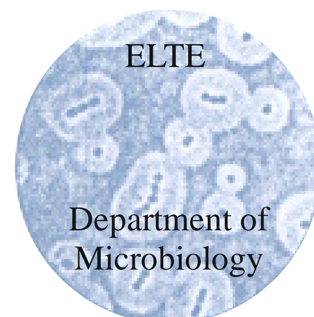
Director: Tamás Turányi, DSc

Biology programme head: Erika Tóth, DSc

Supervisor:

Erika Tóth, DSc.

Department of Microbiology, Institute of Biology, Eötvös Loránd University, Budapest



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List of Abbreviations

AAI: Average amino acid identity

AGL: Aminoglycolipid

ANI: Average nucleotide identity

AOA: Ammonia-oxidizing archaea

AOB: Ammonia-oxidizing bacteria

APL: Aminophospholipid

ATP: Adenosine triphosphate

CPR: Candidate phyla radiation

dDDH: digital DNA–DNA hybridization

DPG: Diphosphatidylglycerol

DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen

EDTA: Ethylenediaminetetraacetic acid

GBDP: Genome blast distance phylogeny analysis

HPLC: High performance liquid chromatography

MALDI-TOF-MS: Matrix-assisted laser desorption/ionization-time of flight

NCBI: National Centre for Biotechnology Information

NGS: Next generation sequencing

OTU: Operational Taxonomic Units

PCA: Principal Component Analysis

PCR: Polymerase Chain Reaction

PG: Phosphatidylglycerol,

PL: Phospholipid

QUAST: Quality assessment tool

Rast: Rapid Annotation System Technology

SPAdes: Iterative short-read genome assembly module

SRA: Sequence Read Archive

TOC: Total Organic Carbon

TYGS: Type strain genome server

WGS: Whole genome sequence

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1. *Introduction*

Oligotrophic environments are characterized by low nutrient flux and low concentrations of organic material. The inhabiting microorganisms are adapted to these extreme conditions; therefore, they often distinguish disparate genomic features and mechanisms for adaptation. Even though, not all strategies employed for survival in such environments are fully clarified, it is known that these organisms have adapted biomolecules (such as exopolysaccharide and extremophilic enzymes) and distinctive biochemical pathways which are of great interest for biotechnological purposes. In order to survive the stress of low nutrient concentrations, oligotrophic microorganisms possess different strategies e.g., physiological manifestations to exploit nutrient poor environments with low energy flows. They also play important role in the ecosystem by regulating the accumulation, export, remineralization, and transformation of organic carbon. Their impact is well observed in the global element cycles, in addition to regulating the activity of the existing microbes within the environment. Although, a complete study of the capabilities of these microorganisms is challenging due to their hard isolation and cultivation within laboratory cultivation conditions. It is of great importance to explore the microbial diversity as it can lead to revealing novel biochemical's and by-products useful for humans, such as enzymes, proteins, drugs, biofuels, understanding the global microbial diversity and its evolution, also identifying the genetic variation and the functionality of existing microbes within their ecosystems.

The aim of the present work

The aim of this study is to explore the diversity of prokaryotes in oligotrophic aquatic environments across 14 sites located in Hungary and Romania. Our study addresses several objectives, which are as follows:

1- Determining the physiochemical characteristics of the different samples collected from these environments and understand how these characteristics influence the microbial diversity and distribution within the studied sites.

2- Utilizing both cultivation and cultivation-independent methods to characterize the structure of the microbial communities found within the samples. Additionally, we aim to estimate the ecological roles of these microbial communities based on information available in previous scientific literature.

3- Identifying and characterizing novel bacterial taxa that thrive in oligotrophic conditions through polyphasic approach, we aim to gain a better understanding of a previously unexplored microbe.

2. *Literature review*

2.1. *Definition of oligotrophic environments and microorganisms*

On Earth, low nutrient content environments are abundant and widespread, sea or ocean water, ground waters, springs usually have low concentrations as 1-10 mg/l (Hirsch 1986). Because the concentration of dissolved organic substances represents the most important ecological feature in the growth of oligotrophic microorganisms. S. I. Kuznetsov et al in 1979, stated that the biological niche for oligotrophic microorganism contains a total content of dissolved and suspended organic substances ranging from 1 to 26 mg of C/l, and averages 1.36 mg of C for suspended organic substance and 15.24 mg of C for dissolved organic substance per litre. Oligotrophic microorganisms are characterized by the ability to grow in low nutrient content environments (Phung et al. 2004). .This feature allows them to surpass the other slowly growing microbes and to dominate the existing environment (Kuznetsov et al. 1979). However, many bacterial taxa isolated from oligotrophic environments could acquire the ability to grow on rich media as well, therefore they are considered as facultative oligotrophic microbes. Based on previous studies, oligotrophs were defined as microbes that can be isolated and maintained on media containing 1-15 mg organic C/l, in addition to the possibility to be able to grow on rich media, either spontaneously or due to adaptation (Poindexter 1981) (Gao et al. 2018). In nature, oligotrophs can be defined as those microbes in which can multiply in low nutrient flux habitats (from near zero to a fraction of 1 mg C/l*day^{-1}), contrary to species whose growth is depending on habitats where the nutrient flux is at least 50 times higher and do not drop to zero for long periods (Poindexter 1981). The concept of Kuznetsov does not apply to bacteria that have been isolated from naturally occurring non-carbonated mineral water. Hence, it would be more correct to refer to them as 'tolerant to', or 'preferring' low nutrient content. The designations oligocarbotolerant or oligocarbophile have been suggested by (Schmidt-Lorenz et al. 1990)

2.2. *Adaptation mechanisms of microbes to oligotrophic environments*

In low nutrient content environments, the members of the microbial communities strongly depend on each other, many are uncultivable microorganisms often presented in viable but non cultivable state due to starvation (Barer et a. 1999), others can grow only on multiple substrates (Lengeler et al. 1999). Some require the presence of a mutualistic partner, like iron reducers, sulphate reducers, methanogens, or methanotrophs (Flynn et al. 2013) and often require special co-aggregations to multiply (Zhang et al. 2016).

In order to survive the stress of low nutrient content, oligotrophic microorganisms possess different strategies to exploit nutrient depletion with low energy flows. A higher substrate utilization efficiency is demonstrated by yielding a higher biomass for each unit of substrate consumed (Ho et al. 2017). Another possibility of the starving survival lifestyle is the rapid change of the life style from the state of dormancy and low activity when a substrate is suddenly added to the environment (Hobbie et al. 2013). The study of Harder et al. 1982 showed that in nutrient limited natural ecosystems, a simultaneous utilisation of the various compounds present in the mixture appears to be the general response of the existing microbial community. Low nutrient content environments can trigger high affinity uptake systems e.g. potassium and ammonia high affinity incorporation systems (Pereira et al. 2017). Also many bacteria developed increased transport capacities e.g. the surface to volume ratio of a typical oligotrophic bacterium is fivefold greater than that of a typical copiotroph (Norris et al. 2021). Another physiological manifestation seen within low nutrient content communities is a so called efficient or "economical" metabolism, characterized by an extremely low maintenance energy required for survival (Saifuddin et al. 2019). Moreover, they have the ability to control the rate at which they metabolize by having one or more "master reactions" or "rate-determining steps" (Miyake 2020). Table 1. summarizes the starvation responses within aquatic microbes and their biological implications.

Table 1. The starvation response in aquatic microbes (Sigee 2005).

Cell characteristic	Starvation response	Biological implications
Genetic changes		
- Formation of starvation-specific transcription factor (RpoS)	- Activation of the rpoS gene leads to formation of RpoS	- Stimulation of rpoS-controlled genes - Gene expression connected to starvation phenotype
- Stimulation of RpoS-controlled genes	- Activation of 30-50 starvation genes	
Biochemical composition		
- Cytoplasmic membrane	- Decrease in the lipid content of the cell membrane. - Changes in fatty-acid composition and fluidity	- Decrease in cell size. - Ability to utilize a greater range of external substrates.
- ATP content (energy state)	- Reduction of adenylate energy charge (AEC)[1] from 1 to value of 0.5-0.75.	- Reduced AEC[1] permits metabolic maintenance but not cell growth.
- Internal storage compounds	- Utilization of specific storage compounds (e.g., glycogen & PHB[2])	- Integration of carbon availability over time
- Internal non-storage molecules	- Loss of free amino-acids Degradation of proteins and RNA	- Internal metabolites and structural molecules become substrates
Cell size and shape	- Decrease in size. Elongate bacteria become spherical	- Increased surface area to volume ratio, increase the potential for nutrient uptake
Bacterial populations		
- Total count - Viable count	- In most cases an initial increase followed by decline - Major decrease in viable count, with	- Reduced food availability for bacterial consumers

	survival of a few dormant cells	- Long-term survival allows future nutrient exploitation

[1] AEC = [ATP] + ½[ADP]/[ATP] + [ADP] + [AMP].

[2] PHB - polyhydroxybutyrate.

2.3. *Role of oligotrophic microorganisms in the environment*

Despite the fact that oligotrophic environments are characterized by low levels of nutrients, microbial counts persist at around 0.5-5 10^5 cells*ml^{-1} (Whitman et al. 1998). They play important roles in the cycling of nutrients such as carbon, nitrogen, and phosphorus in the environment.

In fact, they have an essential impact on regulating the accumulation, export, remineralization and transformation of organic carbon (Cole et al. 1988). Oligotrophic microorganisms contribute to the cycling of carbon in the environment include also using photosynthesis to convert CO_2 into organic matter, which can then be consumed by other organisms in the food chain (Azam et al. 2007). Their role can also be seen in the decomposition of the organic matter and then the release of the CO_2 through respiration (Ravn et al.2020). It is observed that many oligotrophic microorganisms convert organic matter into stable compounds that are stored in sediments for long periods of time (Sheng et al. 2018). Other oligotrophic microorganisms, including methanotrophic bacteria, possess the ability to not only consume methane, a highly potent greenhouse gas, but also effectively convert it into carbon dioxide (Dunfield 2007).

Oligotrophic microorganisms contribute also to the nitrogen cycling in several ways. Previous study showed for example that some microbes characterized with slow growth in oligotrophic environments are very competitive in the nitrification processes by possessing specific metabolisms such as comammox (defined as the capability of certain genera of converting ammonia to nitrate in a single organism) (Dimitri Kits et al. 2017). Others, such as cyanobacteria and certain species of bacteria are capable of nitrogen fixation. This process is an important source of nitrogen in nutrient-limited environments (Karl et al. 1997). Oligotrophic microorganisms can also perform denitrification and ammonification. All these processes are contributing to the availability of nitrogen for other organisms and maintaining the balance of nitrogen in the ecosystem.

Oligotrophic microorganisms utilize common, geochemically important, low molecular organic and inorganic substances, resulting in the incorporation of these substances into the global geochemical cycles of the elements. As a result, oligotrophic activities maintain low concentration of nutrients in the habitat to a point that they become inaccessible to eutrophs. This phenomenon impose a regulatory effect on the activity of eutrophic microbes. (Brodhagen et al. 2015).

2.4. *Cultivation of oligotrophic prokaryotes*

Many authors pointed the difficulty of isolating oligotrophic prokaryotes from the environment and adapting them to the laboratory conditions. Oligotrophs are characterized by their slow growth rate, low biomass production and specialized nutritional requirements. In addition, some oligotrophic microorganisms are often in a viable but non-culturable (VBNC) state, which means that they are metabolically active but are not able to grow on standard laboratory media. Several factors are behind the inability to grow these microbes including their intolerance to high concentrations of nutrient levels in the typical culture media or the usage of unsuitable growth compounds (Akita et al. 2019). Oligotrophic microbes may also require unusual nutrients due to the streamlining selection and gene loss (microelements and or vitamins) (Carini et al. 2013) which occurs as a result of the selective pressure in low nutrient content environments. In fact, to adapt to this environment, the metabolic pathways and genes that are not essential for survival are lost. Previous attempts of cultivation faced also the challenge of the presence of undetected growth inhibitor substrates such as secondary metabolites produced by other microbes (organic acids, toxins and signalling molecules) (Niranjan et al. 2013). Other attempts failed because of the inability of many microbes to grow in close proximity or in the absence of other cells (Thrash, et al. 2015). In natural environments, oligotrophic microorganisms may have evolved mutualistic or antagonistic relationships with other microorganisms. Under laboratory conditions, however, these relationships may not be present, or the microorganisms maybe exposed to novel competitors or pathogens that they are not adapted to. (Gorbatyuk at al. 2005).Oligotrophic microorganisms are often sensitive to changes of the temperature, pH, salinity, and other physical and chemical factors. Laboratory conditions may not accurately mimic the natural environment in terms of these factors, leading to reduced growth, death or entering a dormant or persistent state (Burgess 1997). Most research on oligotrophic bacteria have used conventional laboratory media that have been diluted but otherwise include carbon substrate concentrations of the order of 100 $\mu g\ ml^{-1}$. Oligotrophs

have also been isolated using plain water agar. Taking into consideration that agar itself contains potential nutrient, other jellifying materials are preferable to present oligotrophic conditions. Other commonly used media to cultivate oligotrophs are the minimal media. They contain only the essential nutrients required for microbial growth and are often used to simulate oligotrophic conditions. Examples of minimal media include M9 medium (Song et al. 2010)

It is also seen in previous studies that deionized water or even ultra-pure water has been known to support the growth of some microorganisms. Kulakov et al. (2002) found that many industries such as semiconductor, pharmaceutical, food, and beverage suffer from the microbial contamination of ultrapure water. *Ralstonia pickettii*, *Bradyrhizobium sp*., *Pseudomonas saccharophilia*, and *Stenotrophomonas* bacteria were present in high quantity in these waters. (Walker et al. 2000) conducted a study to identify bacteria present in soft, hard, deionized, or distilled water from dental unit water systems where they found out that these waters harbour bacterial biofilms.

Under these circumstances, oligotrophs are still able to obtain the nutrients they need by scavenging air volatiles and gases as well as traces of nutrients found in water or even on glassware (Sedeek et al. 2022). Oligocarbophile character can be seen in case of a wide range of bacteria that have been isolated from different habitats. 90% of the isolated bacteria belong to the genera *Alcaligenes, Corynebacterium, Hyphomicrobium, Hyphomonas, Listeria, Nocardia, Pedomicrobium, Planococcus, Sphaerotilus, Streptothrix* and *Streptomyces* (Anon 2011).

2.5. *Cultivation independent methods to reveal oligotrophic microorganisms*

Epifluorescence microscopy shows that only 0.1% of all the microbial cells from aquatic environments could be cultivated (Sedeek et al. 2022). Advances in DNA, RNA and protein sequencing techniques, without the need of cultivation discovered several uncultivable prokaryotic taxa (Vaz-Moreira et al. 2014). The earliest sequencing methods were based on chain synthesis termination using the 16S rRNA gene as a molecular marker. Nowadays, among the widely used cultivation-independent techniques is the next generation sequencing (NGS). This technology is able to allow the analysis of many environmental samples through mass sequencing of DNA, enabling the identification of previously hidden microbial diversity (Osman et al. 2019).

The utilization of Next-Generation Sequencing (NGS) techniques has facilitated the cultivation of numerous previously uncultivated microbes. In fact, by analyzing the obtained genomes from metagenomic or single-cell sequencing, researchers have the ability to identify potential genes involved in the utilization of specific substrates, resistance to environmental stresses, or production of bioactive compounds. This knowledge, which offers hints about the needed growth requirements, helps grow targeted previously uncultivated microbes. As an example SARll and SAR11 clades, which were previously identified as uncultivable, was finally possible (Rappé et al. 2002). More recent research could cultivate numerous novel bacteria belonging to *Proteobacteria, Planctomycetes, Bacteroidetes, Acidobacteria* and *Verrucomicrobia* and the discovery of previously unknown microbes such as *Pelagibacter ubique*. The identification of these taxa through the cultivation-independent methods is believed to be a major factor in this achievement (Anon 2011).

Other commonly used technique is the single-cell genomics. This method involves sequencing the genome of individual microbial cells that have been isolated from environmental samples. Single-cell genomics has been used to study the metabolic potential and functional diversity of oligotrophic microorganisms that are difficult to cultivate using traditional culture-based methods (Gawad et al.2016). As an example, *Candidatus Rokubacteria* was first discovered in a freshwater lake using single-cell genomics (Becraft et al. 2017).

It can be also mentioned that fluorescent in situ hybridization (FISH) is a technique which is used to visualize and quantify the abundance of specific microbial taxa in situ, without the need for cultivation. It involves labelling microbial cells in environmental samples with fluorescent probes that target specific DNA or RNA sequences (Liehr 2017). Species such as *Acidithiobacillus ferrooxidans* (Mahmoud et al. 2005), and *Candidatus Brocadia anammoxidans* (Jetten et al. 2001) have been discovered using the fluorescent in situ hybridization (FISH) technique.

However, cultivation independent methods still have their own restrictions and biases, thus, the most reliable results can be obtained by combining cultivation-based and molecular techniques as part of a polyphasic approach (Bohus et al. 2010).

2.6. *Low nutrient content freshwater environments*

Freshwater environments provide diverse ecological habitats and important environmental resources. They contain around 250 000 km^3 of freshwater on the Earth, presented in the forms of lakes, inland seas, or rivers. These environments contain a microbial diversity similar to what has been discovered in open oceans (An 2013).

Among the variability in aquatic ecosystems, many can be considered as oligotrophic environments, such as the deep ocean, low level nutrient lakes and rivers, groundwater drinking water systems etc. (Richards et al. 2005).

2.6.1. *Different groundwater formations*

In a clastic basin, groundwater can be found in layers of sand and gravel that are coarser than the surrounding sediment. With higher depth (30 to 150 meters) below the surface, sandstone can replace the loose sandy layers. At these depths, the pressure from the overlying sediment can cause the loose sand to become more compacted, and with time, the sand can become cemented together to form sandstone. This sandstone can then serve as an aquifer, providing a pathway for groundwater to flow through. Representation of these formation can be found in the report of Bexfield et al (2011). More than three-quarters of the Hungary is underlain by these aquifers, increasing the likelihood of producing local drinking water. Bank-filtered water is the result of wells being drilled into the shallow gravel aquifers that are along with the riverbanks. Water found in deposits close to the surface is referred to as shallow groundwater, whereas water found in deeper clastic sediments is referred to as deep groundwater. Thermal deep groundwater, a subset of thermal waters, is deep groundwater with a temperature higher than 30 °C.

The group of karstic rocks, which make up about half of the hilly regions encompassing one fifth of Hungary's land, is the other major category of groundwater aquifers. These Mesozoic calciferous marine sediments (limestones, dolomites), which were formed during the process of karstification, frequently have a high conductivity along faults, fractures, and holes (Worthington et al. 2017). The karstic waters are effectively recharged because precipitation usually directly and quickly penetrates the karstic rocks (also known as "open karst"). The recharge of karstic waters is efficient because the karstic reservoir is frequently covered by clastic sediments of large (sometimes several km) thickness, generally impermeable lying directly above the karstic formations. The karstic formations are frequently covered by geological formations of low

conductivity (less than 600 μS/cm) (Krawczyk et al. 2006), (covered karst). These formations have low porosity and low permeability, which makes it difficult for electrical current to flow through them (Kaçaroğlu 1999) (Figure 1).

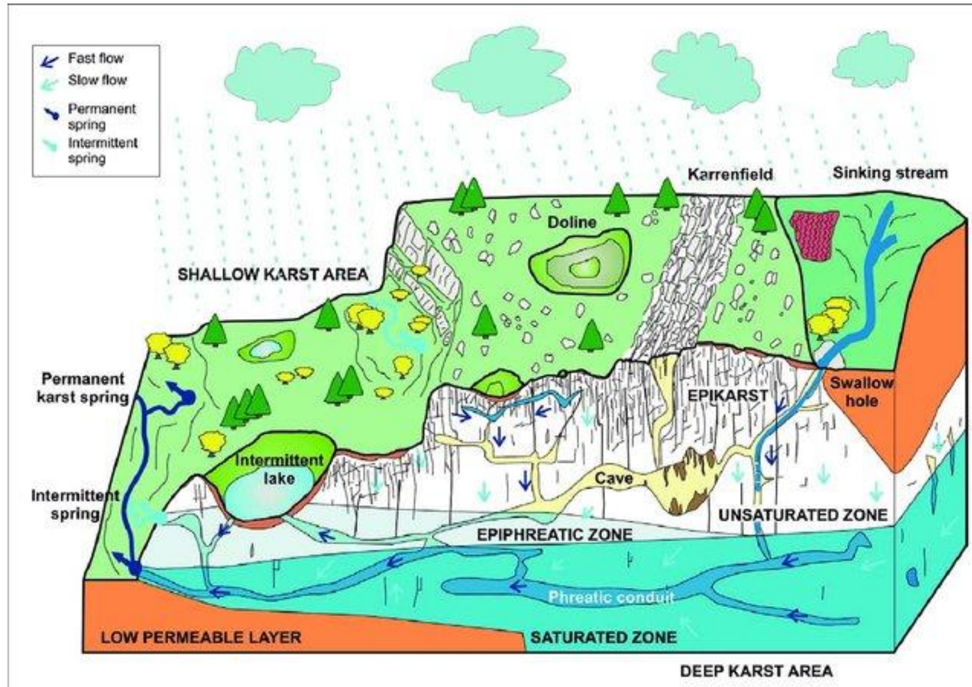


Figure 1. Conceptual three-dimensional model of karst aquifer and groundwater flow. This illustration is taken from Ravbar (2013).

Thermal waters often can be found in the karstic formations. In fact, the water can flow through conduits and fractures in the rock, which can allow it to meet hot rocks and geothermal reservoirs. This can result in the heating of the groundwater and the formation of thermal springs, geysers, and other features (Goldscheider et al. 2010). A portion of these waters rise to the surface as the well-known thermal karst springs. (Héviz, Budapest, Eger).

Non-karstic rocks (crystalline, volcanic, or sedimentary formations of lesser yield) can be found in the hilly regions, from which smaller springs important for local usage arise (Ministry for Environment and Water 2006). The behaviour of non-karstic rocks groundwaters can be quite different from karstic waters due to differences in the structure, porosity, and permeability of the rock formations (Bonacci 2015). For example, sandstone formations typically have high porosity and high permeability, which can result in relatively rapid groundwater flow and the formation of productive aquifers (MacDonald et al. 2005). In contrast, shale formations (composed primarily

of clay minerals and other minerals) typically have low porosity and low permeability, which can make it more difficult for groundwater to flow and can result in lower rates of recharge and discharge (Neuzil 2019).

The temperature and the hydrodynamic-hydrochemical parameters of the water bodies were used also to further categorize them. Cold (below 30°C) and thermal (over 30°C) waters are separated into two groups, allowing for a further division into the three categories indicated above:

– porous or basin-type cold water bodies,

– porous or basin-type thermal water bodies,

as well as,

– karstic cold-water bodies,

– karstic thermal water bodies.

The different formations in Hungary and Romania are visible on Figure 2 and Figure 3.

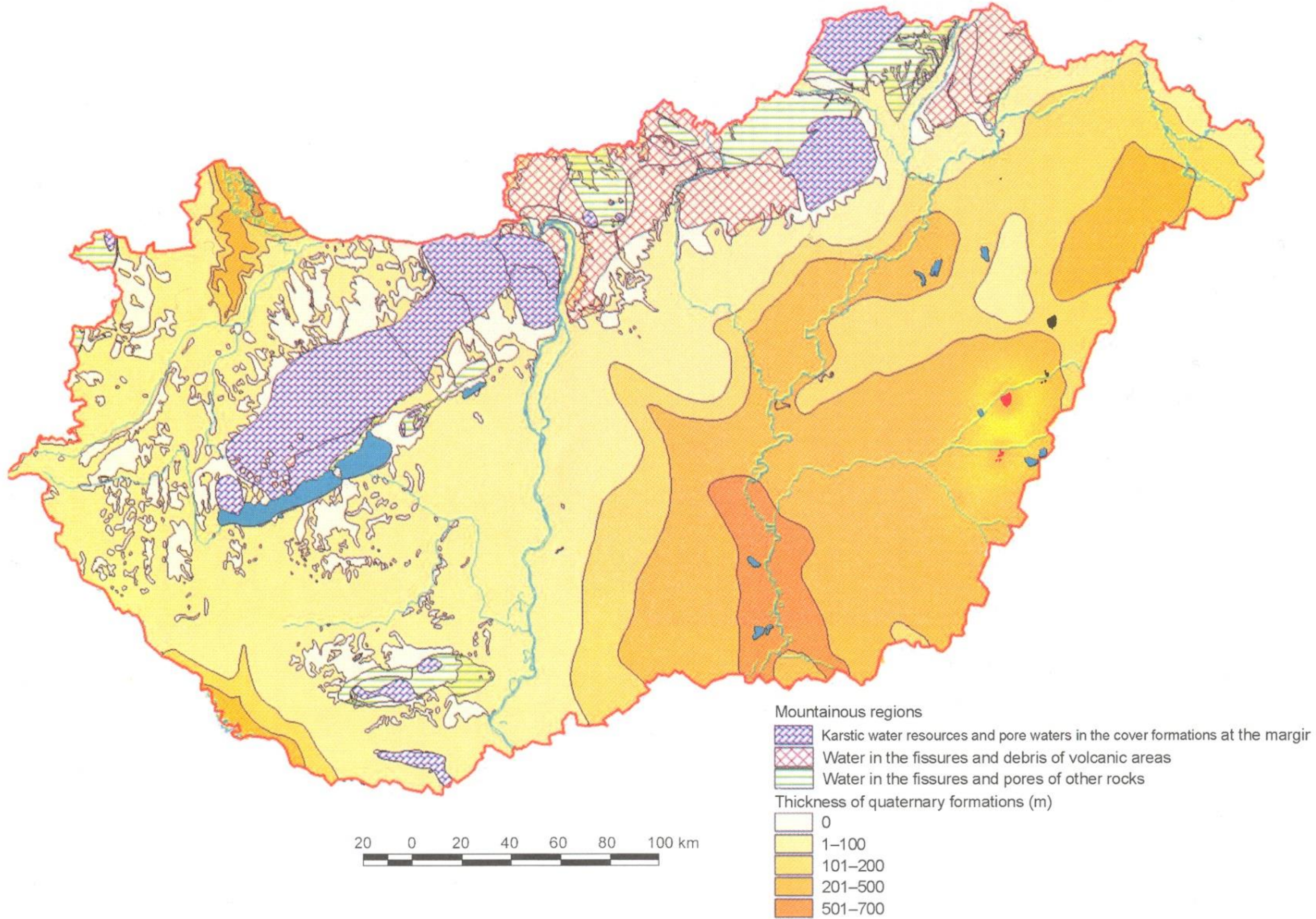


Figure 2. Water bearing formations in Hungary (without geothermal aquifers). This map is taken from the Ministry for Environment and Water 2006.

