

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

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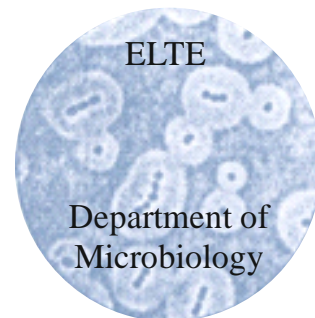
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I. Introduction and objectives

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. 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.

II. Material and Methods

The current study employed a comprehensive sampling approach starting from 2017 to 2021 to investigate various aquatic oligotrophic environments. The investigation focused on determining physical and chemical parameters (Temperature, pH, conductivity, TOC, NO_3^- , SO_4^{2-} , Fe). Additionally, the study employed both cultivation-independent methods, including total cell count using an epifluorescent microscope on a 200 ml from each sample after being filtered and fixed, and amplicon sequencing using the total DNA extracted from 250 ml of water sample after filtration. As well as cultivation-dependent methods. The bacterial strains were isolated after direct spreading of water samples and after enrichment using a newly developed medium called M5.

The isolates 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 1).

Finally, a polyphasic approach incorporating morphological analysis, biochemical profiling, and molecular genetics, was used in order to describe a new bacteria genus.

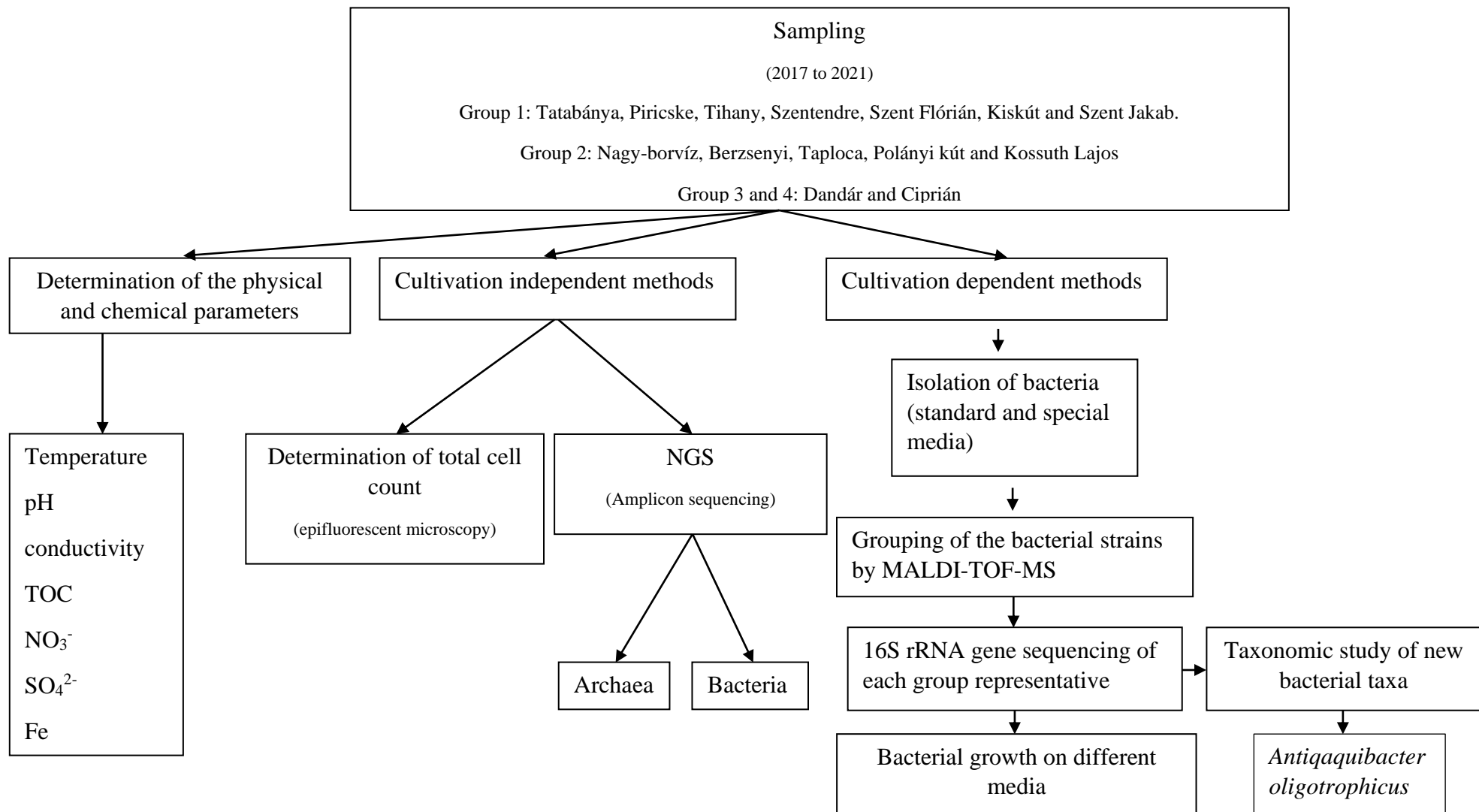


Figure 1. Graphic workflow showing the different steps of the used materials and methods

III. Results and Discussion

III.1. Microbial communities' composition based on amplicon sequencing

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 2, Figure 3).