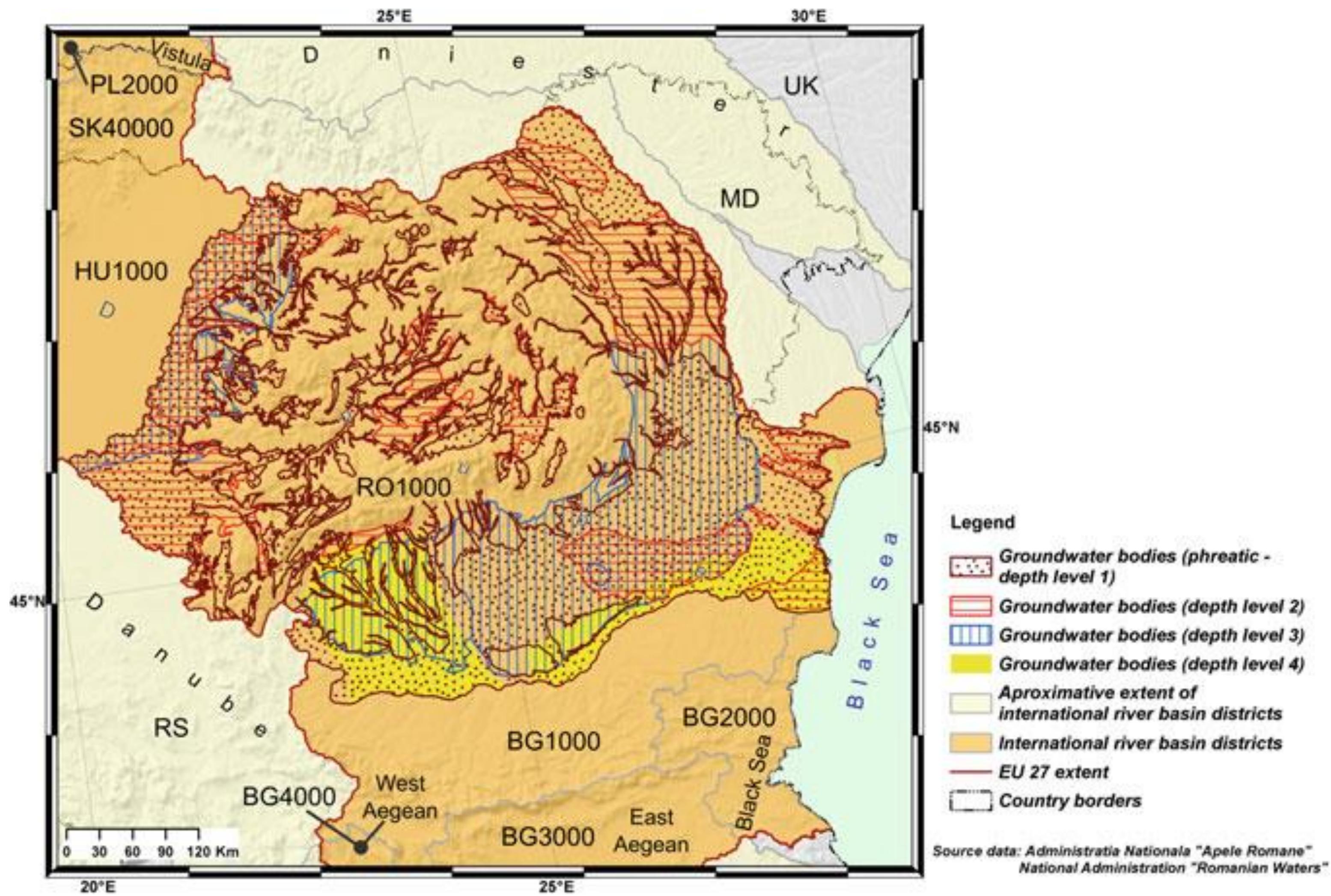


Figure 3. Water bearing formations in Romania. This map was taken from Negm et al. (2019).

Groundwater ecosystems due to the lack of light are generally lack photosynthetic activity and easily available organic carbon source. These factors are key characteristics of these ecosystems comparing to surface aquatic environments. As a result, microbial communities within aquifers are containing large fraction of heterotrophic organisms that are well adapted to the nutrient-poor and oligotrophic groundwater environment, and lithoautotrophs, which are able to fix carbon dioxide and reach their energy demands by oxidising inorganic electron donors (Kováč 2018). Groundwaters are characterised by hydrological, chemical, and geological variabilities. However, within different zones of the strata layers, environmental conditions can be very stable (Danielopol et al. 2000).

Due to the previously mentioned factors/circumstances these systems are considered also as extreme habitats. Nevertheless, groundwater microbial communities are well adapted to these conditions, and they may find strong environmental fluctuations challenging (Griebler et al. 2009). First studies of groundwater microbial communities mostly applied cultivation-based techniques. These methods could reveal only limited diversity of microbes, where the isolated bacteria were close relatives of well-known heterotrophic microorganisms, such as members of the *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes* phyla (Clark 1993). However, with the advantage of cultivation-independent techniques, a new set of novel microbial lineages have been revealed as fundamental components of indigenous microbial communities in the groundwater environments (Ludington et al. 2017). More efforts are still needed in the future in order to improve our understanding of the role of microbial function and biodiversity in groundwaters, especially their role in biogeochemical processes and in the resistance and resilience of aquifers against anthropogenic effects.

2.6.2. *Oligotrophic lakes*

Lakes can be classified based on the amount of available nutrients (phosphorus and nitrogen) for organisms. The table below (Table 2) shows the general features of oligotrophic lakes.

Table 2. General features of oligotrophic lakes (Sigee 2005).

Parameter	**Oligotrophic lakes**
Morphology and hydrology	Often have steep sides and a small surface area relative to their volume, which allows for less mixing of nutrients from the bottom sediments and less opportunity for the wind to bring nutrient-rich water to the surface.
Nutrient availability	The low productivity in oligotrophic lakes results in relatively low levels of organic matter accumulation.
General productivity	Low primary and secondary productivity
Phytoplankton species	Species adapted to low nutrient lakes: Cyanobacteria, green algae, diatoms
Light penetration	High, due to transparent water. Often reaching below thermocline.
Oxygenation	Saturation in epilimnion, with little variation through water column
Macrophyte vegetation	Poorly developed or absent

In Hungary, lake Balaton is considered the largest lake in Central Europe, during the period between 1960 and 1990, the lake went through severe eutrophication processes due to phosphorus discharges from external anthropogenic sources (Hajnal et al. 2008). Because the phosphorus is an important limiting factor in freshwater and marine environments, its increase may lead to the acceleration and extension of eutrophication (Pilmis et al. 2018).

However, from 2001 to 2017 the lake underwent a re-oligotrophication phenomena lead by the subsequent and complex eutrophication control and lake restoration program (Bernát et al. 2020). As a result, Kis-Balaton Water Protection Reservoir system could be reconstructed, new wastewater treatment units aiming the removal of phosphorus has been introduced and sewage direction from the watershed were implemented. In addition to the political reform in 1990 that lead to the collapse of agriculture which resulted in a drop of fertilizer usage (Istvánovics et al. 2007). Currently lake Balaton could restore its former general meso-eutrophic aspect, and meso-oligotrophic aspect in some parts of its eastern basin (Bernát et al. 2020).

2.7. *Importance of oligotrophic organisms*

The ability of an organism to grow in oligotrophic conditions might be followed by evolving new potentials to use many complex substrates. This can be seen by the production of small amounts of extracellular enzymes followed by more production of further enzymes and a rapid degradation of complex solid materials. Moreover, previous studies demonstrated that the microbes isolated from oligotrophic environments on low nutrient content media have the ability to use a significant wider range of compounds as growth substrates than the microbes that were isolated on high nutrient content media (Gupta et al. 2017).

Oligotrophs can also have many medical implications. In fact they can pose a health risk because of their ability to grow in unsterilized and bottled drinking water, mainly in case of a contamination with trace amounts of organic materials, some of which are antibiotics resistant (Pontes et al. 2009). Other species are often found in food industry where they can grow without any signs of foodstuff deterioration that can be seen by the consumer (Bore et al. 2005)

As a result, the knowledge of oligotrophy may be advantageous in order to screen many environments for potential novel antibiotics or bioactive compounds. It may also provide a better understanding of how microorganisms cope with these environments. Finally, many biotechnological discoveries may result from such studies as a result of isolating and growing novel species that may show the ability to produce novel bioactive products with considerable economic importance (Wainwright et al. 1991).

3. *Materials and methods*

The current study employed a comprehensive sampling approach starting from 2017 to 2021 to investigate various locations. The investigation focused on determining physical and chemical parameters. Additionally, the study employed both cultivation-independent methods, including total cell count and amplicon sequencing, as well as cultivation-dependent methods. The bacterial strains were grouped using MALDI-TOF-MS and representative strains from each group were subjected to 16S rRNA gene sequencing. Bacterial growth on different media was also assessed as part of the analysis (Figure 4).

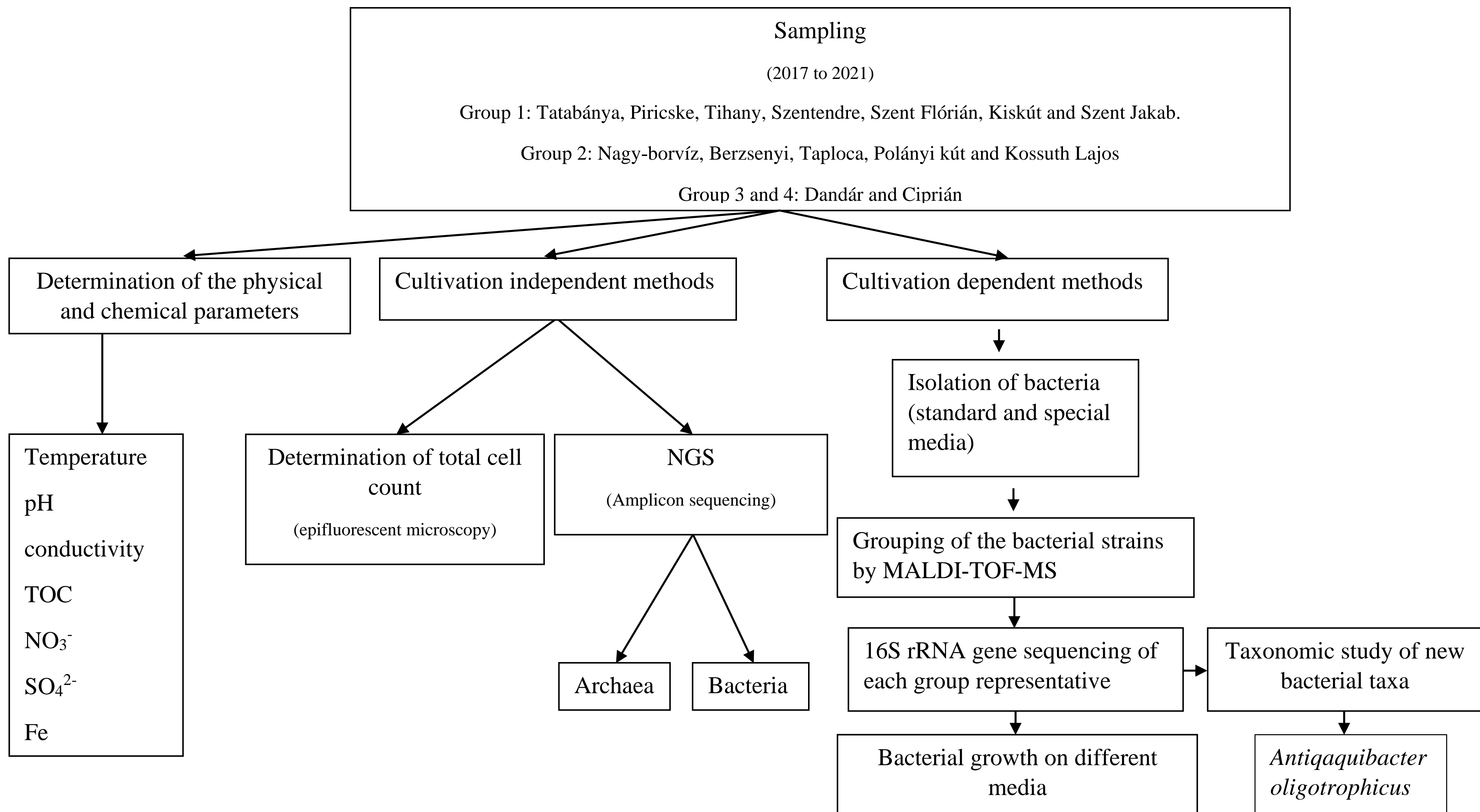


Figure 4. Graphic workflow showing the different steps of the used materials and methods.

3.1. *Description of the hydro-geological properties of the sampling sites*

The study encompassed multiple sampling sites, each offering a unique perspective on the physiochemical parameters present in the site. A comprehensive understanding of these environments required sampling from various locations, spanning both Hungary and Romania. The sampling effort contained a total of 14 sites, having different types of habitats, such as surface water, carbonate (karst, thermal karst and not karst) and non-carbonate aquifers. By strategically selecting these sites, we aimed to capture a representative snapshot of the microbial diversity and ecological dynamics present in each site. In the following sections, we will delve into the specific details of each sampling site.

Dandár well (47.4764° N 19.0709° E) is located at the southern discharge zone of the thermal karst region of Budapest (Figure 5). All the springs in this zone are hot springs characterized with a temperature ranging between 33°C and 47°C (Eross et al. 2008). The seasonal discharge variations are negligible. Due to the long travel time of the water through the pores of the host rock within the aquifer and the high temperature, the water is characterized by high SO_4^{2-} (600 mgl^{-1}) content and high conductivity (1710 μScm^{-1}).

Figure 5. Sampling site of Dandár well.

Ciprián groundwater (6.9215° N 17.8860° E) is located at the northern shore of Lake Balaton on the Tihany Peninsula (Figure 6). The rocks above the groundwater are formed by sands and silts with remarkable porosity and permeability (Jobbágy et al. 2011), therefore the retention time of the water is short. Moreover, intensive agricultural activity is observed at this region.

Figure 6. Sampling site of Ciprián groundwater.

Szentendre spring (47.6987° N 19.0471° E) is located within the Dunazug Mountains (Figure 7) where the aquifer is formed by agglomerates of andesite.

Figure 7. Sampling site of Szentendre spring.

Szent Flórián (47.3963° N 18.9858° E) is an artesian well near the centre of Nagytétény, Budapest (Figure 8), where the host rock is a Miocene carbonate (Izápy 2002).

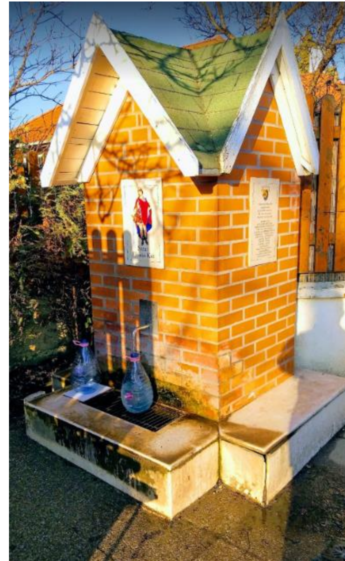


Figure 8. Sampling site of Szent Flórián well.

Tatabánya well (47.5692° N 18.4048° E) belongs to the Komárom – Štúrovo reservoir, this area is situated in the north-eastern part of the Transdanubian Range in Hungary (Figure 9): limestones and dolomites karst aquifer, from the area of outcrops of the Upper Triassic rocks (Status et al. 2013). At the time of sampling the whole aquifer was full of gas bubbles.

Figure 9. Sampling site of Tatabánya well.

Three of the analysed springs are located in the southern part of Harghita county, Romania: Taploca spring (46.3697° N, 25.8055° E) is located in the Csíktaploca (Toplița Ciuc) (Figure 10), Nagy-borvíz spring (46.3753° N, 25.8193° E) (borvíz ["wine water"] means CO_2 rich mineral water) is located in Csíksomlyó (Şumuleu Ciuc) near the pilgrimage site (Figure 11). Today Csíktaploca and Csíksomlyó villages are part of the Csíkszereda (Miercurea Ciuc) town. Piricske spring (46.3696° N, 25.7954° E) is found in the forest close to Csíkszereda. Piricske is a freshwater spring (Figure 12). Taploca and Nagy-borvíz springs are CO_2 rich mineral waters, (Máthé et al. 2010) (Kis et al. 2014).The hydrochemical character of these discharging groundwater is influenced and determined by the geological structure and groundwater-rock interaction resulting in high mineralization of NaCl, sulfate and dissolved gases (Kis et al. 2014).

Figure 10. Sampling site of Taploca spring.

Figure 11. Sampling site of Nagy-borvíz spring.

Figure 12. Sampling site of Piricske.

Five of the investigated springs are situated in Balaton Highland region: Szent Jakab spring of Vászoly (46.9433° N, 17.7580° E) and Kiskút of Szentantalfa (46.9126° N, 17.6745° E) are located in the elevated hills (~280 and ~190 m above sea level) and the Kossuth Lajos (46.9561° N, 17.8950° E), Polányi kút (46.9434° N, 17.8669° E) (Szekér Ernő outflow) and Berzsenyi spring (46.9465° N, 17.8739° E) are situated in Balatonfüred near the shoreline of the Lake Balaton (~105–115 m above the sea level [Figure 13, Figure 14, Figure 15, *Figure 16*, *Figure 17*]). These springs issue from carbonate aquifers or at the boundary of sandstone, metamorphic and carbonate formations, where a hydraulic barrier forces groundwater discharge (Budai et al. 1999). The water

temperature in the elevated parts is close to the mean annual temperature of the air (~10-11°C) because these springs are fed by local and shallow groundwater flow. In turn, the springs of Balatonfüred receives a deeper groundwater flow component which is responsible for the higher water temperature (~14–17°C) and the natural occurrence of the CO_2 (Tóth et al. 2016).

Figure 13. Sampling site of Kiskút of Szentantalfa.

Figure 14. Sampling site of Berzsenyi spring (Balatonfüredi savanyúvíz spring).

Figure 15. Sampling site of Polányi kút spring.

Figure 16. Sampling site of Kossuth Lajos.

Figure 17. Sampling site of Szent Jakab spring.

At the eastern part of Balaton Lake, water sample was collected from the coastal location at Tihany (65.9190° N, 17.88903° E) (Figure 18).

Figure 18. Sampling site of Tihany (lake Balaton).

The figure bellow (Figure 19) shows the location of the sampling sites on map.

Figure 19. Map of the sampling sites.

3.2. *Collection of water sample*

The water sample of Dandár bath well was collected from the underground pipe alimenting the thermal bath, Szentendre, Szent Flórián, Ciprián in addition to water samples located in Romania (Taploca, Nagy-borvíz, Piricske) and in Balaton Highland region (Szent Jakab, Kiskút, Kossuth Lajos, Polányi kút and Berzsenyi) were collected from the spring's water outflow. From Tatabánya, the water sample was collected from a former mine aquifer. Before sampling from Dandár and Tatabánya water samples, the water was allowed to run for 3 minutes from the underground pipe tap, after that the sample was taken from the sampling tap. Tihany surface water sample was collected from 10 cm subsurface. The sampling was carried out during the period from 2017 to 2021 during the dates mentioned in the table below (Table 3). The water samples (2-2 L) were aseptically collected into clean, sterile, glass bottles according to ISO 19458:2006 standard,

transferred at 4° C in a cooler bag and filtered for cell count determination and molecular studies immediately upon arrival at the laboratory.

Table 3. Sampling sites and dates of sampling.

Sample name	**Sampling date**
Dandár	2 April 2018
Szentendre and Szent Flórián,	6 September 2018
Ciprián and Tihany	31 August 2018
Tatabánya	27 November 2017
Balaton Highland region samples	22 June 2021
Hargita county, Romania samples	18 October 2018

3.3. *Determination of the physical and chemical parameters*

The pH and temperature were measured on site using a Hach HQ40D portable multimeter (Hach, Loveland, CO, USA). All other parameters were determined in the laboratory according to standard methods (Rodger et al. 2017). Nitrate ion (ASTM 4500-NO_3^- B) was measured by applying the UV-spectrophotometric screening method using a Perkin Elmer Lambda 35 UV/VIS spectrophotometer (Waltham, MA, USA). Sulfate ion was precipitated in an acidic medium with barium chloride and the absorbance of the resulting barium sulfate suspension (ASTM 4500-SO_4^{2-} E) was measured with a Hach DR2000 spectrophotometer (Loveland, CO, USA). Iron (3500-Fe B) was brought into the ferrous state by boiling with acid and hydroxylamine, then 1,10-phenantroline was added. The absorbance of the resulting red complex was determined using the Hach DR2000 spectrophotometer (Loveland, CO, USA). The amount of total organic carbon (TOC) was measured after the removal of inorganic carbon by acidification and sparging applying the combustion-infrared method (ASTM-5310B). The samples were injected into a heated reaction chamber packed with platinum group metals, where their organic carbon content was oxidized to carbon dioxide and water. The amount of the carbon dioxide was measured by an infrared detector. The type of TOC analyzer was a Multi N/C 2100S (Analytik Jena, Jena, Germany). Hardness was measured using the EDTA titrimetric method applying a Eriochrome Blact T indicator (ASTM-2340 C Hardness).

3.4. *Determination of total cell count*

In order to determine the total cell counts of the samples, 200 ml from each water sample were filtered on a polycarbonate membrane filter (0.2 µm GTTP, Millipore, Burlington, MA, USA). Then, the filters were fixed in a solution of 2% paraformaldehyde (Sigma-Aldrich, Darmstadt, Germany) dissolved in 0.1 M phosphate buffer (NaH_2PO_4 3.2 g, Na_2HPO_4 10.9 g in 1000 ml distilled water, pH 7.2) overnight. The obtained filters were stored at −20° C until further analysis. Microscopic cell counts were determined using Nikon80i epifluorescent microscopy and NisElements program package according to (Kéki et al. 2019).

3.5. *Molecular analysis for microbial community identification*

3.5.1. *DNA isolation and identification of the isolated bacterial strains*

In order to extract the DNA from the isolated bacterial strains following the protocol of Szuróczki et al. (2016). 72 hrs bacterial cultures from every isolate were prepared. After that, 3 loops of biomass from every culture were added to Eppendorf tubes. These tubes were containing RN-ase free water (dH_2O) treated with 100-100 DEPC (diethylpyrocarbonate) and 0.1 ml of sterile glass beads. Subsequently, the obtained aliquots were shaken with a MM301 cell mill instrument (Retsch, Haan, Germany) at 30 Hz for 2 minutes. After the cell digestion, the tubes were centrifuged at 5000 g and the digested biomasses were denatured in a PCR instrument (GeneAmp PCR System 2700) for 5 minutes at 98° C. This step was followed by a second centrifugation of the tubes for 5 minutes at 10 000 g. At last, the DNA samples were stored at -20° C until further processing.

After the DNA extraction, a PCR amplification targeting the 16S rRNA gene was done using the primers 27F (5’-AGA GTT TGA TCM TGG CTC AG-3’) and 1492R (5’-TAC GGY TAC CTT GTT ACG ACT T-3’) following the protocol of (Kalwasińska et al. 2015). The 16S rRNA gene sequencing was carried out at LGC Genomics (Berlin, Germany). The quality of the obtained chromatograms was checked manually with the help of the Chromas software (Technelysium Pty Ltd., Australia). The identification of the sequenced strains was performed using EzBioCloud’s online identification system as described by (Yoon, S. M. Ha, et al. 2017). The sequences of the

bacterial strains were deposited in the NCBI GenBank database and are available under the accession numbers from MN684211 to MN684320.

3.5.2. *DNA extraction from the water samples and amplicon sequencing*

The total DNA was extracted from 250 ml of water sample after filtration, using a 0.22 µm pore size sterile mixed cellulose filter (MF-Millipore GSWP04700, Billerica, MA, USA) using a DNeasy® PowerSoil® DNA Isolation Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The mechanical cell disruption was performed by shaking at 30 Hz for 2 minutes using a Retsch Mixer Mill MM400 (Retsch, Haan, Germany). For PCR reactions, a 3 µl quantity of the template DNA was used. The PCR reaction to amplify the 16S V4 region was done based on the following protocol: 98° C for 3 min; 25 cycles: 95° C for 30 s, 55° C for 30 s and 72° C for 30 s; and 72° C for 5 min for bacteria and 98° C for 3 min; 25 cycles: 95° C for 30 s, 60° C for 30 s and 72° C for 30 s; and 72° C for 10 min for archaea using the following primers: CS1-TS-B341F and CS2-TS-805NR (Herlemann et al. 2011) for bacteria and CS2-TS-Arch-855R and CS1-F-A519F (Klindworth et al. 2013) for archaea. Before sequencing, DNA concentration of the PCR products was determined using a Qubit Fluorometer (Invitrogen Life Technologies, CA, USA) and a minimal concentration of 4 ng/µl and 50 µl of PCR product was respected. Sequencing was performed on an Illumina MiSeq platform using MiSeq standard v2 chemistry by the Genomics Core Facility RTSF, Michigan State University. The forward and reverse fastq files obtained from the Illumina sequencer were processed and analysed using the Mothur v1.40.5 software (Schloss et al. 2009). The contigs were obtained using the **make.contigs** with a deltaq value of 10, in order to keep the sequences with high quality scores. To keep only the sequences fulfilling the expected length and number of polymers and ambiguous bases, the **screen.seqs** command was used. The sequences were aligned to the Silva database (silva.nr_v132.align) using **align.seqs** command (Quast et al. 2013), and the non-aligned sequences and columns containing only "." were removed by using the **screen.seqs** and **filter.seqs**, based on the position of the archaeal and bacterial primers within the 16s rRNA gene. To remove the sequences obtained because of the Illumina sequencing errors, the **pre.cluster** command was used. The chimeric sequences were removed by using the UCHIME algorithm represented with the command **chimera.uchime** (Edgar et al. 2011). Only the abundant sequences were kept using the command **split.abund** which split the sequences into two groups, with a cutoff value equal to 1. The

taxonomic classification of the sequences was done using the Silva database silva.nr_v132.tax, and the non-archaeal and non-bacterial sequences were removed from the analyses based on the taxonomic classification output following the **classify.seqs** and **remove.lineage**. The OTUs (Operational Taxonomic Units) were calculated using a distance matrix with distances larger than 0.15 obtained by using the **dist.seqs** and later the cluster commands to assign sequences to OTUs (Operational Taxonomic Units), and eventually the consensus taxa were determined using the **classify.otu**. At the end, the data were normalized using the **sub.sample**, and **rarefaction.single** and **summary.single** were used to calculate the rarefaction curve data and the values of the diversity indices. Sequence reads were deposited in the NCBI SRA database and are accessible through the BioProject ID: PRJNA628507 and BioSample ID: SAMN14732952 for Dandár, SAMN14732956 for Szentendre, SAMN14732979 for Szent Flórián, SAMN14732951 for Tatabánya, SAMN14732957 for Ciprián, SRS6537389 for Taploca, SRS6537391 for Piricske, SRS6537423 for Nagy-borvíz, SRS9983636 for Berzsenyi, SRS9983634 for Polányi kút, SRS9983633 for Kossuth Lajos, SRS9983477 for Kiskút, SRS9983632 for Szent Jakab and SAMN14732963 for Tihany. Shannon-Weaver and inverse Simpson (1/D) diversity indices and Chao-1 and ACE richness metrics were calculated using Mothur software (Klindworth et al. 2013).

3.6. *Isolation of bacterial strains*

To isolate bacterial strains, a new medium (named M5) was developed using 0.05 g/l yeast extract, 0.05 g/l proteose peptone, 0.05 g/l casamino acids, 0.05 g/l glucose, 0.05 g/l soluble starch, 0.03 g/l sodium pyruvate, 0.03 g/l K_2HPO_4, and 0.005 g/l $MgSO_4$▪$7H_2O$, adding 15 ml/l of growth factor solution (composition: sodium acetate: 0.5 g, sodium formiate: 0.5 g, sodium succinate: 0.5 g, L-D glucosamine: 0.5 g and glycerin: 0.5 ml dissolved in 100 ml of distilled water) and 15 ml^{-1} of trace element solution ($FeSO_4$▪$7H_2O$: 2 g, H_3BO_3: 0.03 g, $MnCl_2$▪$4H_2O$: 0.1 g, $CoCl_2$▪ $6H_2O$: 0.19 g, $NiCl_2$▪$6H_2O$: 0.024 g, $CaCl_2$▪$2H_2O$: 0.002 g, $ZnSO_4$▪$7H_2O$: 0.144 g, Na_2MoO_4▪$2H_2O$: 0.036 g and EDTA: 5.2 g dissolved in 1 litter of distilled water). The pH was adjusted to 7.0 – 7.2 and tap water was added to the medium until the final volume of 1 l was reached, and then finally autoclaved at 121° C for 20 minutes. The media were solidified with either agar or gellan gum, with a quantity of 16 gl^{-1} and 12 gl^{-1}, respectively. Isolation happened in a random manner from two parallels: after direct spreading of 100 µl water samples and also after enrichment of 50 ml of water sample in 250 ml of M5 media for 2 weeks using polyurethane foam-based traps (Szuróczki

et al. 2016). In order to enrich the present bacteria, we employed polyurethane foam-based (PUF) traps immersed in a 250 ml liquid medium comprising a combination of 10% R2A and minimal synthetic media. Prior to use, the PUF blocks underwent sterilization in an autoclave at 121 °C for 20 minutes. Subsequently, the blocks were soaked with melted agar and gellan gum and introduced into the liquid media. Following an incubation period of 3 weeks at 25 °C, the PUF blocks were carefully extracted from the enrichment media and subjected to gentle pressing using sterile mortar. The liquid extracted from the PUF blocks was then spread onto M5 medium after serial dilution. The plates were subsequently incubated at 25 °C for 4 days to allow for bacterial growth.

Cultivation was performed only from the water samples of: Dandár, Szentendre, Szent Flórián, Ciprián and Tatabánya. The plates were incubated (9 days at 25° C) and the isolates from the different samples were purified and grouped based on their MALDI-TOF profile (Carbonnelle et al. 2011) where the group representatives and ungrouped bacterial strains were subjected to the 16S rRNA gene sequencing.

3.7. *Grouping of the isolated bacterial strains using MALDI-TOF-MS*

The isolates were grouped using the Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) protein analysis according to (Carbonnelle et al. 2011). At first, a standard solution was prepared. It contains 50 Vol% acetonitrile (ACN), 2.5 Vol% 2,2,2-Trifluoroacetic acid (TFA) and 47.5 Vol% HPLC quality water. Later, a matrix solution was prepared using α-cyano-4-hydroxycinnaminic acid (HCCA) dissolved in the standard solution. The final concentration of matrix solution is 10 mg HCCA/ml. A single colony was directly placed on 4 spots of the stainless-steel target plate of the MALDI-TOF. After drying, 1 µl of matrix solution per spot was applied. MALDI-TOF MS was conducted using a Microflex LT mass spectrometer (Bruker Daltonics) equipped with an N2 laser. All spectra were recorded in linear, positive ion mode. The acceleration voltage was 20 kV. Spectra were collected as a sum of 500 shots across a spot (40 to 50 shots per second). A mass range of 2000–20000 m/z was used for analysis. Bacterial spectra were obtained through the flexControl version 3.0 software, and spectra analysis was carried out with Biotyper version 3.0 software.

To group similar bacterial isolates using MALDI-TOF MS, a spectral database of known bacterial species is first established. Then, the spectra of the unknown bacterial isolates are compared to

those in the database using a software algorithm. The algorithm compares the peaks in the spectra, looking for similarities and differences that can be used to determine the identity of the unknown isolate. Once the identity of the bacterial isolate has been determined, it can be compared to other isolates in the database to group similar isolates together. This is typically done using clustering algorithms that group isolates based on similarities in their spectral profiles.

After that a main dendrogram was created showing the grouping of all the isolates together, using all the generated spectra. This allowed us to obtain the representative groups of all the isolates. Following this step, two members from every group were selected to sequence their 16S rRNA genes.

3.8. *Study of the bacterial growth on different media*

In order to determine the growing capability of the sequenced 100 bacterial strains, 96-well microtiter plates were applied using different concentrations of nutrients, per- formed in three replicates in the following order: 100%, 10% and 1% R2A (Reasoner et al. 1985); 100% and 10% M5; 100%, 10% and 1% glucose added to minimal medium, and 100%, 10%, 1% and 0.1% yeast extract added to minimal medium. The minimal medium was composed of: 1 g/l K_2PO_4, 0.5 g/l $MgSO_4$, 0.5 g/l KCl, 0.01 g/l $FeSO_4$ and 2 g/L of $NaNO_3$. The stock solutions of yeast extract and glucose media contained 30 g/l yeast extract or glucose respectively. The microtiter plates were incubated at 25° C for 7 days, and the optical density was measured every day using an Elisa reader (SUNRISE Tecan, Grödig, Austria) at a wavelength of 620 nm.

3.9. *Statistical Analyses*

The relationship between the environmental variables (physical and chemical parameters) diversity indices, cell counts and the obtained OTUs (archaea and bacteria) were revealed by principal components analysis ordination (PCA) combined with vector-fitting. The “envfit” function from the vegan package was used in order to fit the variables as vectors (Oksanen et al. 2019) onto the ordination of OTUs, and the significance of fittings was tested with random permutations in program R (R Core Team 2016; http://www.r-project.org/, accessed on 4 April 2020). Shannon diversity index was calculated in order to describe the population diversity in the

analyzed samples based on operational taxonomic units (OTUs). It is calculated with the following formula:

$$H = -\sum_{i=1}^{S}(P_i \ln P_i)$$

Where P is the proportion (n/N) of individuals of one particular species found (n) divided by the total number of individuals found (N), ln is the natural log, Σ is the sum of the calculations.

3.10. *Polyphasic approach to study the novel bacterial taxa isolated from the water sample of Szentendre*

During this study, a new bacterial genus was described using the polyphasic taxonomic approach along with its closest relatives *Salinibacterium hongtaonis* MH299814 (97.77 %) followed by *Leifsonia psychrotolerans* GQ406810 (97.57 %), *Herbiconiux ginseng* jgi.1076294 (97.48 %), *Leifsonia bigeumensis* EF466124 (97.48 %) and *Leifsonia kafniensis* AM889135 (97.48 %).

3.10.1. *DNA-based analysis of the new bacterial genus*

3.10.1.1. *Determination of the complete 16S rRNA gene of the new bacterial strain SG_E_30_P1*

The new bacterial genus formed a unique group member in the MALDI-TOF-MS obtained dendrogram (dendrogram is not shown). Therefore, as previously stated in the section titled "DNA isolation and identification of the isolated bacterial strains," the 16S rRNA gene of the novel genus was sequenced. As this isolate was a new genus candidate, both ends sequencing (27F and 1492R) was performed. The 2 sequences were used to obtain the complete 16S rRNA gene by complementing them to each other's using the MEGA software 11 (Tamura et al. 2021).

Following the determination of the 16S rRNA gene sequence of the bacterial strain, it was aligned with its closest relatives using the SILVA project algorithm (http://www.arb-silva.de) (Quast et al. 2013). The phylogenetic analysis was performed using the MEGA software 11 (Tamura et al. 2021), the evolutionary distances were calculated based on Kimura's two-parameter model

(Kimura 1980), the phylogenetic dendograms were calculated using the maximum-likelihood (Felsenstein 1981) and neighbor-joining (Gascuel et al. 2006) methods.

3.10.1.2. *Determination the whole genome sequence of bacterial strain SG_E_30_P1*

To determine the whole genome sequence of the novel bacterial strain SG_E_30_P1, DNA isolation was performed as mentioned in the section "DNA extraction from the water samples and amplicon sequencing" using a DNeasy® PowerSoil® DNA Isolation Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. At the exception of the mechanical cell disruption that was performed by shaking at 30 Hz for 2 min using a Retsch Mixer Mill MM400 (Retsch, Haan, Germany). The concentration of DNA was measured using the Qubit Fluorometer™ dsDNA HS Assay Kit and adjusted to a final concentration of 50 ng μl^{-1}.

The full genome sequencing of the strain SG_E_30_P1 was performed on a NovaSeq S4 platform (Illumina) using patterned flowcells and a reformulated NextSeq 2-colour SBS chemistry. The sequencing was provided by the JGI, U.S. Department of Energy (DOE), Office of Science User Facility, the University of California, USA. The bioinformatics analysis was performed by Dr. Gorkhmaz Abbaszade (Department of Microbiology at ELTE) following Chun et al. (2018).

Sequence read quality was checked by FastQC (Leggett et al. 2013) and de novo assembly of raw reads was performed using SPAdes 3.15.2 tool in careful mode (Bankevich et al. 2012).

The assembly and coverage depth qualities were evaluated by QUAST 5.1.0. Contigs with less than 500 nt were removed from the assembly.

The 16S rRNA gene coming from the whole genome sequencing was checked using the ContEst16S platform for possible contamination. Raw sequence reads are deposited in the NCBI Sequence Read Archive (SRA) and can be accessed through the BioProject ID PRJNA762240, the whole genome project is deposited in the GenBank database and is accessible with the accession number CP085036.1. The 16S rRNA gene obtained by Sanger sequencing (96.1% completeness) was compared to the 16S rRNA gene obtained from the full genome assembly and resulted in 100% similarity. In order to draw the balanced minimum evolution tree, closely related type strains were identified, and their whole genome sequences are obtained from Genome Server (TYGS) (Meier-Kolthoff et al. 2019). The phylogenomic tree was created based on the intergenomic

distances that were calculated from the Genome Blast Distance Phylogeny analysis (GBDP) with 100 pseudo bootstrap replicates using FastMe 2.0 with a BioNJ starting tree (Lefort et al. 2015).

The average nucleotide identity (ANI), average amino acid identity (AAI) and digital DNA–DNA hybridization (dDDH) values were determined among the genome sequence of SG_E_30_P1 and the reference genomes of the closest relatives (Yoon et al. 2017) (Rodriguez et al. 2016) (Meier-Kolthoff et al. 2013) (*Leifsonia psychrotolerans* [GQ406810], *Galbitalea soli* [DSM 105515], *Amnibacterium flavum* [MJJ-5], *Conyzicola nivalis* [CGMCC 1.12813], *Herbiconiux ginseng*, *Microterricola pindariensis* [DSM 22300] and *salinibacterium hongtaonis* [MH299814]; the different sequences were downloaded from the NCBI genome database).

3.10.2. *Examination of the phenotypic characteristics of bacterial strain SG_E_30_P1*

In order to observe the colony morphology, incubation at 28 °C for 3 days was performed on R2A agar medium (Reasoner et al. 1985) (pH 7). After that, observation of single colonies was realized in native preparations and after Gram-staining using light microscopy (Thorn et al. 2016) and transmission electron microscopy (TEM 7100 Hitachi) (Golding et al. 2016) in order to assess the cell motility.

3.10.3. *Examination of the ecological tolerance of bacterial strain SG_E_30_P1*

The growth of strain SG_E_30_P1 was assessed on R2A agar slants (pH 7) at different temperatures (4, 7, 20, 35, 40 and 45 °C). The tolerance to NaCl and pH was assessed by using R2A broth (pH 7) at different concentrations, (0%, 1%, 2%, 5%) and at different pH values (3, 4, 5, 6, 7, 8, 9, 10, 11), respectively. Catalase and oxidase activity, oxidative and fermentative degradation of glucose, indole production, casease, urease, gelatinase, DN-ase and phosphatase activities, hydrolysis of starch and Tween 80, production of H_2S from peptone, NO_3^- reduction to NO_2, N_2 and NH_3 were checked following the protocol of (Tóth et al. 2008). Additional physiological and biochemical tests were performed using API ZYM and API 50CH kits of bioMerieux according to the manufacturer’s instructions. API ZYM is a rapid diagnostic test that aims to detect the presence of 19 enzymes, API 50CH on the other hand aims to detect the acid formation using 49 different carbon sources.

3.10.4. *Chemotaxonomic study of bacterial strain SG_E_30_P1*

In order to study the chemotaxonomic characters of the new isolate, a large amount of biomass was produced from the bacterial cells in a shaker flask. The chemotaxonomic studies were performed by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) laboratory under the supervision of Dr. Meina Neumann-Schaal.

Chemotaxonomic analysis (peptidoglycan, isoprenoid, quinones, polar lipids and fatty acids) were performed following the protocols of Tóth et al. (2014).

The biomass for the analysis of isoprenoid quinones and polar lipids were obtained by cultivation in R2A broth at 28 °C for 2 days. Isoprenoid quinones were extracted following the protocol of (Collins et al. 1977), further analysis were performed using HPLC (Shimadzu LC 20A) and electron-impact mass spectrometry (Singlequad 320) (Groth et al. 1997).

In order to perform the cellular fatty acids analysis, bacterial cells were cultivated for a total period of 24 hours on R2A at 28 °C to harvest sufficient cells. Fatty acid methyl esters were obtained following the protocol of (Stead et al. 1992) and analysis was done using an Agilent 6850 chromatograph with the MIDI Microbial Identification System (library TSBA40, 4.10; Sherlock software package, version 6.1). Summed feature components were identified thereafter by GC/MS using a Singlequad 320 instrument (Varian).

Polar lipids were obtained following the protocol of (Minnikin et al. 1979) and separated by two dimensional TLC in order to identify spots. Ninhydrin and Zinzadze reagents and molybdophosphoric acid were used following (Rathsack et al. 2011).

4. *Results*

4.1. *Physical and Chemical Parameters of the Water Samples*

Table 4 provides the physical and chemical parameters of the various sampling sites. Although all sites are classified as nutrient-depleted environments based on their TOC values, they exhibit differences in their physical and chemical characteristics in addition to significant difference in their TOC values as well.

Table 4. Physical and chemical parameters and cell count values of the samples.

Sample	**T (°C)**	**pH**	**conductivity (μScm^{-1})**	**TOC[1] (mgl^{-1})**	**NO_3^- (mgl^{-1})**	**SO_4^{2-} (mgl^{-1})**	**Fe (mgl^{-1})**
Nagy-borvíz	14.1	6.18	2785	1.72	< 0.5	3.9	8.63
Piricske	8.5	6.74	163	2.81	3.1	6	0.07
Taploca	17.7	6.08	1490	0.85	< 0.5	2.7	5.42
Berzsenyi	17.2	6.23	1627	0.50	1.7	275	2
Kiskút	15	7.19	913	1.40	22	18	< 0.10
Kossuth Lajos	17.1	6.66	1185	1	1.5	73	1.80
Szent Jakab	19.7	7.04	581	13	19	18	< 0.10
Polányi kút	18.5	6.23	1526	8.40	1.6	210	2.40
Dandár	46	6.70	1710	1.75	<1.5	600	<0.03
Szentendre	8.6	8.09	454	1.97	6.3	71	<0.01
Szent Flórián	11	7.89	388	1.81	7	83	<0.01
Tatabánya	8	7.01	712	2.4	<0.5	44	0.29
Ciprián	12.5	8.1	1205	3.4	160	156	33
Tihany	22	8.6	680	7.7	0.1	119	-

[1]Total Organic Carbon

Applying the PCA on the different samples based on the chemistry dataset showed the existence of two main groups. First group is composed of the samples: Tatabánya, Piricske, Tihany, Szentendre, Szent Flórián, Kiskút and Szent Jakab. They were grouped together based on their

TOC values (Figure 20). As it is a measure of the amount of carbon contained in organic matter, his group can share common information about the organic carbon content of the samples.

The second group is composed of Nagy-borvíz, Berzsenyi, Taploca, Polányi kút and Kossuth Lajos spring. This distinct cluster of samples is observed on the opposite side of the TOC parameter.

In addition, they were grouped together by their conductivity, temperature and the SO_4^{2-} content. Dandár water sample was characterized by very high values of temperature, conductivity and SO_4^{2-}, which made it a distinct sample compared to all the others. This is also seen with Ciprián water samples, which was characterized with high values of NO_3^-. (Figure 20)

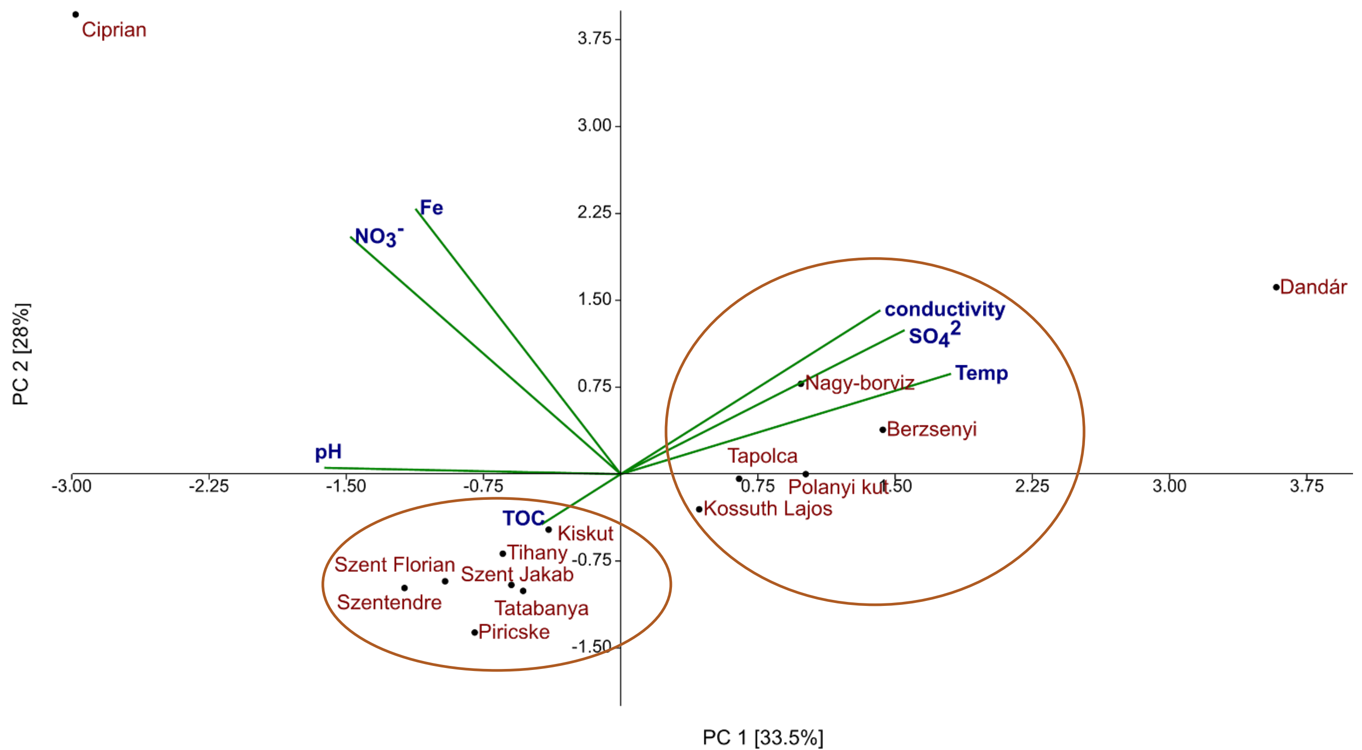


Figure 20. PCA ordination of the water samples based on environmental parameters.

4.2. *Microscopic cell counts and diversity indices of the samples*

The values of the number of bacteria (cell count) and diversity among the samples are shown in Table 5.

Table 5. Microscopic cell counts and diversity indices of the samples.

Sample name	Cell counts*ml^{-1}	Diversity index of archaea	Diversity index of bacteria
Group 1			
Tatabánya	13.3*10^4	4.95644	3.71137
Piricske	20.7*10^4	1.78737	2.44132
Tihany	6.34*10^6	4.48182	3.95352
Szentendre	10.8*10^4	4.95847	7.80743
Szent Flórián	36.7*10^4	3.82095	4.94798
Kiskút	5.9*10^4	4.7969	7.41751
Szent Jakab	2.3*10^4	4.38686	7.15128
Group 2			
Nagy-borvíz	5.9*10^4	0.63732	5.03453
Berzsenyi	5.1*10^4	2.41562	3.89847
Taploca	9.0*10^4	4.10171	6.46526
Polányi kút	1.7*10^4	4.96297	6.01387
Kossuth Lajos	1.5*10^4	5.07818	2.45293
Group 3			
Dandár	36.5*10^4	1.37108	3.7693
Group 4			
Ciprián	1.33*10^6	4.27066	1.92573

The cell count values of Ciprián and Tihany water samples were higher than all the other samples by at least one order of magnitude (1.33*10^6 and 6.34*10^6 respectively) (Table 5). The majority of the samples had significant Shannon diversity index differences between the values of archaea and bacteria. The most diverse sample in term of archaea was Kossuth Lajos water sample, however it was among the least diverse in case of bacteria, similarly to Ciprián water sample.

The opposite was observed in the case of Nagy-borvíz water sample, where it showed high bacterial and lower archaeal diversity.

A general trend was seen within most of the samples (except Kossuth Lajos and Tihany water samples) showing that the cell count values are tending to be lower when the bacterial diversity is higher (Figure 21).