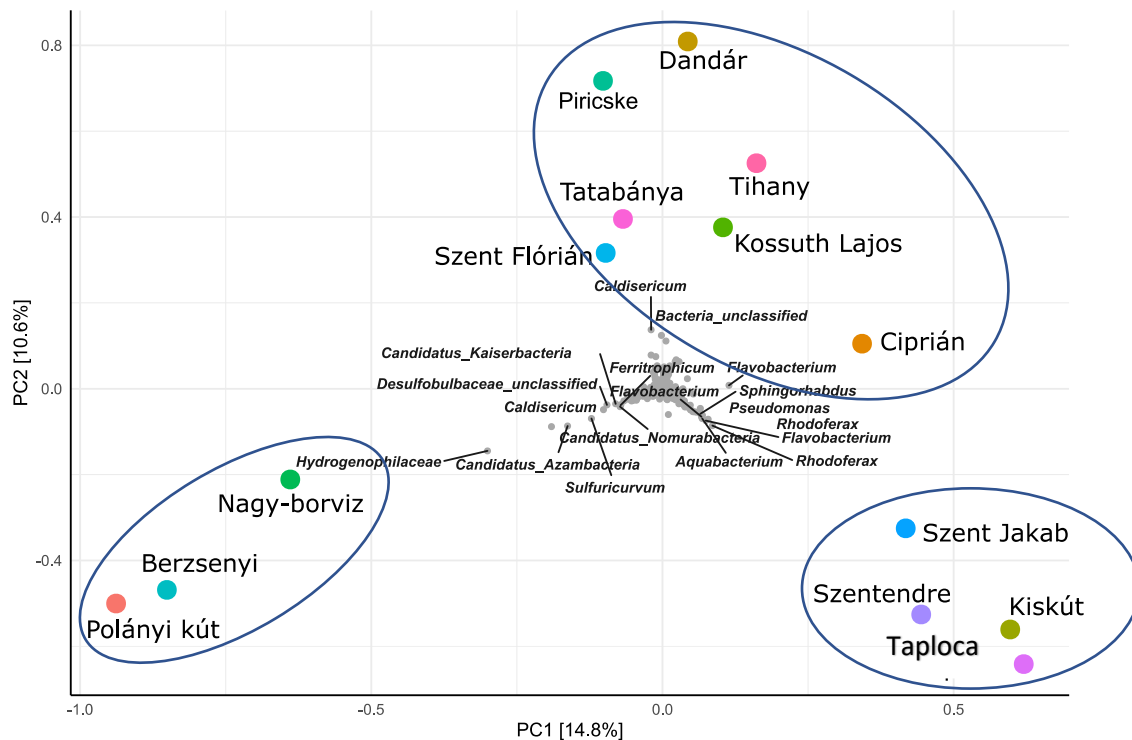


Figure 2. PCA ordination of the water samples based on bacterial community structure and environmental parameters.

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, Berzsényi and Polányi 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.