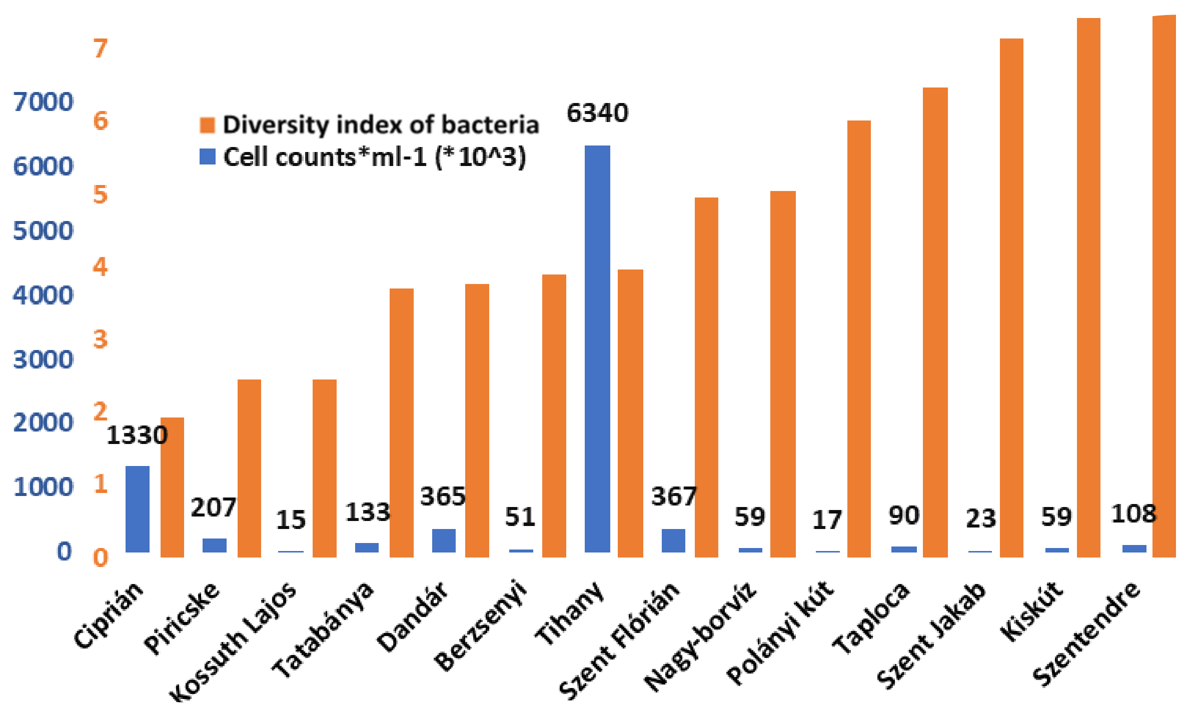


Figure 21. Relationship between the cell count (in 10^3) and the bacterial Shannon diversity indices of the different samples.

4.3. *Microbial communities of the different samples based on amplicon sequencing*

4.3.1. *Bacterial community composition of the different samples based on amplicon sequencing*

The results of amplicon sequencing identified 20 bacterial phyla presented a ratio higher than 2% in at least one of the 14 samples. The results of the rarefaction curves. (Figure S1) showed that the sequencing depth was sufficient to identify the majority of the bacterial taxa.

All samples were characterized by the abundance of *Proteobacteria* and *Patescibacteria*. The phylum *Patescibacteria* contained high ratio of *Parcubacteria* and many candidatus genera like *Falkowbacteria*, *Magasanikbacteria*, *Azambacteria* (Figure 22).

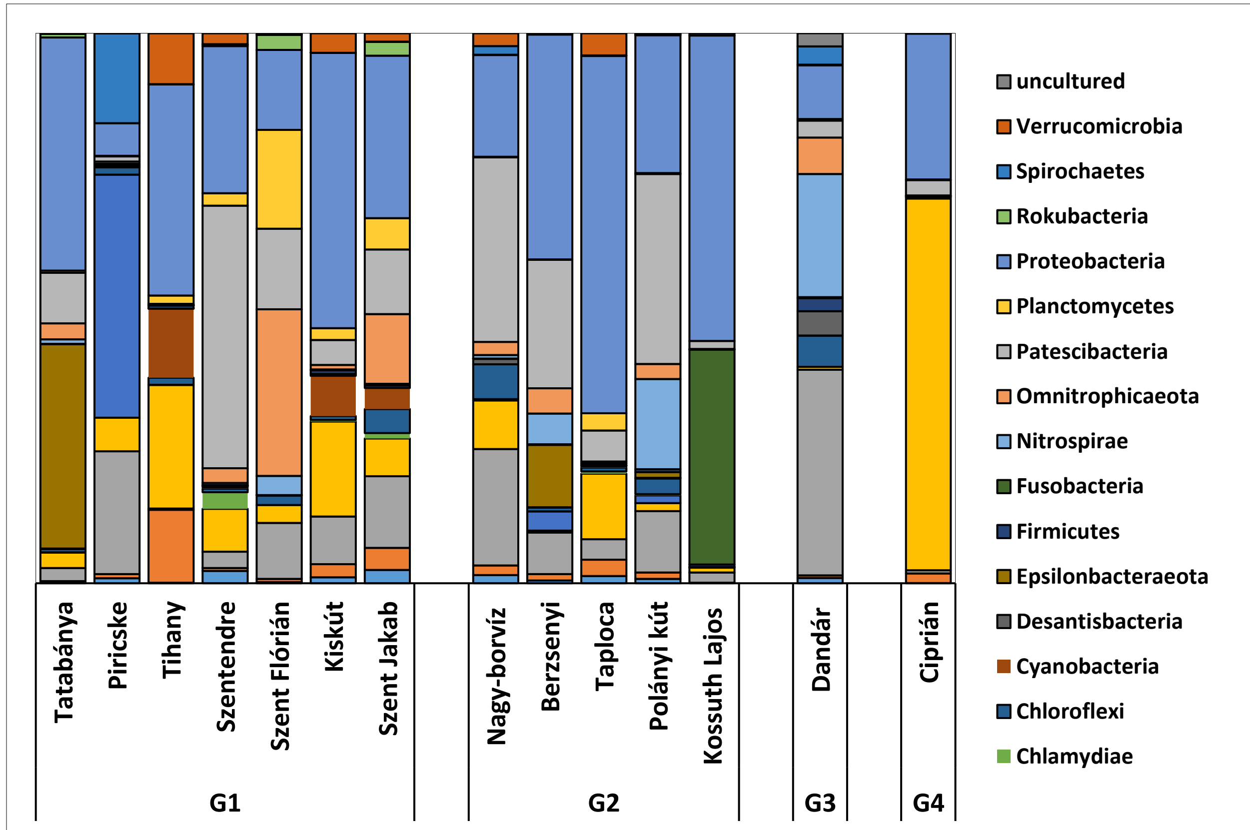


Figure 22. Distribution of the abundant (98%) bacterial phyla based on the 16S rRNA gene amplicon sequencing of the different groups of the water samples (G1: group 1, G2: group 2, G3: group 3 and G4: group 4).

4.3.1.1. Bacterial community composition of the first group of samples (G1) based on amplicon sequencing

The analysis of the water samples from the group 1 locations showed different microbial compositions. Notably, the sample from Tatabánya exhibited a high prevalence of *Desulfocapsa*, *Desulfurivibrio*, *Sulfuricella*, *Sideroxydans*, and several families including *Gallionellaceae*, *Hydrogenophilaceae*, *Methylophilaceae*, *Rhodocyclaceae*, *Methylococcaceae*, and *Methylomonaceae*. Additionally, a high number of *Epsilonbacteraeota* was observed in Tatabánya sample, which was nearly absent in the other samples. The Tatabánya sample also contained

Arcobacter, unclassified *Campylobacterales*, *Sulfurovum*, *Sulfuricurvum*, and *Sulfurimonas* from the *Epsilonbacteraeota* phylum. Piricske water sample was dominated by *Caldisericota*, with a substantial fraction of *Spirochaetota* and *Bacteroidetes*. Tihany water sample was characterized by the presence of *Actinobacteria*, *Bacteroidetes*, *verrucomicrobia*, and *Cyanobacteria*. The Szentendre sample was characterized by the Candidate phylum *Dependentiae* and the phylum *Verrucomicrobia*, with most of the sequences belonging to *Lacunisphaera* and unclassified *Pedosphaeraceae* family. The Szent Flórián sample was dominated by *Rokubacteria* and WOR-1, and characterized by the presence of unclassified *Bacteriovoracaceae*, *Myxococcales*, *Sandaracinaceae*, *Acidiferrobacteraceae*, *Sulfurifustis*, and *Ferritrophicum*. The Kiskút water sample was largely dominated by the common phyla *Proteobacteria* and *Patescibacteria*, and also shared other phyla with other samples such as *Cyanobacteria* and *Bacteroidetes*. Finally, the Szent Jakab water sample contained a unique presence of *Planctomycetota* and *Myxococcota*, as well as *Chloroflexi*, *Cyanobacteria*, *Bacteroidota*, and *Actinobacteriota*.

4.3.1.2. Bacterial community composition of the second group of samples (G2) based on amplicon sequencing

The Nagy-borvíz water analysis revealed the presence of significant proportions of *Chloroflexi*, *Bacteroidota*, *Desulfobacterota*, *Acidobacteriota*, and *Actinobacteriota*. Similarly, *Campylobacterota* was detected in the Berzsenyi sample, whereas Polányi kút exhibited noteworthy fractions of *Nitrospirota* and *Desulfobacterota*. The Taploca sample was found to contain *Bacteroidota*, *Planctomycetota*, and *Myxococcota*. Lastly, the Kossuth Lajos water sample was characterized by a predominance of *Fusobacteriota*, mainly comprising members of the *Hypnocyclicus* genus.

4.3.1.3. Bacterial community composition of the samples group G3 and G4 based on amplicon sequencing

Dandár water sample showed a high ratio of *Chloroflexi* where most of them belonged to *Anaerolineae* while also *Desantisbacteria*, *Firestonebacteria* and *Firmicutes* appeared, the most abundant genera of these phyla were *Desulfotomaculum* and *Thermodesulfitimonas* but still with low abundance, in addition to the presence of *Spirochaetes.*

The most abundant sequences in the Dandár water sample belonged to *Stenotrophomonas*, *Pseudomonas*, *Desulfobacca*, *Desulfomonile*, *unclassified Myxococcales* and *Sphingomonadaceae.*

The Ciprián water sample was dominated by the phylum *Bacteroidetes*, being one order of magnitude higher as compared to the Szentendre Tatabánya, Taploca and Tihany samples and approximately 2 orders of magnitude higher than the rest of the samples. Most bacteria belonged to the genus *Flavobacterium* (Figure 23).

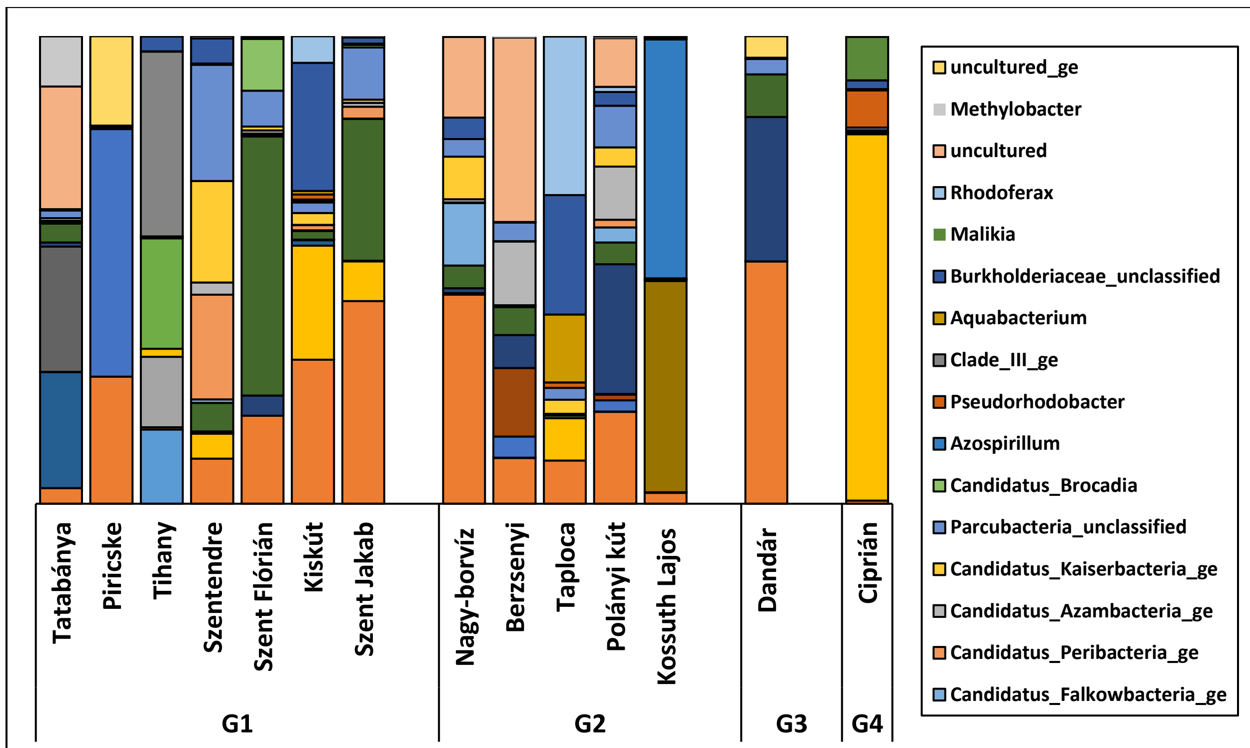
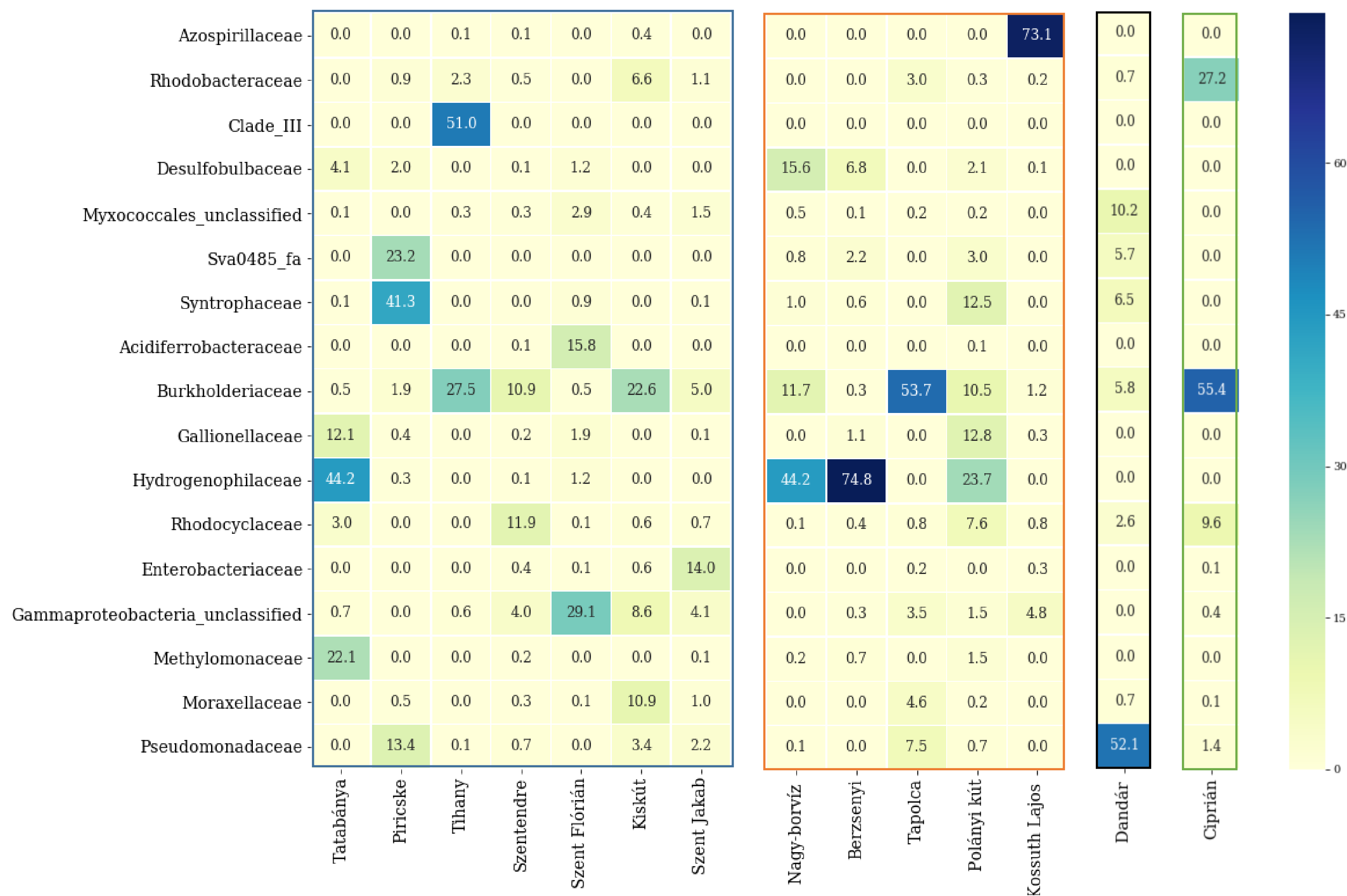


Figure 23. Distribution of the bacterial genera based on 16S rRNA gene amplicon sequencing in the water samples with a ration higher than 10% at least in one of the samples.

4.3.2. *In depth Proteobacteria composition of the different samples based on amplicon sequencing*

The composition of the Proteobacteria phylum can be summarized in the following heatmap.



	Tatabánya	Piricske	Tihany	Szentendre	Szent Flórián	Kiskút	Szent Jakab	Nagy-borvíz	Berzsenyi	Tapolca	Polányi kút	Kossuth Lajos	Dandár	Ciprián
Azospirillaceae	0.0	0.0	0.1	0.1	0.0	0.4	0.0	0.0	0.0	0.0	0.0	73.1	0.0	0.0
Rhodobacteraceae	0.0	0.9	2.3	0.5	0.0	6.6	1.1	0.0	0.0	3.0	0.3	0.2	0.7	27.2
Clade_III	0.0	0.0	51.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Desulfobulbaceae	4.1	2.0	0.0	0.1	1.2	0.0	0.0	15.6	6.8	0.0	2.1	0.1	0.0	0.0
Myxococcales_unclassified	0.1	0.0	0.3	0.3	2.9	0.4	1.5	0.5	0.1	0.2	0.2	0.0	10.2	0.0
Sva0485_fa	0.0	23.2	0.0	0.0	0.0	0.0	0.0	0.8	2.2	0.0	3.0	0.0	5.7	0.0
Syntrophaceae	0.1	41.3	0.0	0.0	0.9	0.0	0.1	1.0	0.6	0.0	12.5	0.0	6.5	0.0
Acidiferrobacteraceae	0.0	0.0	0.0	0.1	15.8	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Burkholderiaceae	0.5	1.9	27.5	10.9	0.5	22.6	5.0	11.7	0.3	53.7	10.5	1.2	5.8	55.4
Gallionellaceae	12.1	0.4	0.0	0.2	1.9	0.0	0.1	0.0	1.1	0.0	12.8	0.3	0.0	0.0
Hydrogenophilaceae	44.2	0.3	0.0	0.1	1.2	0.0	0.0	44.2	74.8	0.0	23.7	0.0	0.0	0.0
Rhodocyclaceae	3.0	0.0	0.0	11.9	0.1	0.6	0.7	0.1	0.4	0.8	7.6	0.8	2.6	9.6
Enterobacteriaceae	0.0	0.0	0.0	0.4	0.1	0.6	14.0	0.0	0.0	0.2	0.0	0.3	0.0	0.1
Gammaproteobacteria_unclassified	0.7	0.0	0.6	4.0	29.1	8.6	4.1	0.0	0.3	3.5	1.5	4.8	0.0	0.4
Methylomonaceae	22.1	0.0	0.0	0.2	0.0	0.0	0.1	0.2	0.7	0.0	1.5	0.0	0.0	0.0
Moraxellaceae	0.0	0.5	0.0	0.3	0.1	10.9	1.0	0.0	0.0	4.6	0.2	0.0	0.7	0.1
Pseudomonadaceae	0.0	13.4	0.1	0.7	0.0	3.4	2.2	0.1	0.0	7.5	0.7	0.0	52.1	1.4

Figure 24. Heat-map representing the percentage of the microbial community of Proteobacteria in the different samples. Only taxa with a ratio higher than 10% in at least one of the 14 samples are presented. The colour intensity in each panel shows the percentage ratio of the given taxon in a sample, referring to the colour key at the right.

4.3.2.1. Proteobacteria composition of the G1 group

The Proteobacteria phylum composition of the G1 group was analyzed. The Tatabánya sample exhibited a notable prevalence of *Methylomonaceae*, *Hydrogenophilaceae*, and *Gallionellaceae*. The Piricske water sample was primarily composed of members of the *Pseudomonadaceae* and *Syntrophaceae* families. In the Tihany water sample, a significant fraction of the family *Burkholderiaceae* and *Clade III* was detected. Within the *Proteobacteria* phylum in the Szentendre sample, sequences of *Burkholderiaceae* and *Rhodocyclaceae* were identified in a higher fraction. The Szent Flórián sample was characterized by the presence of unclassified *Acidiferrobacteraceae*. The Kiskút water sample contained unclassified *Alphaproteobacteria* and *Burkholderiales*, *Rhizobiaceae*, *Sphingomonadaceae*, and *Moraxellaceae*. The Szent Jakab water sample exhibited a high diversity within the *Proteobacteria* phylum, and no family dominated. Nevertheless, significant fractions of *Burkholderiaceae* and *Enterobacteriaceae* were identified, the latter family containing members of the genera *Lelliottia* and unclassified *Enterobacteriaceae* (Figure 24).

4.3.2.2. Proteobacteria composition of the G2 group

The microbial diversity of the G2 group revealed that the Nagy-borvíz water sample exhibited the presence of the families *Burkholderiaceae*, *Hydrogenophilaceae*, and *Desulfobulbaceae*. Similarly, the Berzsenyi water sample was found to be dominated by *Hydrogenophilaceae* and *Sulfuricellaceae*. The Taploca water sample was characterized by a high abundance of the *Burkholderiaceae* family. Furthermore, the Polányi kút water sample contained a relatively significant fraction of the three families *Syntrophaceae*, *Gallionellaceae*, and *Hydrogenophilaceae*. Finally, the Proteobacteria community of Kossuth Lajos water sample was characterized mainly by *Azospirillaceae* and a small fraction of unclassified *Gammaproteobacteria*. (Figure 24)

4.3.2.3. Proteobacteria composition of the G3 and G4 groups

The *Proteobacteria* presented in the Ciprián sample showed a high ratio of *Burkholderiaceae*, *Rhodobacteraceae* and *Rhodocyclaceae.* On the other hand, more than half of the *Proteobacteria*

sequences in Dandár sample were *Pseudomonadaceae* in addition to the presence of *Myxococcales* and *Syntrophaceae* family. (Figure 24)

4.3.3. *Archaeal community composition of the different samples based on amplicon sequencing*

Altogether, 10 archaeal phyla were detected in the water samples. The rarefaction curves of the samples (Figure S2) showed that the sequencing depth was sufficient to recover the majority of the archaeal taxa.

All samples were characterized by a high percentage of unclassified and uncultured OTUs. Unclassified Archaea, *Nanoarchaeota* - belonging to the *Woesearchaeales* order -, *Euryarchaeota* - low fractions in Dandár and Kossuth Lajos water samples -, *Thaumarchaeota* - low fraction within Nagy-borvíz, Piricske and Tihany water samples - were present in all the samples. *Euryarchaeota* phylum contained mainly *Thermoplasmata*, *Methanobacteria* and *Methanomicrobia* classes (Figure 25).

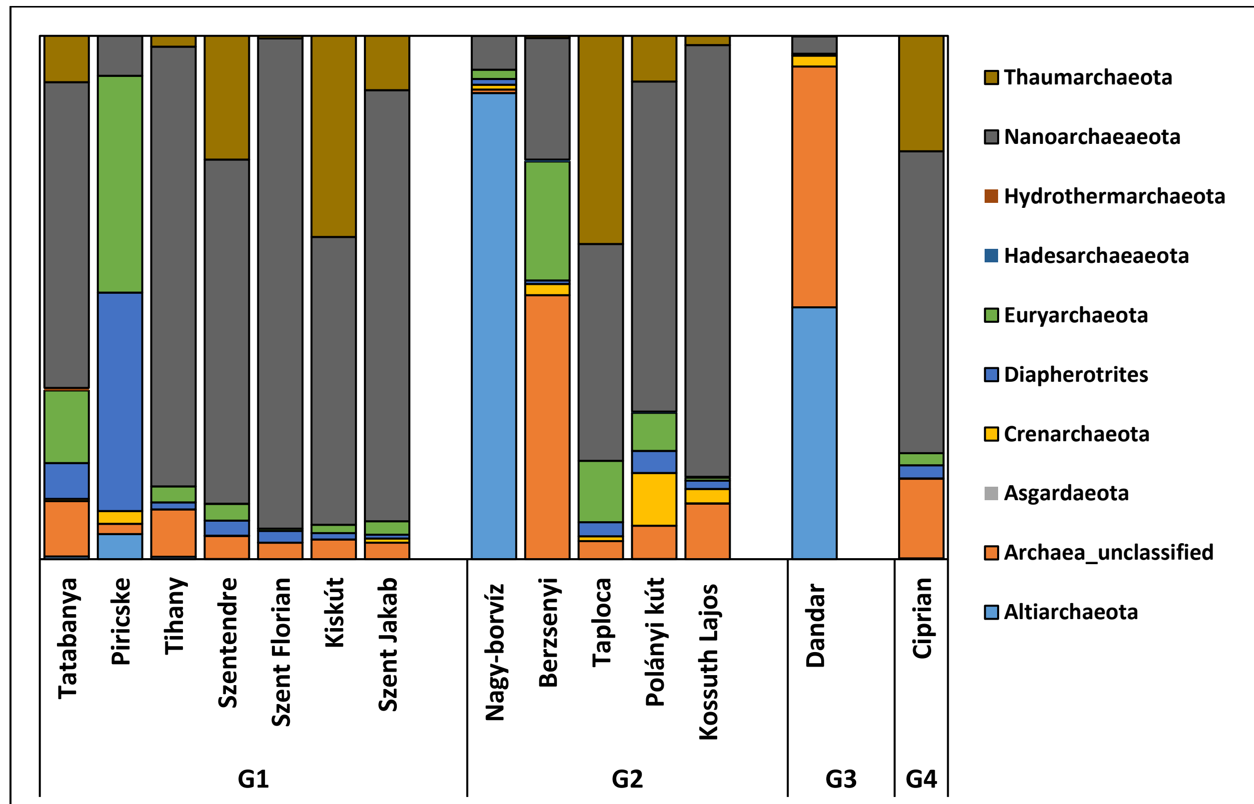


Figure 25. Distribution of the abundant (98%) archaeal phyla based on 16S rRNA gene amplicon sequencing in the water samples.

Seven samples were included in Group 1, consisting of Tatabánya, Piricske, Tihany, Szentendre, Szent Florián, Kiskút, and Szent Jakab. Among these samples, Tatabánya was characterized by the presence of *Methanoperedenaceae* within the *Euryarchaeota* phylum, as well as *Nitrososphaeria* and *Nitrosopumilales* within the *Thaumarchaeota* phylum. Piricske water sample was mainly composed of *Diapherotrites* (*Micrarchaeia*) and *Euryarchaeota* containing *Methanobacteriaceae* taxa. Szentendre water sample had an important fraction of *Nitrosopumilaceae* and *Nitrosotaleales* within the *Thaumarchaeota* phylum, as well as *Thermoplasmata* within the *Euryarchaeota* phylum and *Iainarchaeia* in *Diapherotrites*. Szent Florián had an important fraction of *Diapherotrites*. Kiskút and Szent Jakab showed a significant presence of *Thaumarchaeota*, while Tihany water sample did not exhibit any important fractions of sample-specific archaeal taxa.

Group 2 included five water samples, Nagy-borvíz, Berzsenyi, Taploca, Polányi kút and Kossuth Lajos. Nagy-borvíz water sample was mainly composed of members of *Altiarchaeota* (unclassified *Altiarchaeia* family), while Berzsenyi water sample was dominated by a high proportion of unclassified Archaea in addition to important fractions of *Nanoarchaeaeota* and *Euryarchaeota*. Taploca, Polányi kút, and Kossuth Lajos water samples had a similar composition, consisting primarily of high fractions of *Nanoarchaeaeota*, followed by either *Thaumarchaeota*, *Eukaryota*, or *Diapherotrites*.

Group 3 and 4 included two water samples, Ciprián and Dandár. Ciprián was similar to most of the samples, dominated by *Nanoarchaeaeota* with significant fractions of *Thaumarchaeota*, *Eukaryota*, or *Diapherotrites*. However, Dandár water sample's archaeal community was divided between unclassified bacteria and *Altiarchaeota*.

4.4. *Results of cultivation*

4.4.1. *Isolation of bacteria*

In order to reveal the cultivable diversity, 314 bacterial strains were isolated from the following samples: Dandár, Szentendre, Szent Flórián, Ciprián and Tatabánya. (Their taxonomic position is given in Table 6).

The main dendrogram created by MALDI-TOF MS software to show the grouping of all the isolates. Allowed us to obtain 100 strains considered as groups representatives of the different clusters which were sequenced eventually. The number of isolates grouped in each cluster are shown in Table 6.

Table 6. Results of taxonomic identification of group representative bacterial strains using 16S rRNA gene sequencing.

Strain designation	Closest related type strain (EZbiocloud)	Culture collection number	Similarity (%)	Phylum	Number of isolated strains (Cluster)
SG_E_30_P1*	*Salinibacterium hongtaonis*	DSM 106171	96.33	*Actinobacteria*	3
SG_E_28_P3*	*Paenibacillus sinopodophylli*	KCTC 33807T)	97.97	*Firmicutes*	9
SG_E_25_P2*	*Rhizobium alvei*	DSM 100976	96.44	*Proteobacteria*	1
SG_23_I_P2_1_T	*Moraxella osloensis*	CCUG 350	99.1	*Proteobacteria*	1
SG_18_I_P	*Pararhizobium herbae*	DSM 26427	99.33	*Proteobacteria*	1
SG_16_I_P3	*Streptomyces tateyamensis*	DSM 41969	98.86	*Actinobacteria*	2
SG_6_I	*Microbacterium tumbae*	JCM 28836	98.45	*Actinobacteria*	1
SA_E_32_P2_2	*Brevibacillus nitrificans*	DSM 26674	96.61	*Firmicutes*	4
SA_E_31_P2	*Prosthecobacter algae*	JCM 18053	99.62	*Verrucomicrobia*	2
SA_E_8_P3	*Microbacterium keratanolyticum*	DSM 8606	99.46	*Actinobacteria*	2
SA_E_7_P1	*Ancylobacter rudongensis*	DSM 17131	99.81	*Proteobacteria*	2
SA_E_5_P2	*Paenibacillus tundrae*	DSM 21291	99.72	*Firmicutes*	1
SA_E_2_P2	*Flectobacillus roseus*	BCRC 17834	99.27	*Bacteroidetes*	15
SA_19_I*	*Sphingobium aquiterrae*	DSM 106441	97.74	*Proteobacteria*	2
SA_3_I_P1	*Streptomyces rhizosphaerihabitans*	KACC 17181	99.08	*Actinobacteria*	1

SG_E_30_P3	*Variovorax boronicumulans*	DSM 21722	99.41	*Proteobacteria*	1
SG_E_25_P3	*Pedobacter miscanthi*	KCTC 62786	97.9	*Bacteroidetes*	1
SG_25_I_P1	*Massilia lutea*	DSM 17473	99.03	*Proteobacteria*	2
SG_24_I	*Sphingomonas aerolata*	DSM 14746	100	*Proteobacteria*	1
SG_17_I_P1	*Bacillus simplex*	DSM 1321	100	*Firmicutes*	1
SG_8_I	*Streptomyces scabiei*	DSM 41992	99.87	*Actinobacteria*	1
SG_5_I	*Variovorax paradoxus*	DSM 30034	99.63	*Proteobacteria*	3
SA_E_40	*Ferrovibrio soli*	KACC 19102	99.15	*Proteobacteria*	1
SA_E_39	*Lysinibacillus fusiformis*	DSM 2898	100	*Firmicutes*	1
SA_E_34_P2	*Bacillus mycoides*	DSM 2048	100	*Firmicutes*	1
SA_E_34_P1	*Azospirillum largimobile*	DSM 2294	98.76	*Proteobacteria*	1
SA_E_32_P3	*Caulobacter mirabilis*	DSM 21795	99.12	*Proteobacteria*	1
SA_E_15_P1	*Brevundimonas terrae*	DSM 17329	99.48	*Proteobacteria*	1
SA_E_12	*Paenibacillus typhae*	DSM 25190	99.71	*Firmicutes*	1
SA_E_4_P1	*Pararhizobium giardinii*	DSM 26427	98.25	*Proteobacteria*	2
SA_E_3	*Taonella mepensis*	KACC 16940	99.81	*Proteobacteria*	1
SA_20_I_P	*Bacillus timonensis*	DSM 25372	99.45	*Firmicutes*	1
SA_16_I_P	*Pseudomonas lini*	CFBP 5737	99.89	*Proteobacteria*	1
SA_6_I	*Streptomyces umbrinus*	DSM 40278	99.04	*Actinobacteria*	1
SA_1_I_P1*	*Flavobacterium granuli*	DSM 17797	97.53	*Bacteroidetes*	1
SG_15_I	*Agromyces fucosus*	DSM 8598	99.9	*Actinobacteria*	1
SA_15_I	*Ensifer adhaerens*	CCUG 56773	99.31	*Proteobacteria*	1
SA_9_I	*Pigmentiphaga aceris*	DSM 104072	99.51	*Proteobacteria*	1

FG_E_46	*Microbacterium paraoxydans*	DSM 15021	99.27	*Actinobacteria*	9
FG_E_32_P2	*Microbacterium oxydans*	DSM 20578	99.64	*Actinobacteria*	5
FG_E_20	*Pseudoxanthomonas mexicana*	ATCC 700993	99.17	*Proteobacteria*	1
FA_E_42_P2*	*Paracoccus acridae*	KCTC 42932	97.75	*Proteobacteria*	4
FA_E_40	*Aurantimonas altamirensis*	CECT 7138T	99.55	*Proteobacteria*	9
FA_E_13	*Novosphingobium lindaniclasticum*	DSM 25409	98.83	*Proteobacteria*	4
FG_E_32_P1	*Flavobacterium mizutaii*	NCTC 12149	99.62	*Bacteroidetes*	11
FA_E_42_P2	*Paracoccus haematequi*	LMG 30633	100	*Proteobacteria*	1
FA_E_3_P2	*Bosea robiniae*	DSM 26672	99.43	*Proteobacteria*	1
CG_E_11_P2	*Achromobacter deleyi*	LMG 3458	99.56	*Proteobacteria*	2
CG_E_6_P1	*Bacillus idriensis*	DSM 19097	99.55	*Firmicutes*	2
CG_22_I	*Rhodobacter azotoformans*	CIP 105439	96.91	*Proteobacteria*	2
CG_19_I	*Curvibacter delicatus*	DSM 11558	99.53	*Proteobacteria*	5
CG_14_I*	*Rheinheimera mesophila*	DSM 29723	97.9	*Proteobacteria*	2
CA_E_2*	*Dyadobacter hamtensis*	JCM 12919	97.89	*Bacteroidetes*	1
CA_18_I_P	*Rhodoferax antarcticus*	ATCC 700587	98.55	*Proteobacteria*	1
CA_3_I_P1	*Hydrogenophaga taeniospiralis*	DSM 2082	98.61	*Proteobacteria*	3
CG_E_16_P	*Pseudomonas wadenswilerensis*	LMG 29327	98.95	*Proteobacteria*	1
CG_E_11_P1	*Devosia riboflavina*	ATCC 9526	100	*Proteobacteria*	1
CG_E_5	*Paenibacillus tritici*	LMG 29502T	100	*Firmicutes*	1
CG_E_1	*Paenibacillus populi*	JCM 19843	99.43	*Firmicutes*	7
CG_20_I	*Flavobacterium sasangense*	DSM 21067	98.47	*Bacteroidetes*	1
CG_17_I	*Rhodoluna limnophila*	DSM 107802	100	*Actinobacteria*	1

CG_14_I*	*Rheinheimera aquatica*	BCRC 80081	97.97	*Proteobacteria*	1
CG_11_I	*Brevundimonas denitrificans*	TAR-002	99.51	*Proteobacteria*	1
CG_8_I	*Devosia insulae*	DS-56	100	*Proteobacteria*	1
CG_2_I	*Fictibacillus barbaricus*	DSM 30726	99.82	*Firmicutes*	1
CG_1_I_P1	*Bacillus butanolivorans*	DSM 18926	98.49	*Firmicutes*	1
CA_E_10	*Yonghaparkia alkaliphila*	KSL-113	98.95	*Actinobacteria*	1
CA_E_9*	*Devosia submarina*	NRIC 0884	97.85	*Proteobacteria*	1
CA_E_2	*Dyadobacter koreensis*	WPCB159	98.41	*Bacteroidetes*	1
CA_23_I	*Pseudorhodobacter sinensis*	CGMCC 1.14435	97.12	*Proteobacteria*	11
CA_20_I	*Aurantimicrobium minutum*	JCM 16856	98.86	*Actinobacteria*	1
CA_12_I_P1	*Cellulomonas oligotrophica*	DSM 24482	99.91	*Actinobacteria*	1
CA_10_I*	*Aquabacterium commune*	DSM 11901	97.19	*Proteobacteria*	1
CA_8_I	*Acidovorax temperans*	DSM 7270	99.28	*Proteobacteria*	1
CA_1_I_P2	*Arenimonas aquaticum*	KACC 14663	99.03	*Proteobacteria*	1
CG_E_13*	*Dyadobacter sediminis*	JCM 30073	97.23	*Bacteroidetes*	3
CG_13_I	*Malikia spinosa*	ATCC 14606	99.81	*Proteobacteria*	2
CG_9_I_P1	*Aquabacterium citratiphilum*	DSM 11968	99.53	*Proteobacteria*	1
CA_E_6_P2_L	*Bosea eneae*	DSM 21596	99.7	*Proteobacteria*	7
CA_9_I_P2	*Altererythrobacter troitsensis*	KCTC 12303	99.15	*Proteobacteria*	1
CA_1_I_P1	*Jeotgalibacillus campisalis*	DSM 18983	99.91	*Firmicutes*	1
B_15	*Sphingomonas koreensis*	JSS26	99.5	*Proteobacteria*	13
B_13	*Microbacterium album*	DSM 104474	98.48	*Actinobacteria*	27
AG_66_I	*Kineococcus radiotolerans*	SRS30216	98.71	*Actinobacteria*	2
AG_57	*Methylorubrum pseudosasae*	ICMP 17622	99.59	*Proteobacteria*	2
AG_56_I	*Nevskia ramosa*	DSM 11499	99.56	*Proteobacteria*	2

AG_45_E	*Pseudoxanthomonas mexicana*	ATCC 700993	99.83	*Proteobacteria*	19
AG_9_E	*Sphingopyxis chilensis*	DSM 14889	99.34	*Proteobacteria*	3
AA_11_I	*Sphingopyxis solisilvae*	KEMB 9005-451	100	*Proteobacteria*	1
AA_10_I	*Sphingomonas hunanensis*	JSM 083058	99.74	*Proteobacteria*	6
AA_6_I	*Sphingopyxis fribergensis*	DSM 28731	98.89	*Proteobacteria*	3
AA_66_E_P2	*Pseudomonas psychrotolerans*	LMG 21977T	100	*Proteobacteria*	1
AG_11_E_P2	*Methylorubrum pseudosasae*	ICMP 17622	99.64	*Proteobacteria*	2
AW_35_I	*Acinetobacter pittii*	CCUG 61664	99.91	*Proteobacteria*	1
AW_39_I	*Paracoccus yeei*	CIP 108092	99.56	*Proteobacteria*	2
AW_40_I	*Micrococcus yunnanensis*	DSM 21948	99.35	*Actinobacteria*	3
GW_2_I	*Pseudomonas zeshuii*	DSM 27927	99.74	*Proteobacteria*	15
WA_10_E	*Bacillus licheniformis*	ATCC 14580	99.34	*Firmicutes*	13
WA_63_E	*Roseomonas mucosa*	CCUG 48654	99.82	*Proteobacteria*	1
WG_2_E	*Bacillus circulans*	ATCC 4513	100	*Firmicutes*	6

*Potential novel bacterial taxon

Based on the complete 16S rRNA gene sequence similarities most bacterial strains showed between 98 and 100% similarity values to the reference sequences of the type strains of the given species. Nine bacterial strains had lower than 98% similarity to their closest relative, presenting them as novel taxa among the isolated bacteria. The isolated bacteria were affiliated into four phyla, with most of them being *Proteobacteria* (59%) followed by *Actinobacteria* (21%), *Firmicutes* (17%) and *Verrucomicrobia* (1%). In the case of amplicon sequencing, the *Proteobacteria* phylum was dominant, *Firmicutes* were present in high numbers in the Dandár water sample, and *Actinobacteria* were represented by less than 5%. The cultivable microbial communities showed similarities between the different samples, e.g., *Micrococcus*, *Pseudomonas*, *Bacillus* and *Pseudoxanthomas* genera were present in the majority of the samples where the

isolation were performed. The members of the facultative chemolitothrophic genera of *Sphingobium*, *Sphingomonas*, *Sphingopyxis* and the heterotrophic *Microbacterium* were characteristic only of the Szentendre, Szent Flórián and Tatabánya samples. Many detected taxa known to thrive under nutrient-depleted circumstances, e.g., *Acinetobacter*, *Novosphingobium* and *Nevskia*, were also detected. The distribution of the different cultivated genera is given in Figure 26.

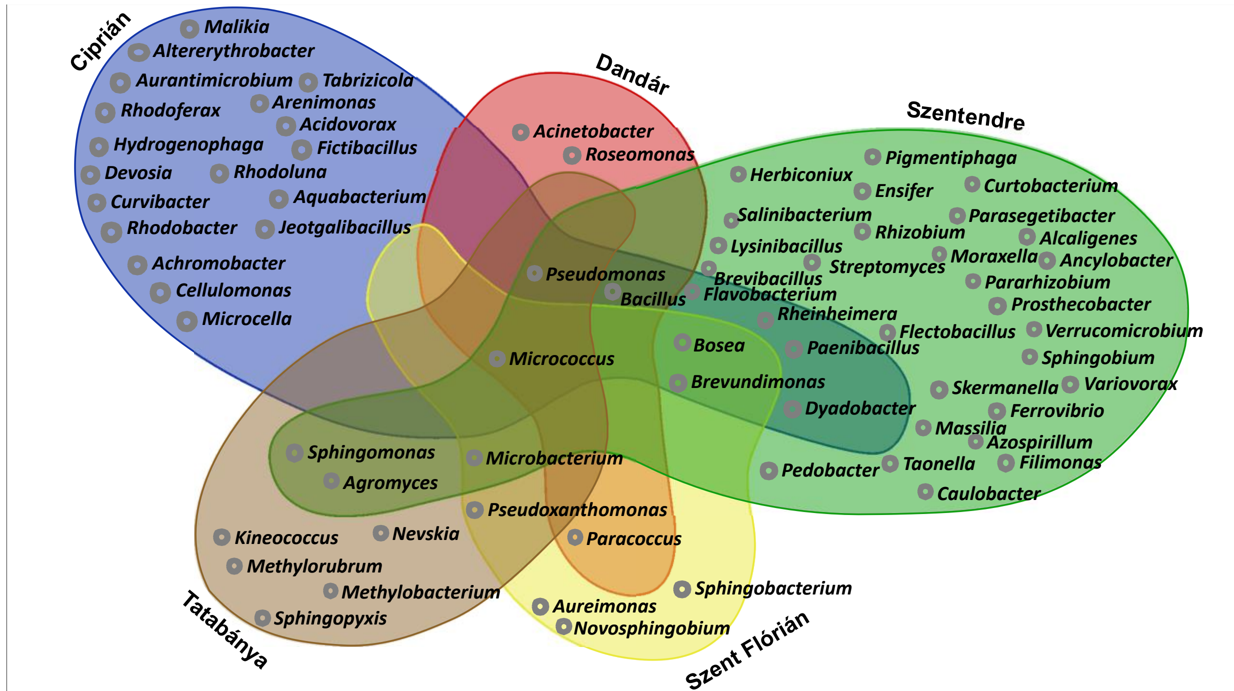


Figure 26. Distribution of the cultivated taxa among the water samples.

4.4.2. *Bacterial growth in different media - testing of oligotrophic characters*

The number of bacterial strains that were able to grow at the different nutrient concentrations was calculated, and results are shown in Figure 27.

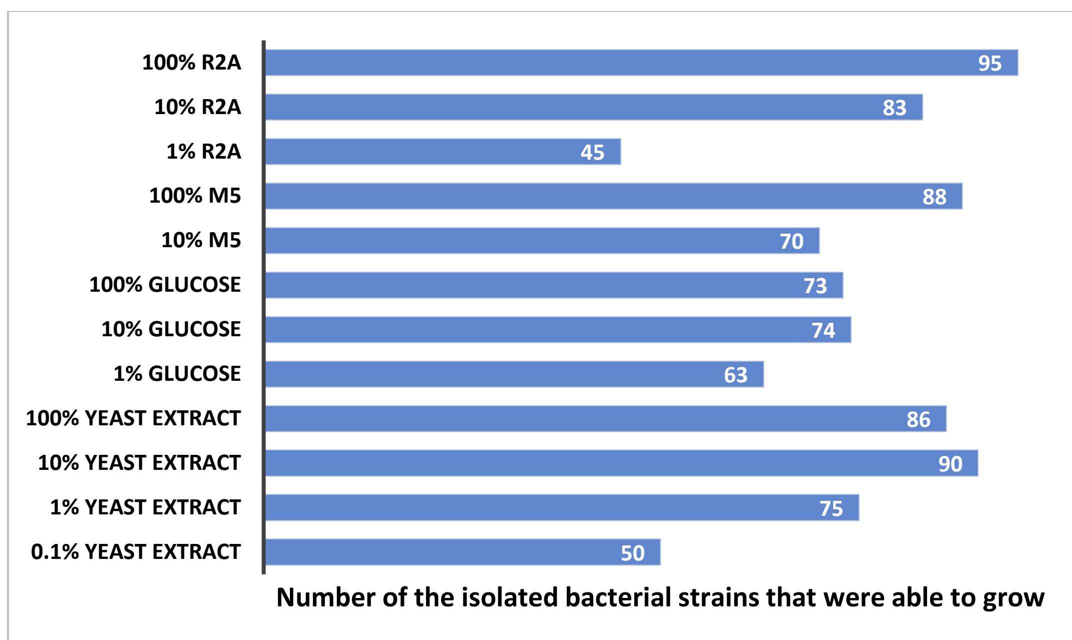


Figure 27. Number of the isolated bacterial strains that could grow in the different nutrient content media.

24 of the 100 cultivated strains could thrive under all the used media, while the rest of them were absent in one or more media. Only 10 of the tested bacteria were able to grow in one or more oligotrophic media and unable to survive in the 100% yeast extract media (Figure 27). The list of these bacteria is given in Table 7, and four of them belong to novel bacterial taxa, as their 16S rRNA gene sequence homology is below 98% (Pronk, Goldscheider, and Zopfi 2009) (Giovannoni et al. 1990).

Table 7. The list of bacteria able to grow only in nutrient-depleted conditions.

Sign of the bacterial strain	**Closest relative based on 16S rRNA gene sequencing**	**Similarity values (%)**
SG_E_30_P1	*Salinibacterium hongtaonis* (194)	96.33
CG_13_I	*Malikia spinosa* (ATCC 14606)	99.81
SA_6_I	*Streptomyces umbrinus* (NBRC 13091)	99.04

SA_E_31_P2	*Prosthecobacter algae* (EBTL04)	99.62
CG_19_I	*Curvibacter delicatus* 146	99.53
CG_E_13	*Dyadobacter sediminis* (Z12)	97.23
CG_14_I	*Rheinheimera aquatica* (GR5)	97.97
CG_9_I_P1	*Aquabacterium citratiphilum* (B4)	99.53
CA_10_I	*Aquabacterium commune* (B8)	97.19
SA_E_40	*Ferrovibrio soli* (A15)	99.15

4.5. *Polyphasic approach to study new bacterial strain isolated from the water sample of Szentendre*

Strain SG_E_30_P1 was isolated from the groundwater sample, of Szentendre. In order to characterize the new isolated taxa, a polyphasic approach was used in order to identify and describe the genetic, morphological, and physiological features of the new bacterial taxa isolated from the water sample using a combination of molecular and culture-based methods.

The strain SG_E_30_P1 belongs to the family *Microbacteriaceae* which belongs to the order *Microbacteriales*, class *Actinomycetia* within the *Actinobacteria* Phylum. This family contains 69 genera at the time of writing and 2 Candidatus taxa (https://lpsn.dsmz.de/family/microbacteriaceae). The members of this family are chemoorganotrophic, their metabolism is primarily respiratory with oxygen as the terminal electron acceptor, they are mostly aerobic, microaerophilic or facultative anaerobic bacteria (Evtushenko 2015). The natural habitats of the members of this family are various terrestrial and aquatic environments. Some species are associated with plants (Behrendt et al. 2002), animals (Kämpfer et al. 2000), algae (Alvarado et al. 2018), fungi (Cardinale et al. 2011), in addition to clinical specimens (Evtushenko 2015). In this study, a novel genus of this family, isolated from a groundwater sample in Hungary is described based on a polyphasic approach.

The 16S rRNA gene sequence of SG_E_30_P1 comparison within the EzBioCloud database indicated that the isolated bacterium is a member of the family *Microbacteriaceae*. The closest relative in term of sequence similarity is *Salinibacterium hongtaonis* MH299814 (97.77 %) followed by *Leifsonia psychrotolerans* GQ406810 (97.57 %), *Herbiconiux ginseng* CGMCC 4.3491 (97.48 %), *Leifsonia bigeumensis* EF466124 (97.48 %) and *Leifsonia kafniensis* AM889135 (97.48 %).

The Maximum likelihood phylogenetic tree was generated using the 16S rRNA sequences of each of the 21 species included in this study. The phylogenetic analysis of the 16S rRNA gene sequences of the novel strain showed that SG_E_30_P1 has been positioned near the type strains of two independent genera, *Leifsonia psychrotolerans* (97.57 % similarity) and *Galbitalea soli* (97.04% similarity) and separated from all other close relatives (Figure 28).

The full genome sequencing of the strain SG_E_30_P1 resulted in a full genome with 2 895 655 nt length, the percentage of GC content is 65.55 %, with 433 x genome coverage. SG_E_30_P1 is characterized with a phylogenetic placement within the family *Microbacteriaceae*, this is confirmed by the results of the phylogenomic analysis (pseudo-bootstrap value was 100%; Figure 29), where the novel genus is separated from all the other relatives, far grouping together with *Amnibacterium flavum* MJJ-5. The phylogenetic analysis showed that the bacterium SG_E_30_P1, form a completely separate lineage between other clades of the family *Microbacteriaceae*, it is strengthening also by the phylogenomic analysis (Figure 29)

The average nucleotide identity (ANI), average amino acid identity (AAI) and digital DNA–DNA hybridization (dDDH) values were determined among the genome sequence of SG_E_30_P1 and the reference genomes of the closest relatives (Yoon et al. 2017) (Rodriguez et al. 2016) (Meier-Kolthoff et al. 2013) (*Leifsonia psychrotolerans*, *Galbitalea soli*, *Amnibacterium flavum, Conyzicola nivalis, Herbiconiux ginseng* (CGMCC 4.3491)*, Microterricola pindariensis* and *salinibacterium hongtaonis;* the different sequences were downloaded from the NCBI genome database). The results are shown in the table below (Table 8). The ANI (85 %) (Kim et al. 2014), AAI and the genome to genome distance calculations (GGDC) (70%) (Luo et al. 2014) values are at a lower level than the genus delineation threshold. According to the ANI, AAI and dDDH values including DNA G+C content differences between the genome sequence of SG_E_30_P1 and the

reference genomes, it is advised that SG_E_30_P1 does not represent a member of any existing genera of the family *Microbacteriaceae*.

Analysis of SG_E_30_P1 genome through Rast server 2.0 have shown the presence of different resistance genes to antibiotics and toxic compounds (copper homeostasis, cobalt-zinc-cadmium resistance, fluoroquinolones resistance and mercury reductase). Genes encoding for toxin-antitoxin systems were also found. The genome also included ammonia and organic sulfur assimilation genes. Moreover, genes included in vitamin biosynthesis were also revealed (thiamin, menaquinone, riboflavin and pyridoxin).

In order to better understand the genetic difference of the new bacterial taxa within the context of microbial diversity. The closest relatives of the new taxa were chosen based on the 16 S rRNA gene sequence similarity using the EZbiocloud algorithm. Using these taxa, the genetic traits were compared like the Table 8 is showing.

Table 8. Difference between the genome sequence of SG_E_30_P1 and the reference genomes of its closest relatives in term of average nucleotide identity (ANI), average amino acid identity (AAI), digital DNA–DNA hybridization (dDDH) and DNA G+C content differences.

Strain	**AAI (%)**	**dDDH (%)**	**DNA G+C content difference (%)**	**G+C content**
***Amnibacterium flavum* (M8JJ-5)**	60.2	20.2	3.09	68.6
***Conyzicola nivalis* (CGMCC 1.12813)**	64.6	19.3	2.62	68.2
***Herbiconiux ginseng* (CGMCC 4.3491)**	60.6	20.1	2.88	68.4
***Microterricola pindariensis* (DSM 22300)**	60.4	20.2	4.23	69.8
***Salinibacterium hongtaonis* (DSM 106171)**	62.5	20.2	1.32	64.1
***Leifsonia psychrotolerans* (DSM 22824)**	59.2	19.9	1.31	64.2

***Galbitalea soli* (DSM 105515)**	63.6	19	3.64	69.2

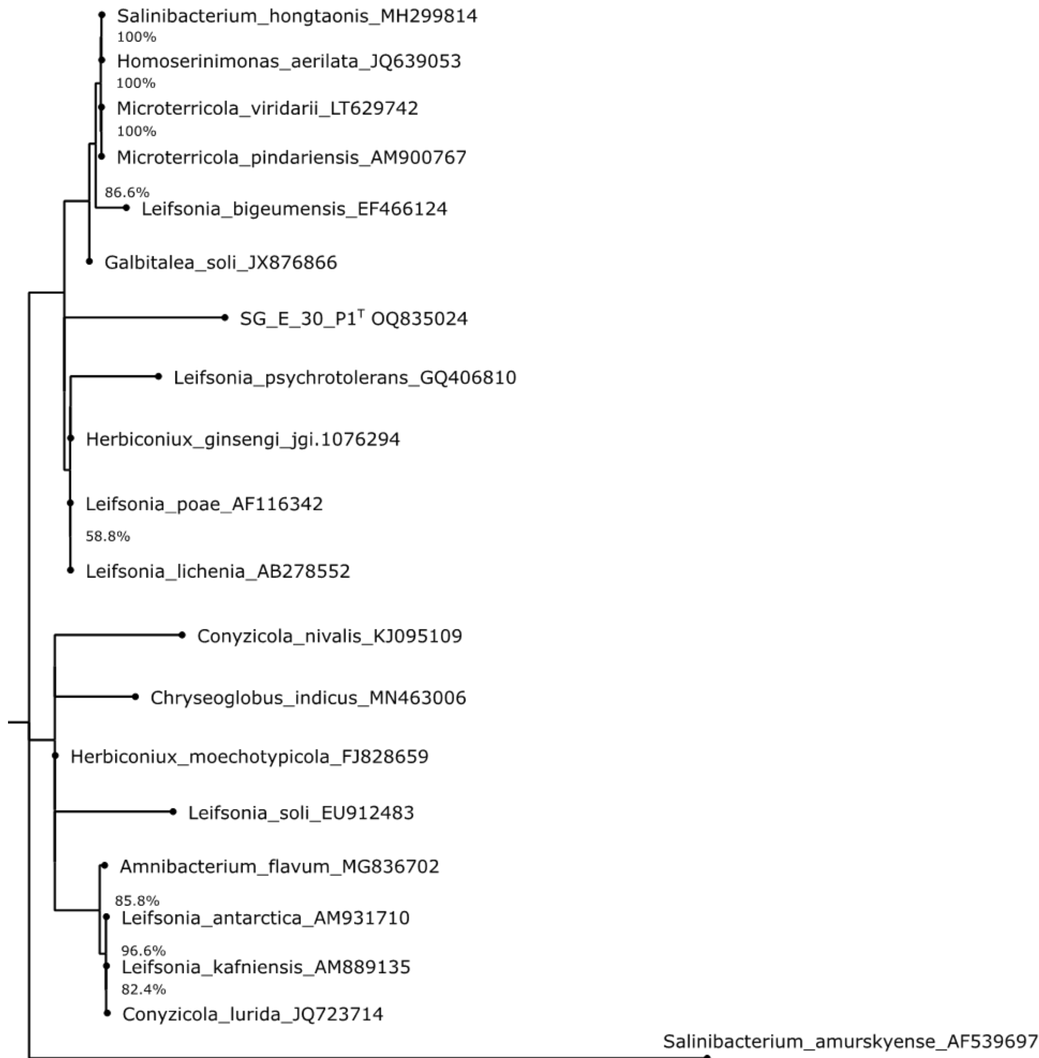


Figure 28. Maximum likelihood phylogenetic tree based on the complete 16S rRNA gene sequences showing the phylogenetic positions of the strain SG_E_30_P1 with closely related taxa. Numbers at nodes indicate the percentage of 1000 bootstrap replicates.

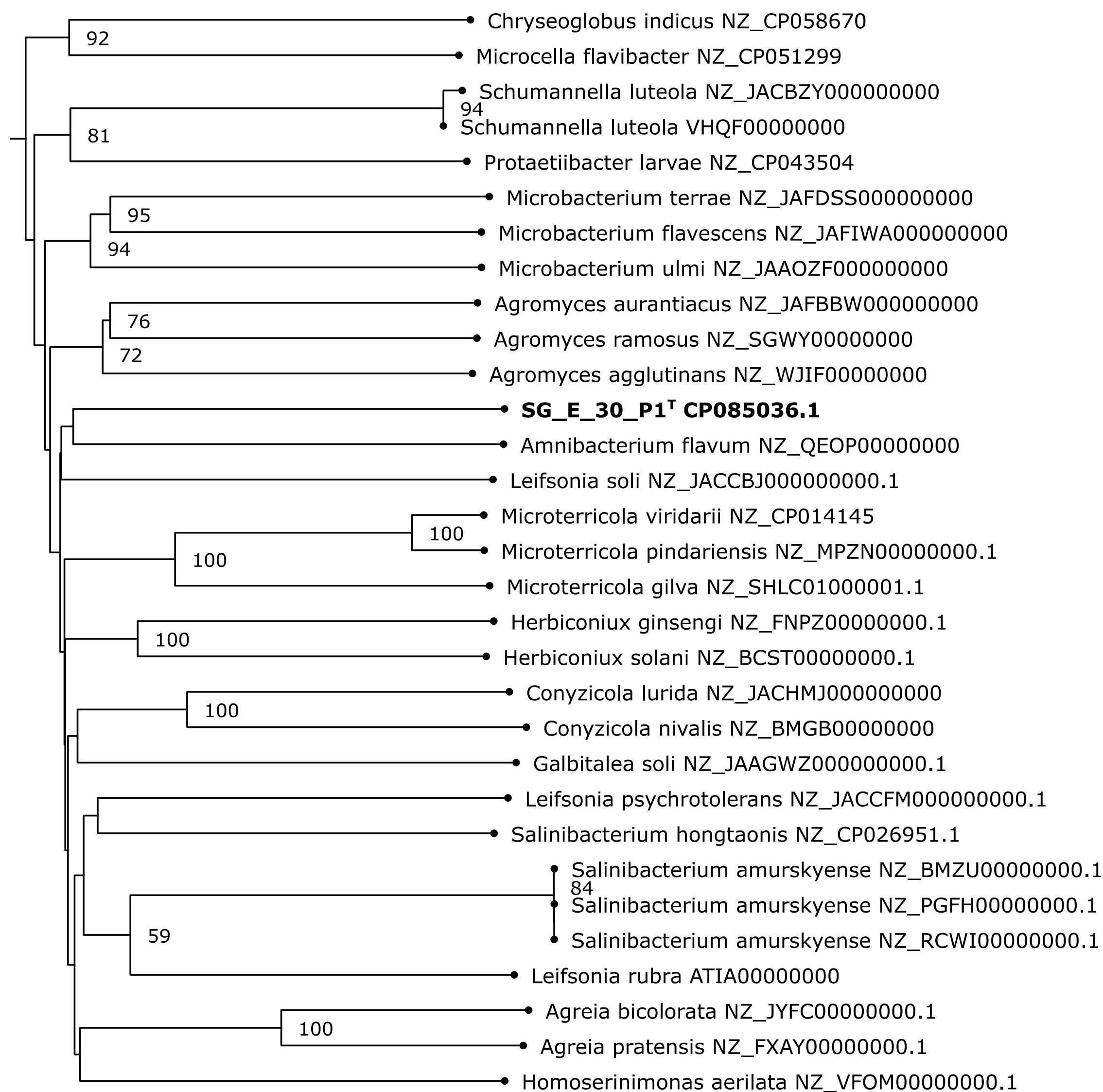


Figure 29. Balanced minimum evolution tree of SG_E_30_P and type strains of closely related taxa based on their genome sequences. The tree was reconstructed based on data from the Type (Strain) Genome Server (TYGS). The tree was inferred with FastME 2.1.6.1 from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d5. The numbers above branches are GBDP pseudo-bootstrap. Values under 50 are not given. Bar, 0.02 substitutions per site.

Based on the 16S rRNA gene sequence similarities observed in the phylogenetic tree, *Galbitalea soli* (DSM 105515) and *Leifsonia psychrotolerans* (DSM 22824) were chosen as side-by-side

analysis for phenotypic characteristics. The cells of the novel bacterium were non motile and stained Gram positive. Their shape varied from single rods to elongated forms (2µm-7µm) (Figure 30) On R2A agar medium light yellow pigment production was observed.

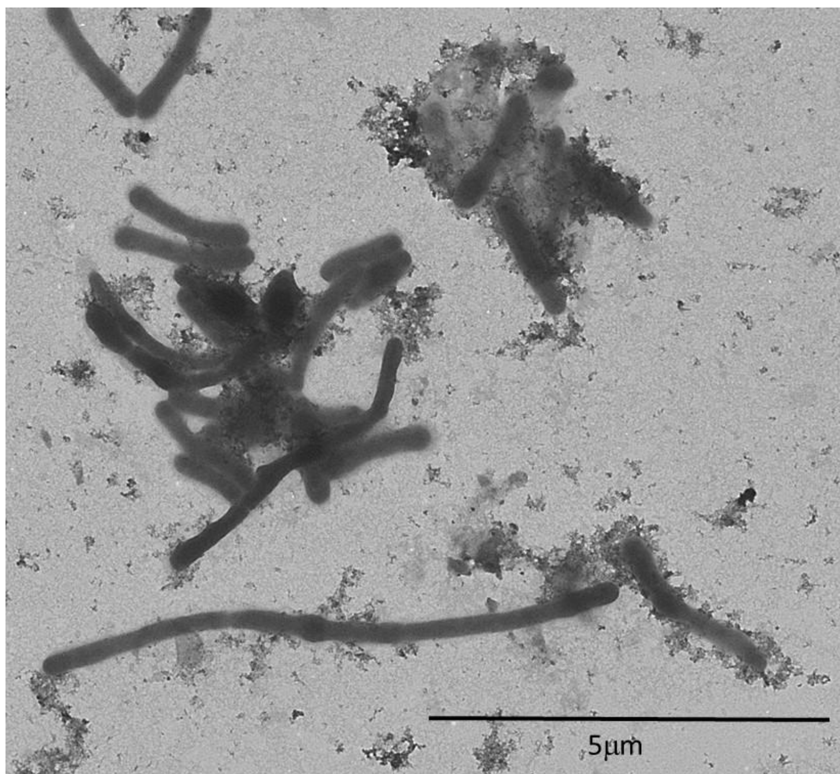


Figure 30. Transmission electron microscopic image of bacterial strain SG_E_30_P1.

Table 9. Differential characteristics of strain SG_E_30_P1 with members of closely related taxa.

Strains: 1, SG_E_30_P1; 2, *Leifsonia psychrotolerans* DSMZ 22824; 3, *Galbitalea soli* DSMZ 105515.

Data presented here are based on analyses performed during this study, except of those indicated with a star(*), +, Positive; −, negative; w+, weak positive reaction; ND, no data.

Characteristic	**1**	**2**	**3**
Isolation source	Groundwater	* Soil	□ Soil

Colony pigmentation	Light yellow	Yellow	Light yellow
Shape	short to elongated rod (Fig. 3)	irregular rod- or filament-shaped	rod
Motility	non motile	motile	motile
Temperature (°C)			
Range	4 - 28	4 - 35	4 - 45
Optimum	20 - 28	20 - 28	7 - 28
pH for growth			
Range	6 - 8	5 - 7	6 - 10
Optimum	7	6 - 7	8
NaCl tolerance (%,w/v)			
Range	0 - 2	0 - 1	0 - 2
Optimum	1 - 2	1	0 - 1
Utilization as sole source of			
L-arabinose	-	+	-
D-ribose	-	+	-
D-xylose	-	+	-
L-rhamnose	-	+	-
Aesculin	-	-	+
Salicin	-	-	+
Potassium 5_ketogluconate	+	-	+
Enzyme activities			
Esterase (C4)	-	+	-
Valine arylamidase	-	+	+
Cystine arylamidase	-	+	-

Acid phosphatase	-	+	-
β-galactosidase	-	+	+
α-glucosidase	-	+	+
β-glucosidase	-	+	-
Hydrolysis of:			
Catalase	-	+	-
Casease	-	+	+
Phosphatase	-	-	+
Gelatinase	-	+	+
Urease	-	-	-
NO_2	+	+	-
Major fatty acids+	anteiso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{14:0}$	*anteiso-$C_{15:0}$, $C_{18:0}$, $C_{16:0}$ anteiso-$C_{17:0}$	□anteiso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{14:0}$
Major polar lipids*	DPG, AGL, APL, PG, PL	*PG, DPG	□DPG, PG, GL
DNA G+C content (%)	65.55	*64.50	□69.19

DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; †ai, Anteiso-branched; i, iso-branched.

* Data from Ganzert et al. 2011.

□ Data from S. J. Kim et al. 2014

Cells are Gram stain positive, elongated shaped, non-motile, oxidase and catalase negative. The cells contain MK 7 quinone. The predominant fatty acids are anteiso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{14:0}$. The major polar lipids are DPG, AGL, APL, PG, PL.

SG_E_30_P1 contained MK 7 as a sole respiratory quinone, peptidoglycan structure analysis revealed B type with a DAB: D-/L-Ala: L-DAB: D-Glu with a ratio of 1.6 Ala, 1.0 Gly, 1.0 Glu, 0.6 DAB. The phospholipids profile of SG_E_30_P1 is different from its closest relatives due to

the presence of AGL, APL, and PL and similar due to the presence of DPG and PG. The fatty acid profile showed common presence and importance of anteiso-$C_{15:0}$, iso-$C_{14:0}$ and iso-$C_{16:0}$ (Table 9 and Figure 31) between SG_E_30_P1 and *Galbitalea soli*, however some differences in the amounts of the components were present (Figure S3, Table S1).

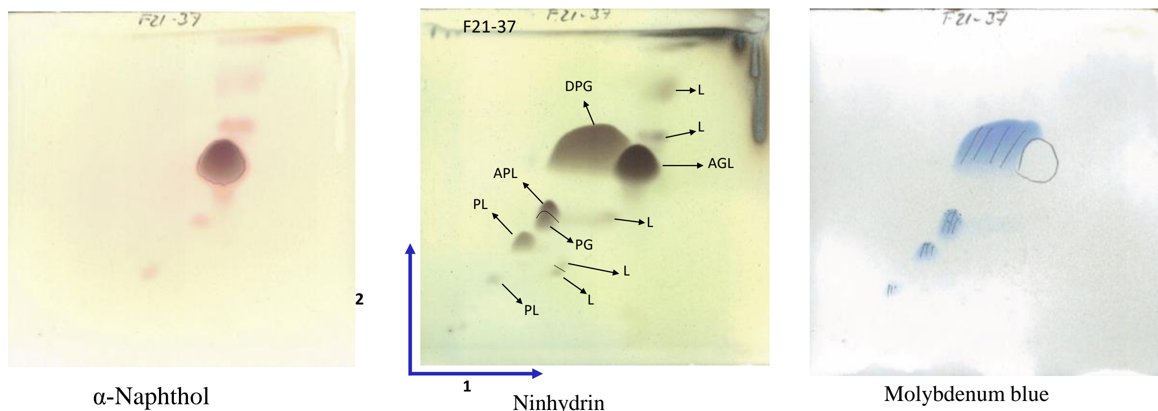


Figure 31. Two-dimensional TLC of polar lipids of strain SG_E_30_P1 after spraying with α-naphthol reagent and heating at 100 °C for 10 minutes (A). After spraying with dodecamolybdophosphoric acid and heating at 140 °C for 15 minutes (B). After spraying with ninhydrin (circles) and molybdenum blue (blue spots) (C)

On the basis of the phenotypic data presented in Table 9, SG_E_30_P1 is characterized by the the inability to tolerate higher temperatures than 28 °C. Other differences were seen by the inability to utilize valine arylamidase, β-galactosidase and α-glucosidase or to produce casease and gelatinase enzymes also differentiate our bacterium to its closest relatives. The non-motile characteristic of bacterial strain SG_E_30_P1 differentiates it from the rest of the members of the genera *Leifsonia and Galbitalea.*

Based on the phenotypic, chemotaxonomic, phylogenetic and phylogenomic data presented, bacterial strain SG_E_30_P1 represents a novel genus within the family *Microbacteriaceae,* for which the name *Antiqaquibacter oligotrophicus* gen. nov. is proposed.

DESCRIPTION OF *ANTIQAQUIBACTER* GEN. NOV.

Antiq.aqui.bacter. “Antiq”, referring to the name of the ground water Sztaravoda (Szentendre water sample) in Serbian language meaning old water, where the strain was isolated from, “aqui”

is referring originating from water and bacter “bacter” is a rod shape bacterium; N. L. masc. n. Antiqaquibacter rod-shaped bacterium from Szentendre groundwater.

DESCRIPTION OF ANTIQAQUIBACTER OLIGOTROPHICUS SP. NOV.

Antiqaquibacter oligotrophicus (oligotrophicus. L. masc. adj., refers to the low nutrient content of the isolation site) possess the following properties in addition to those given at the genus level: grows well on R2A agar and M5 media (Toumi et al. 2021). The colonies on R2A are small, circular, light yellow, non-motile, elongated. Growth occurs at 4-28°C (optimum 20-28 °C), pH 6-8 (optimum 7), NaCl concentration at 0-2%. Positive for NO_3^- reduction and negative, casease, phosphatase, gelatinase, urease, starch, indol, H_2S production. Negative for acid production from any carbon sources of API 50CH except Potassium 5_ketogluconate. In API ZYM kit the results are positive for esterase lipase (C 8), leucine arylamidase and naphtol-AS-BI-phosphohydrolase. The type strain SG_E_30_P1 was isolated from a water sample collected from Szentendre groundwater in Hungary. The DNA G+C content of the type strain is 65.55% (calculated from the genome sequence). The GenBank accession number for the 16S rRNA gene sequence is OK362296.1. The whole genome shotgun project has been deposited in NCBI database under the accession number SAMN21381085 in the Bioproject number PRJNA762240.

5. *Discussion*

5.1. *Physical and Chemical Parameters of the Water Samples*

It is known that hydrogeological and other ecological factors influence the microbiological processes and composition of microbial communities. On the other hand, the metabolism of microorganisms usually affects the water quality of groundwater systems. In addition, the fate and transport of microorganisms in groundwaters are the result of their physicochemical characteristics (size, inactivation rate, and surface electrostatic properties) and the groundwater media (flow velocity, gain, size, porosity, solid organic carbon content, temperature, pH, and other chemical characteristics of the water) (Edberg et al. 1997).

Ciprián water sample is characterized by high cell counts, most probably due to the intensive agriculture activity above its catchment area provides several nutrients. The nitrate content and TOC values of this sample were also high, indicating the human influence within this region. The

negative correlation in cell counts and diversity indices in case of the Szentendre sample can be explained by the location area: Stravoda region is located within the Visegrád mountains with many forests; therefore, many soil bacteria with the ability to tolerate low nutrient content could infiltrate into the groundwater and thus increase the diversity within the sample. This assumption is in accordance with the results of (Herrmann et al. 2019). Balaton Highland region and both of Dandár and Szent Flórián water samples had similar Shanon diversity index values showing low external influence on the waters. These are confined and semi-confined aquifers that protect their groundwaters from external influence. Moreover, previous reports indicated that a high concentration of SO_4^{2-} in the Dandár water sample significantly influenced the microbial diversity due to the negative interaction of several sulfate-reducing bacteria with other microorganisms (Lemos et al. 2011). Tatabánya water sample had a relatively high diversity index in case of archaea, and this can be explained by the high abundance of methanogenic prokaryotes, where they can be connected to dolomite formation (S.J. Mazzullo 2000) or precipitation in shallow groundwater (Roberts et al. 2013), such as in the case of the Tatabánya well which belongs to a dolomite karst aquifer.

It is worth mentioning that before isolation, in the media of the Tatabánya water sample, many bubbles were observed (

Figure 32), indicating strong gas production of the cultivated bacteria.

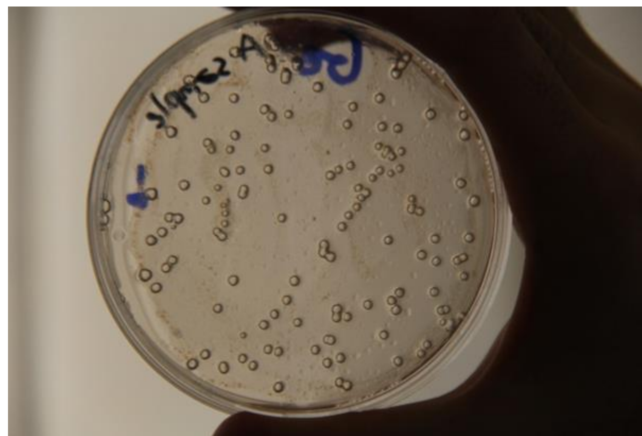


Figure 32. Bubbles observed during cultivation after spreading the Tatabánya water sample on the Petri plates.

The observation that when the cell count values are tending to be lower, the diversity is higher (Table 5) can be explained by the dominance of only few taxa in the samples due to their adaptability to the specific environment conditions.

5.2. *Microbial communities of the different samples based on amplicon sequencing*

Previous studies showed that low nutrient content environments and groundwaters discharge data sets are usually dominated by unknown taxa. Previous studies of Lopez-Fernandez et al. (2018) and Gayner (2018) showed that almost 50% of the identified phyla in groundwater samples were archaeal or bacterial candidates, moreover, the percentage of unknown and candidate phyla increased with depth, which highlights the importance of further studies to characterize deep biosphere microbial communities.

These literature findings were endorsed by the presence of many unclassified taxa, among them unclassified *Parcubacteria* occurring in all samples. Candidatus *Parcubacteria,* is considered as a part of CPR superphylum, members of this phylum are known to harbour a variety of metabolisms with the possibility of acquiring fermentative processes able to produce acetate, ethanol, lactate, and hydrogen (Gayner 2018). Previous genomic analyses of *Parcubacteria* revealed the existence of nitrite reductases which can transform nitrite to produce nitric oxide (gene nirK) and ammonium (gene nirB) (Castelle et al. 2018).

The predominance of the superphylum *Patescibacteria* in groundwaters is often related to their mobilization from soils and their good survival under oligotrophic conditions (Herrmann et al. 2019). Co-occurrence network analysis pointed to potential associations of *Patescibacteria* with specific organisms involved in nitrogen, sulfur and iron cycling (Herrmann et al. 2019). Other capability of Candidatus *Patescibacteria* members in oligotrophic habitats is their abundance under ultra-small cells and acquirement of reduced genome size (pass through 0.2 µm pore size filter) (Miyoshi et al. 2005) (Luef et al. 2015). These features are thought to be evolutionarily advantageous, as the increased surface-to-volume ratio optimizes the uptake of the sparse nutrients (Sowell et al. 2009) and the loss of expendable genes leads to a lower metabolic cost of reproduction (Giovannoni et al. 2014), these characteristic are also seen in *Caldisericota* genome (Rodríguez-Gijón et al. 2021) which was revealed in Piricske water sample.

The predominance of *Woesearcheota* in all the samples can be explained by a syntrophic metabolic model (Liu et al. 2018), which removes the thermodynamic bottlenecks and enables several metabolic reactions under nutrient-depleted conditions (Lau et al. 2016). These results were confirmed by a co-occurrence network analysis (Andersen et al. 2018), and indeed a short distance was shown between many *Woesearcheota* OTU and both *Methanomicrobia* and *Nitrososphaeria*. These results suggest that *Woesearchaeota* might form a common consortium with methanogens in anaerobic environments. Moreover, *Woesearchaeota* may have a role in the processes of denitrification, nitrogen fixation, or even dissimilatory nitrite reduction. These findings are in accordance with (Liu et al. 2018). The widespread presence of *Omnitrophicaeota* in the studied samples is in accordance with previous studies showing their presence in groundwaters and drinking water treatment plants (Bruno et al. 2018).

Amplicon sequencing showed the presence of members of *Thermoplasmata*, known previously of inhabiting not only extreme environments but also a wide range of environments (Hu et al. 2021). Taxa existing within this phylum could be classified only in case of Nagy-borvíz and Piricske water samples where mainly they belonged to *Marine_Benthic_Group_D (Thermoprofundales)* and *DHVEG-1 (Thermoplasmata).*

Based on few available cultures and genomes of *Thermoplasmata*, researchers could have an insight on their myxotrophic lifestyle (Zhou et al. 2019) which is crucial for their sustainability in oligotrophic ecosystems in order to compensate the lack of organic nutrients (Hartmann et al. 2012).

Microbial community of group 1 (G1)

The Tatabánya sample is characterized by many hydrogenotrophic methanogens, among them *Methanobacteriac*eae and *Methanoperedenaceae*. The latter is often found at oxic-anoxic interfaces where they are involved in nitrate-dependent anaerobic oxidation of methane. This reaction links carbon and nitrogen cycles (Guerrero-cruz et al. 2018). The presence of *Micrarchaeia* is reported in several oxygen-poor aquatic environments e.g., shallow groundwater (Gayner 2018), oxygen-minimum zones of Arabian Sea, Bay of Bengal (Fernandes et al. 2020), or estuarine water (Zemskaya et al. 2019). The group of Marine Benthic Group D and A were found previously in oxygen-depleted water columns (Takai et al. 2001). They have the ability to play important roles in the sedimentary carbon cycle (Zhou et al. 2019). The Deep Sea *Euryarchaeotic*

Group is reported to occur together with anaerobic methanotrophic archaea, and many of them were present in the Tatabánya water sample (Inagaki et al. 2006). *Methylorubrum pseudosasae*, a methylotrophic bacterium, was even cultivated. The presence of *Arcobacteraceae* in the Tatabánya sample can be explained by having high survival rate in nutrient-limited groundwater (Mcelwain et al. 2002). Some species are capable of autotrophic carbon dioxide fixation via the reverse tricarboxylic acid cycle (Waite et al. 2017). In accordance with the archaeal community, the bacterial community is characterized by the presence of members able to metabolize molecular hydrogen as a source of energy, among them *Hydrogenophilaceae* (Stöhr et al. 2001) (Vésteinsdóttir et al. 2011) and *Sulfurimonas* belonging to *Thiovulaceae* family (Han et al. 2014) (Cai et al. 2015). Therefore, the experienced gas bubbles are most probably the result of microbial actions, by H_2 production of bacteria, or by methane production of archaea. To reveal precisely the source of gas production at the sampling site, further analysis would also be needed.

Piricske water sample (dominated by *Caldisericota*, with a substantial fraction of *Spirochaetota* and *Bacteroidetes*). Previous literature showed that *Caldisericota* belong to the phyla with small genome sizes (< 2 Mb) (Rodríguez-Gijón et al. 2021) and *Spirochaetota* is characterized with a spiral shape at a diameter lower than 0.2 µm (Herrmann et al. 2019). the small genome sizes of microorganisms in oligotrophic environments may contribute to their ability to thrive in nutrient-poor conditions. In fact, they may require fewer resources to replicate and maintain, faster growth and reproduction and more adaptable to changing environmental conditions (rapidly acquire or lose genes through lateral gene transfer) (Eguchi et al. 2001). Species from the phyla candidate division *Zixibacteria* found especially in Piricske water sample - and *Chloroflexi* are known to contain nitrification genes (Chen et al. 2021). Methanogenic archaea were present with the members of *Methanoperedenaceae*. They are known to be able to thrive in nutrient-poor and low ionic-strength environments (Bräuer et al. 2015).

Bacterial communities characterizing Tihany water sample was also present in many fresh water oligotrophic lakes. Such as, the presence of both *Ilumatobacteraceae* and *Sporichthyaceae* (Bashenkhaeva et al. 2020) (Özbayram et al. 2021) (Newton and McLellan 2015) (Cabello-yeves et al. 2020). The presence of *Cryomorphaceae* family in similar environments showed that they have roles in aquatic ecosystem secondary production, (Bowman 2014). While much evidence suggests that high nutrient loads promote cyanobacterial blooms in lakes, there is also widespread

evidence that blooms occur in oligotrophic systems as well. Many adaptations of *cyanobacteria* allow them to thrive under a wide range of environmental conditions, including low nutrient content environments. Such as, the ability to efficiently take up and store nutrients in various forms (Reinl et al. 2021).

The Szent Flórián sample was characterized by distinctive families, the most abundant among them being *Candidatus Kaiserbacteria, Candidatus Magasanikbacteria, Candidatus Uhrbacteria, Candidatus Azambacteria* and the family *Brocadiaceae*. Many members of the family *Brocardiaceae* can be responsible for anaerobic oxidation of ammonium (anammox bacteria). This can suggest that in this environment some ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) are present, oxidizing the ammonium to nitrite, while anammox bacteria such as *Brocadia* would convert what remains from the ammonium. In addition, nitrite reducer bacteria such as *Kaiserbacteria* (Danczak et al. 2017) would convert what remains from the nitrite to dinitrogen gas (Strous et al. 1997). The action of these organisms is very important in these environments where the nitrogen is often in limited concentrations. Members of the taxa *Brocadiaceae, Parcubacteria, Peribacteria* and *Saccharimonadales* are characterized by small genomes and a high degree of specialization. These features are often associated with microorganisms performing a limited range of metabolic activities (Nelson et al. 2015) (Anantharaman et al. 2016) (Lemos et al. 2019). The reduced genome size is often related to strong dependent conditions (e.g., interactions between various microbial populations).

The water sample from Szentendre revealed the presence of certain phyla that are typically found in groundwater. One of these phyla includes *Dependentiae*, which is an ultra-small bacterium that has a minimalistic metabolism. This bacterium is known for its genetic flexibility, which allows it to adapt to various groundwater conditions (Gios et al. 2023). Another phylum that was identified in the sample is *Verrucomicrobia*. This phylum has been previously isolated from different oligotrophic environments (Tran et al. 2018).

Szent Jakab and Kiskút water samples showed the presence of overlapping oligotrophic microorganism presented in the previous samples.

Microbial community of group 2 (G2)

Nagy-borvíz water sample which is characterized by high ammonia content, contained members of *Nitrosomonadaceae* family which are lithoautotrophic ammonia oxidizing bacteria (Rosenberg 2013).

Polányi kút water sample contained important ratio of the family *Gallionellaceae* where its members belong to *Gallionella* and *Sideroxydans* genera. Both are adapted to chemolithoautotrophy (Emerson et al. 2013). Chemistry measurements showed important fraction of iron within this sample.

Hydrogenophilaceae family members, known as chemolithotrophic showed high abundance in Nagy-borvíz, Polányi kút and Berzsenyi water samples - characterized with medium to high SO_4^{2-} content comparing to the other samples – these taxa are using various inorganic electron donors such as reduced sulfuric compounds or hydrogen (Gayner et al. 2018). They can give an insight about their potential role as sulfate reducers. This is endorsed also by the presence of members of sulphur oxidizing bacteria "*sulfuricellaceae*" in the mentioned samples.

Azospirillum genus dominated Kossuth Lajos water sample, it can use NH_4^+, NO_3^-, amino acids and N_2 as nitrogen sources for growth. They can grow under anaerobic conditions using nitrate as electron acceptor, microaerobic (N_2 or NH_3 as nitrogen sources) and fully aerobic conditions with combined nitrogen only (NH_3, NO_3^- and amino acids) (Okon 1985).

Methanogenic archaea were present in Nagy-borvíz, Berzsenyi and Polányi kút water samples with the presence of *Methanoperedenaceae*. They are able to thrive in nutrient-poor and low ionic-strength environments (Bräuer et al. 2015).

The water sample collected from Taploca contained predominantly taxa, such as *Burkholderiaceae*, that have been demonstrated to be capable of functioning as either obligate or facultative chemolithotrophs. (Yavari-bafghi et al. 2023).

Microbial community of group 3 and 4 (G3 and G4)

The dominance of *Altiarchaeia* within the archaeal community of Dandár water samples can be explained also by their adaptation to this environment. Based on literature data, *Altiarchaeia* have evolved specific structural and metabolic features, e.g., developing nanograppling hooks. This anchor allows it to stay stationary on the top of the water despite the water current (Probst et al.

2014). Moreover, their presence is common in anaerobic groundwaters; in addition, relatives of *Altiarchaeia* were found to be widespread in sulfide springs in Europe (Rudolph et al. 2004). Their role is important, often being a carbon dioxide sink (Probst et al. 2014). The presence of *Sphingomonadaceae* and *Rhodocyclaceae* families in the Dandár water sample can be explained by their capability to degrade many substrates. They can possess a variety of metabolic pathways catalyzing various organic compounds, which is an important feature in oligotrophic environments (Oh et al. 2019) (Táncsics et al. 2018). Unclassified members of *Thermodesulfovibrio* were isolated earlier from terrestrial hot springs and deep aquifers. They are able to reduce sulfate, thiosulfate or sulfite (Frank et al. 2016). The families *Pseudomonadaceae*, *Burkholderiaceae* and both the phyla *Omnitrophicaeota* and *Desantisbacteria* were described from different aquifers in previous studies (Probst et al. 2017).

In the Ciprián sample, an important fraction of ammonia-oxidizing archaea was detected (*Nitrosopumilaceae, Nitrosotaleaceae and Nitrososphaeraceae*). Compared to ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA) have the ability to inhabit a wide range of extreme environments (Zhou et al. 2015). This can explain their dominance in nutrient-depleted environments. The sequences assigned to the genus *Flavobacterium* are shown to be widespread in nature including groundwaters, rivers and oligotrophic lakes (Yi et al. 2006). Moreover, many of the *Flavobacterium* species are able to reduce nitrate to nitrite (Bernardet et al. 2015). This can explain their predominance in the Ciprián water sample, which is characterized by a high NO_3^- content. *Rhodobacteraceae* and *Rhodocyclaceae* can overcome oligotrophic conditions by photoheterotrophic metabolism. *Methanoregulaceae, Methanobacteriaceae* and *Methanosarcinaceae* are methanogenic bacteria, and by their metabolism they are able to thrive in nutrient-poor, low ionic-strength environments (Bräuer et al. 2015).

Grouping of the water samples based on their microbial community

Applying the PCA on the different samples based on the OTU datasets showed the existence of three main groups. They were grouped each with distinct microbial communities (Figure 33, Figure 34).

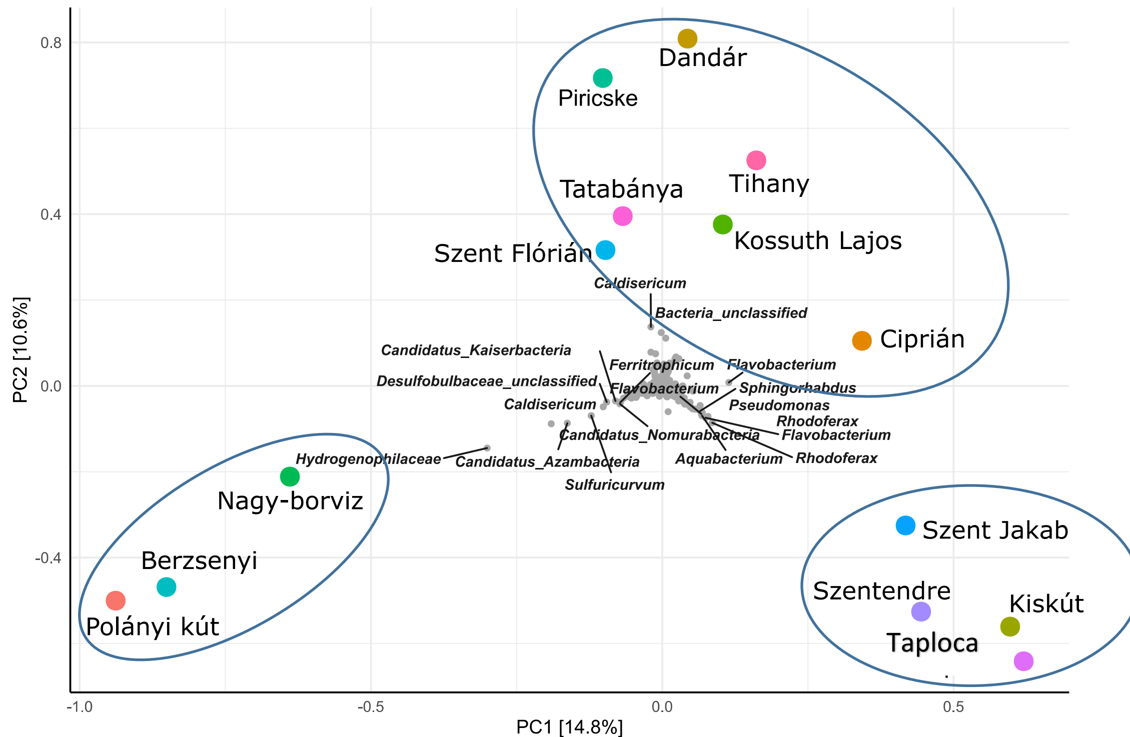


Figure 33. PCA ordination of the water samples based on bacterial community structure and environmental parameters.

The most characterizing genera in Ciprián sample were *Flavobacterium* and *Pseudomonas*, among their members some species are able to use nitrate as an electron acceptor (e.g., *Pseudomonas denitrificans, Brevundimonas denitrificans*), these results endorse the findings at the level of archaea.

Nagy-borvíz, Berzsenyi and Polány Kút were grouped together with the presence of *Hydrogenophilaceae*, these samples are characterized with high conductivity values that can provide chemolithotrophs with the needed electron donors.

Szent Jakab, Szentendre, Kiskút and Taploca grouped together with the presence of microbes commonly found previously in aquatic systems, and oligotrophic aquatic environments as an example *Aquabacterium* (Kalmbach et al. 1999). The rest of the samples had many unclassified bacteria as a grouping taxon.

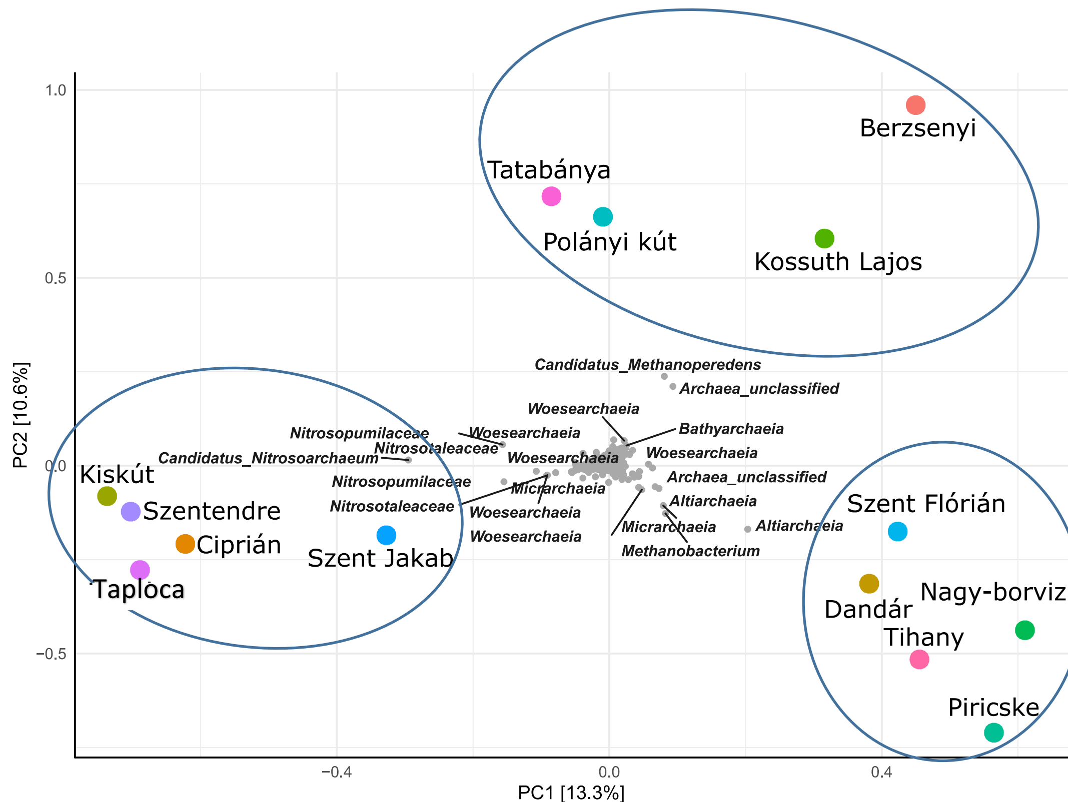


Figure 34. PCA ordination of the water samples based on archaeal community structure and environmental parameters.

In case of the Dandár and Nagy-borvíz groundwaters, the uptake of ions by the water flow from the host rock results in high conductivity. The PCA ordination of the results show that it moves together with the higher presence of many unclassified archaea and *Altiarchaeia*. Previous studies did not determine yet the exact electron donor and acceptors for this archaeon; however, it is assumed to be an autotrophic organism (Perras et al. 2015). This finding highlights the importance of the ions existing in the water as potential electron donors for the many existing unclassified archaea. The rest of the water samples grouping together with the latter two samples, showed many unclassified archaea as well.

Kiskút, Szentendre, Ciprián, Taploca and Szent Jakab were grouped together with a high presence of taxa involved in the nitrogen cycle such as (*Nitrosopumilaceae* and *Nitrosotalea*). At the exception of Taploca water samples, chemical analyses showed that the rest of the samples were characterized with the highest NO_3^- values.

The rest of the samples grouped together with the presence of unclassified archaea, *Woesarchaea*, and *Methanoperedens* in the case of Tatabánya water sample.

5.3. *Microbial community composition based on cultivation*

Genera of *Micrococcus*, *Pseudomonas*, *Bacillus* and *Pseudoxanthomas* are widespread in different aquatic environments and they are shown to survive also in nutrient-depleted conditions using different strategies; e.g. most of these bacteria have the ability to form biofilms (Marsden et al. 2017). Previous literature data showed that many of them have been isolated from an oligotrophic aquifer in West Bengal, and they were characterized by high metabolic flexibility, such as the ability to utilize multiple hydrocarbons and using different electron acceptors (Mohapatra et al. 2018). Though *Bacillus* species are widespread in nature, they are able to produce endospores but often can show extremely slow growth as alternative strategy to survive starvation (Gray et al. 2019). *Sphingomonas* and *Brevundimonas* species also have the ability to survive in low concentrations of nutrients, as well as to metabolize a wide variety of carbon sources (Fegatella et al. 2000) (Barton et al. 2007). *Microbacterium* species are able to convert ammonium to nitrogen under aerobic conditions (Zhang et al. 2013). From the Szentendre water sample, potential nitrogen-fixing bacteria could be isolated, e.g., *Herbiconiux* (Puri et al. 2020), *Rhizobium* (Tilak et al. 2006), *Ensifer* (Rogel et al. 2001) and also ammonia-oxidizing bacteria, e.g. *Prosthecobacter* (Gonzalez-Martinez et al. 2016). In oligotrophic environments, nitrogen fixation can be an important feature due to limited nitrogen sources. In addition, some archaeal OTUs were found, which can be responsible for ammonia oxidization (e.g., *Nitrosopumilaceae*, *Nitrosotaleaceae* and *Nitrososphaeraceae*). *Rhodobacter azotoformans* in the Ciprián water sample is a denitrifying phototrophic bacterium (Hiraishi et al. 1996), and *Fictibacillus* is able to perform ammonification and also iron reduction (Zvb 2017). The Dandár water sample contained members of *Acinetobacter*. Many species of this genus are able to mobilize inorganic phosphate, and so have a key function for nutrient acquisition in these starved ecosystems (Barton et al. 2007). Many of the cultivated species from the Szent Flórián and Tatabánya water samples were isolated previously from groundwaters, suggesting their adaptation to nutrient-depleted environments. The genus *Sphingopyxis* was represented by different species (*S. fribergensis, S. chilensis* and *S. solisilvae*), and it is commonly isolated also from freshwater and marine habitats - many of them

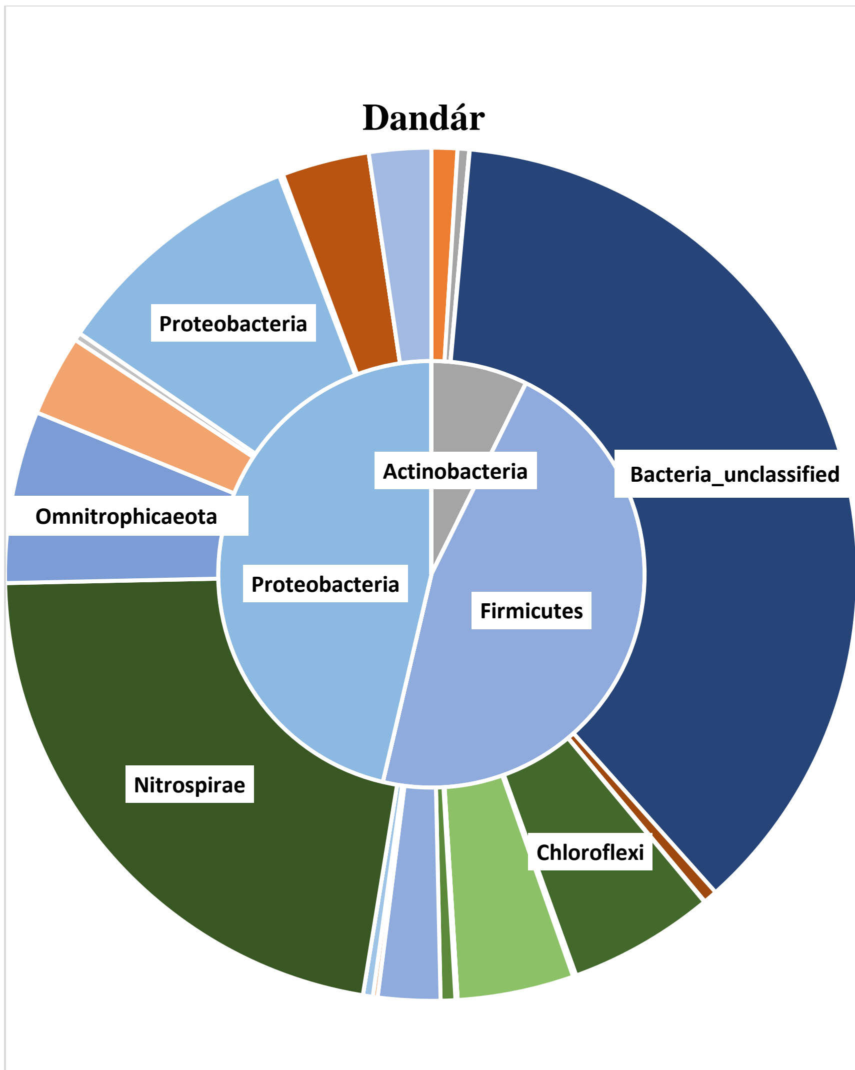
are facultative chemolithotrophs, often producing H_2 during their metabolic processes. These bacteria could also be responsible for the observed gas production.

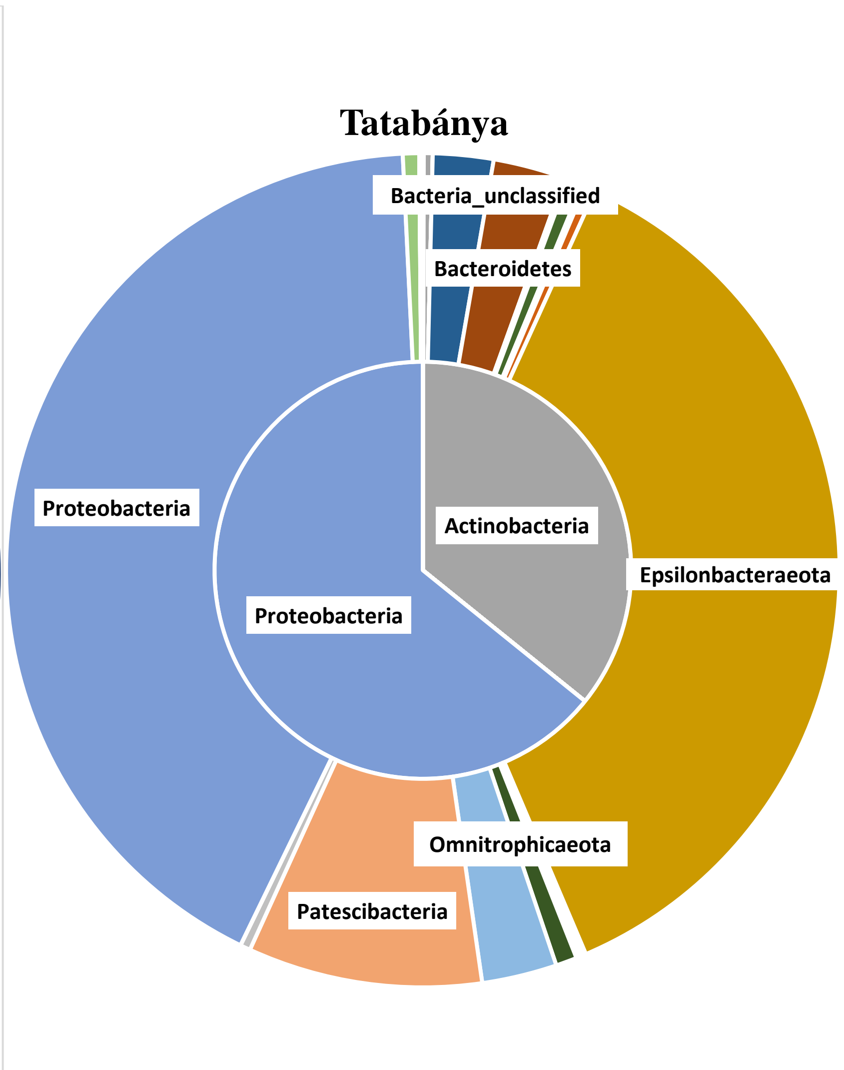
5.4. Comparison between cultivation and amplicon sequencing results

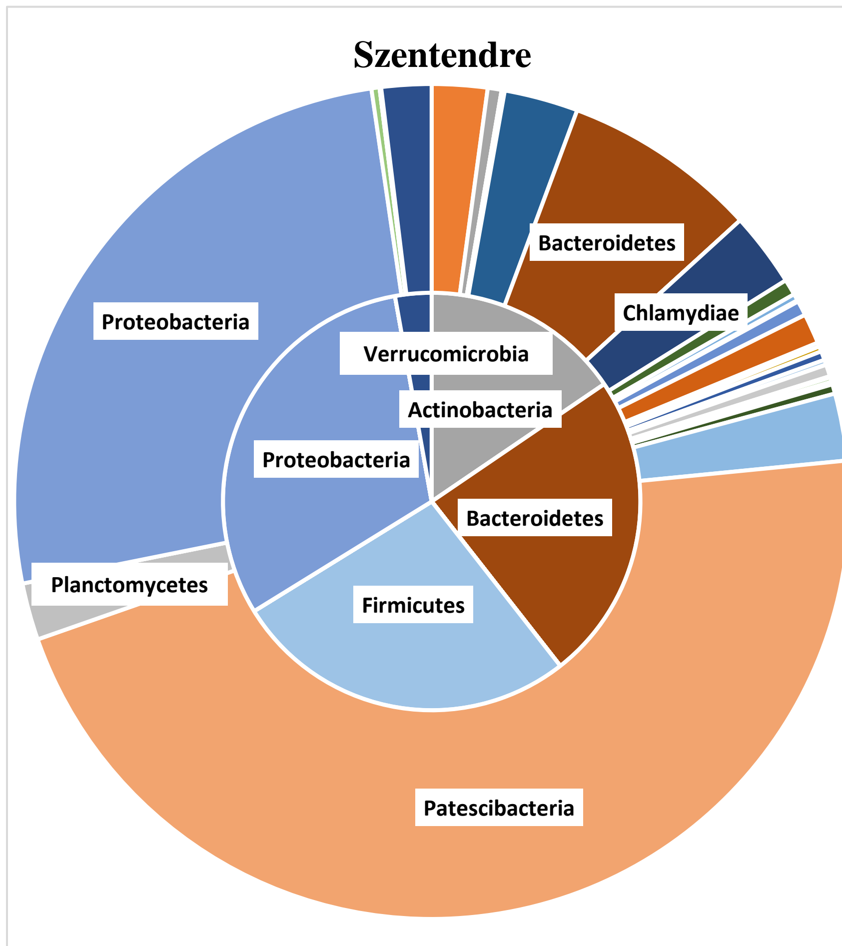
The comparison between the microbial community revealed by amplicon sequencing and cultivation was conducted at the phyla level. The obtained results align with previous findings, comparing the reliability and consistency of both approaches. Amplicon sequencing proved to be highly effective in identifying and characterizing a wide range of microbial taxa, including rare and uncultivable organisms. Conversely, the cultivation method exhibited selectivity and bias towards organisms capable of thriving under controlled laboratory conditions, thereby inadequately representing the diverse variability of microorganisms observed through amplicon sequencing.

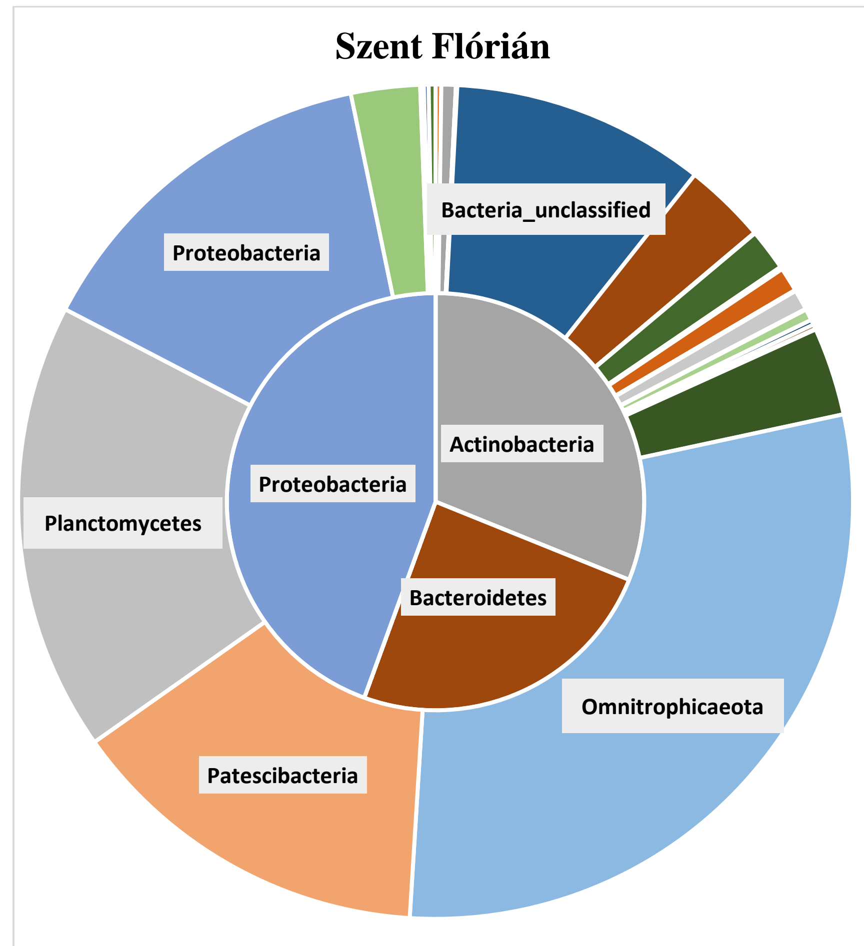
Nevertheless, it was observed that several phyla cultivated in the study were also present in significant proportions within the amplicon sequencing results. Specifically, Proteobacteria, Bacteroidetes, and Actinobacteria were prevalent among both methods. However, a majority of the phyla detected through amplicon sequencing could not be revealed using cultivation-dependent techniques, indicating the limitations of the latter in capturing the full extent of microbial diversity.

The figure bellow (Figure 35) shows the comparison of the obtained phyla using cultivation and amplicon sequencing


Dandár
Bacteria_unclassified
Actinobacteria
Proteobacteria
Omnitrophicaeota
Proteobacteria
Firmicutes
Nitrospirae
Chloroflexi


Tatabánya
Bacteria_unclassified
Bacteroidetes
Proteobacteria
Actinobacteria
Epsilonbacteraeota
Proteobacteria
Omnitrophicaeota
Patescibacteria


Szentendre
Bacteroidetes
Chlamydiae
Proteobacteria
Verrucomicrobia
Actinobacteria
Proteobacteria
Bacteroidetes
Planctomycetes
Firmicutes
Patescibacteria


Szent Flórián
Bacteria_unclassified
Proteobacteria
Actinobacteria
Proteobacteria
Planctomycetes
Bacteroidetes
Omnitrophicaeota
Patescibacteria

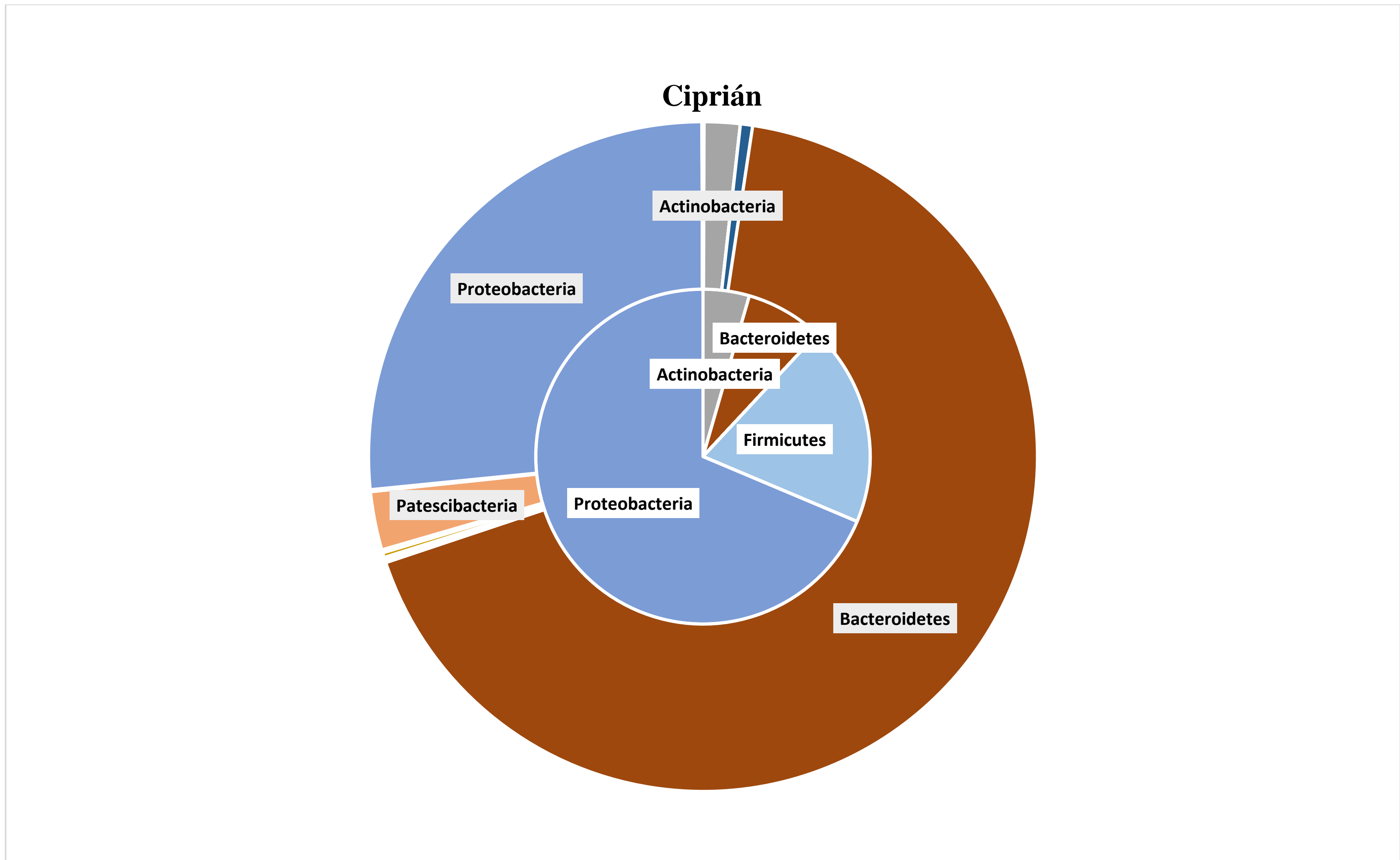


Figure 35. Donut charts comparing the revealed microbial communities in 5 samples at the phylum level using cultivation (inner circle) and amplicon sequencing (outer circle).

5.4. *Bacterial growth in different media - testing of oligotrophic characters*

It is known that bacteria from low nutrient content environments often lose their ability to grow in rich nutrient content circumstances (Schut et al. 1997). In our case, 10 bacterial strains were not able to grow in the presence of higher nutrient content (100% yeast extract) (Table 7). (Flardh et al. 1992) suggested that this is the result of the development of high substrate affinities during nutrient limitations. Based on this, our findings contradict that the limiting factor in the bacterial growth is always the nutrient availability. In fact, the limiting factor is the ability of the cell itself to grow in conditions supporting its growth. Previous literature's data show that a sudden addition of high quantities of nutrient to an organism can lead to rapid death via osmotic swelling (Barton et al. 2004). (Hodgson 2000) described that many members of the genus *Streptomyces* are facultative oligotrophic microorganisms, some species can grow under oligocarbophile conditions. (Semenov 1991) discovered that members of *Prosthecobacteria* have an extremely high affinity for different substrates. *Curvibacter delicatus,* which was characteristic in the Ciprián water sample, was among the bacterial communities that have fouled polyvinylidene fluoride microfiltration membranes, which are used for drinking water treatment (Chon et al. 2009). *Rheinheimera aquatica* was isolated from hot springs from the Jazan region in Saudi Arabia, which is considered as an oligotrophic environment (Yasir et al. 2019). Both *Aquabacterium citratiphilum* and *Aquabacterium commune* were isolated from biofilms of the Berlin drinking water system, where they could resist a severe limitation of low nutrient contents (Kalmbach, Manz, Jörg Wecke, et al. 1999). Different species belonging to the genus *Ferrovibrio* were isolated from a thermal bath in Budapest, where the water contains only limited organic carbon source (Szuróczki et al. 2016). *Ferrovibrio* species are often related to corrosion in different pipelines, while *F. denitrificans* can be responsible also for nitrate reduction (Sorokina et al. 2012). No previous studies reported the cultivation of *Malikia spinosa* and *Salinibacterium hongtaonis* in similar environments. All these findings show that some of the cultivated taxa are true oligocarbophile microorganisms.

6. *Conclusion*

In this study the diversity and abundance of microbial communities in several oligotrophic aquatic environments were investigated. To achieve this, a combination of methods including amplicon sequencing, cultivation, and fluorescent microscopy were employed. The results of the study provided valuable insights into the complex relationships between microbes and their environment. The TOC level in the different water samples divided the samples into 4 groups. Each group was characterized by a unique diversity and composition of its microbial community. Independently of the TOC values and other parameters at each aquatic habitat, the biogeochemical cycles could be completed by various microorganisms.

It was observed that water sample which negatively correlated with TOC values, had higher fraction of autotrophic microbes (e.g. *Hydrogenophilaceae* and *Altiarchaeia)*. However, group 1 and Ciprián water sample (Group 4) which were characterized with higher TOC values, showed a more balanced presence between autotrophs and heterotrophs.

It was also seen that other environmental factors structured the microbial community composition of the samples in a way that they developed unique strategies to obtain necessary nutrients and to survive in these conditions. This was seen by the high presence of nitrifiers in samples with high NO_3^- content, sulphate reducers in samples with high SO_4^{2-} content, and iron oxidisers in the samples characterized with high Fe content.

Our findings (based on using different concentrations of nutrients) indicated that nutrient availability is not the sole factor that impacts microbial growth, as an important bacterial fraction displayed an inability to grow also at high nutrient content conditions. Our experiments had shown that many uncultivable prokaryotes could reside in these low nutrient environments - as evidenced by the discovery of novel bacterial taxa during the study.

7. *Summary*

7.1. The water samples were collected from different oligotrophic aquatic environments in Hungary and in Romania (Transylvania). Based on the dataset of chemical characterisation, two main groups were obtained. First group is composed of the samples: Tatabánya, Piricske, Tihany, Szentendre, Szent Flórián, Kiskút and Szent Jakab. The second group is composed of Nagy-borvíz, Berzsenyi, Taploca, Polányi kút and Kossuth Lajos spring. Dandár and Ciprián water samples made distinct points compared to all the others.

7.2. A general trend was seen within most of the samples, showing that the cell count values are usually lower when the bacterial diversity tend to be higher. Some samples were characterized by the dominance of only few taxa, this is due to their adaptability to the specific environment conditions.

7.3. Amplicon sequencing could identify bacteria and archaea involved in the different biogeochemical cycles. The study found ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) within the nitrogen cycle involved microorganisms, while sulphur/sulphide oxidizers were identified in terms of sulphur cycling. The presence of organisms involved in the iron cycle was also detected. Furthermore, the study characterized the existing microorganisms in the different groups, revealing a variety of metabolic types that enable them to thrive in such conditions.

7.4. The microbial community composition based on cultivation revealed the existence of microbes characterized by different strategies that enable them to survive in nutrient-depleted conditions.

7.5. An important fraction of the isolated bacterial strains was not able to grow in the presence of higher nutrient content. these findings show that some of the cultivated taxa are true oligocarbophile. They have evolved to thrive in nutrient-poor conditions, and exposure to high levels of nutrients can be toxic or inhibit their growth.

7.6. Many previously uncultivated heterotrophic bacteria were cultivated. Among them, a new genus was described based on its genomic, phenotypic, and chemotaxonomic characteristics.

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8. *Appendix*

Table S1. Major cellular fatty acids [%] of strain SG_E_30_P1 (1) and its closest relatives Leifsonia psychrotolerans (2) and Galbitalea soli (3). Fatty acids with values < 1% are not shown. ND – not detected.

Fatty acid	1	2	3
C14:0 iso	9.7	<1	<1
C15:1 anteiso ω10c	ND	4.3	ND
C15:0 iso	4.4	1.1	6.4
C15:0 anteiso	34.5	70.2	56.3
C16:0 iso	39.4	4.1	2.2
C16:0	4.7	1.2	3.7
C17:0 iso	1.2	<1	1.3
C17:0 anteiso	4.5	18.6	28.6

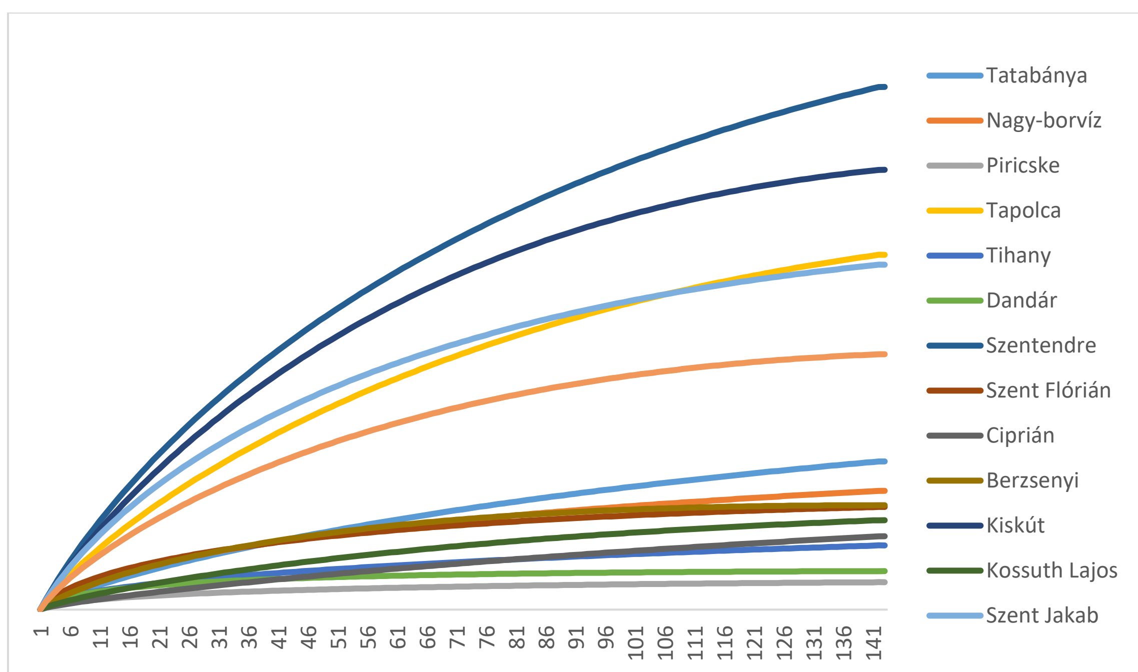

Figure S1. Rarefaction curves of bacterial OTUs of the samples based on 16S rRNA gene amplicon sequencing.

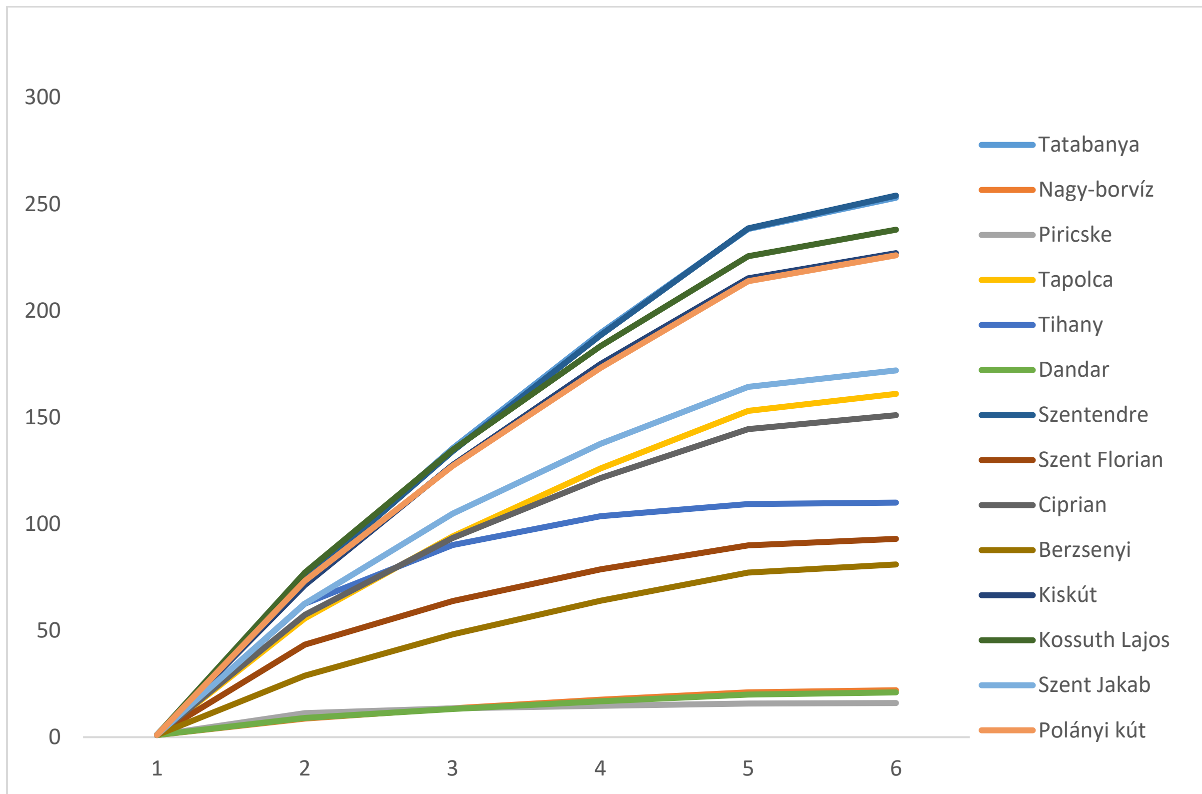

Figure S2. Rarefaction curves of archaeal OTUs of the samples based on 16S rRNA gene amplicon sequencing.

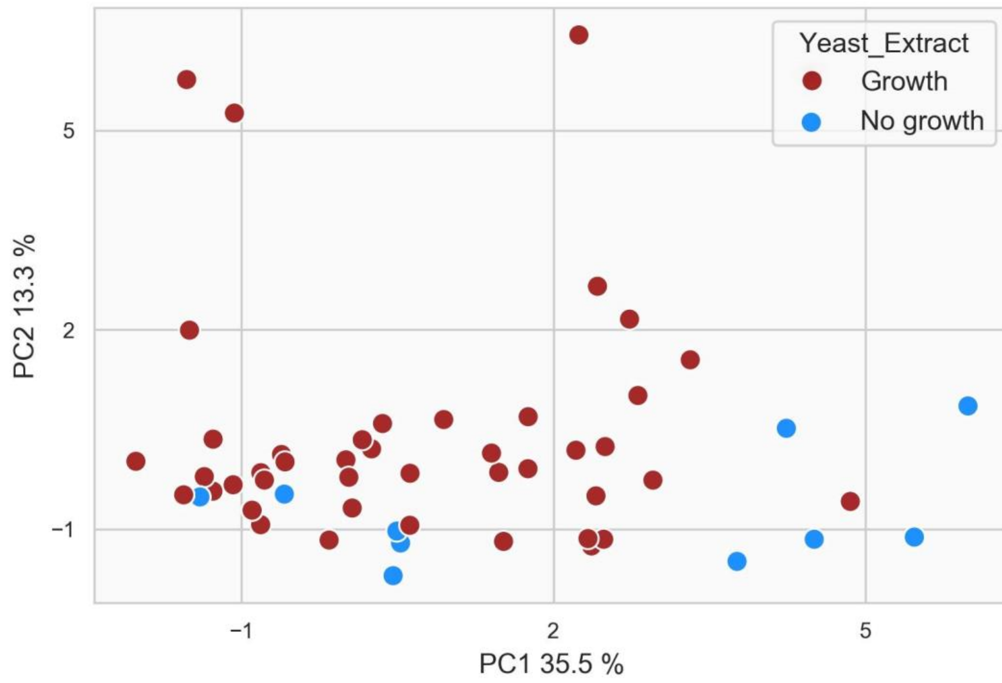

Figure S3. PCA ordination of the bacterial strains' growth in 100% yeast extract medium.