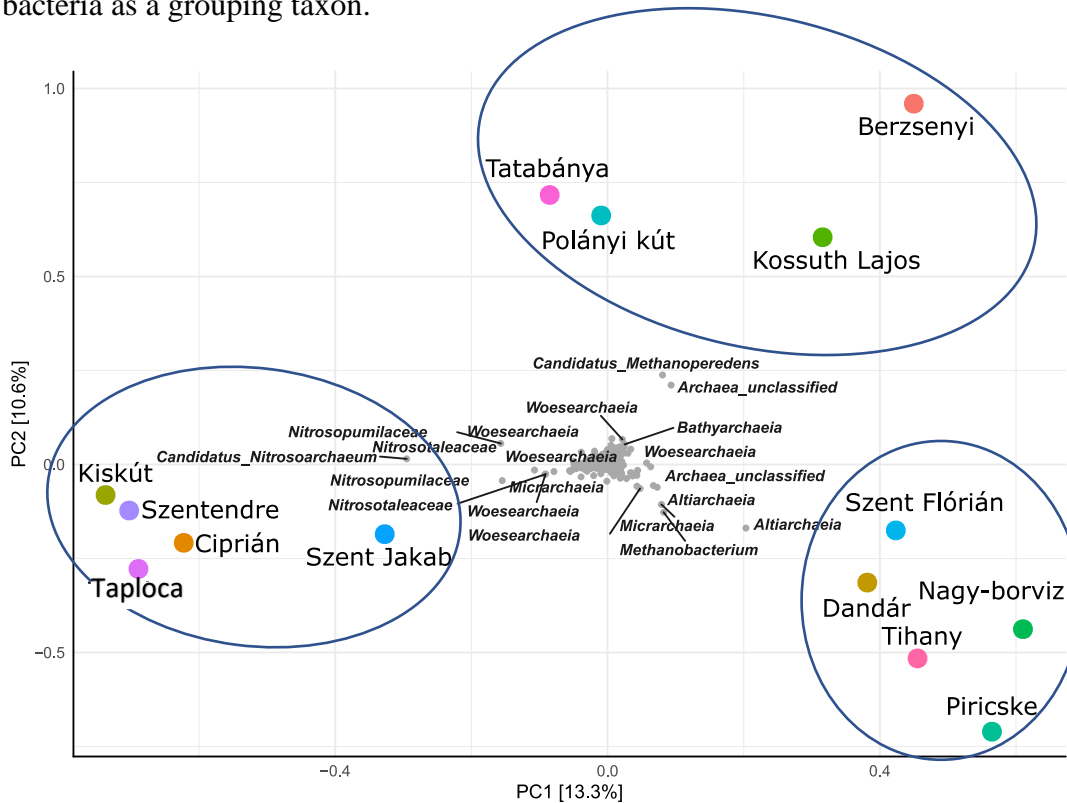


Figure 3. PCA ordination of the water samples based on archaeal community structure and environmental parameters.

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.

III.2. Microbial community composition based on cultivation

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%). 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 chemolithotrophic 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 4.

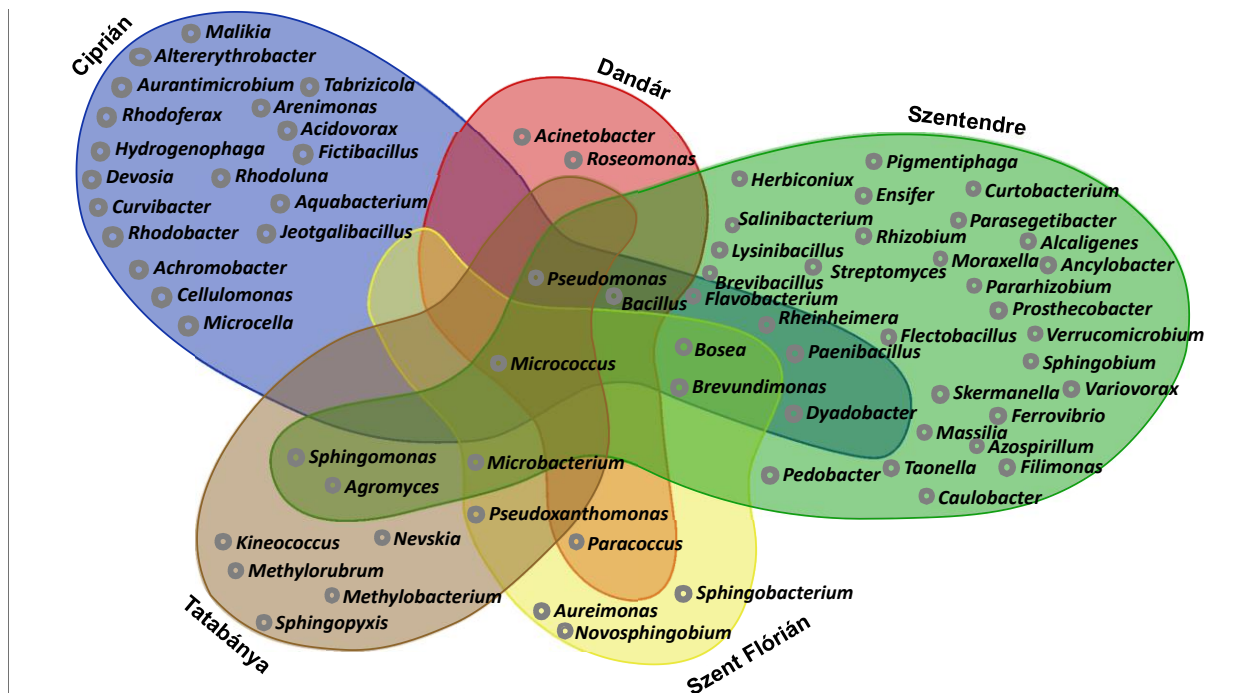


Figure 4. Distribution of the cultivated taxa among the water samples.

Genera of *Micrococcus*, *Pseudomonas*, *Bacillus* and *Pseudoxanthomonas* are widespread in different aquatic environments and they are shown to survive also in nutrient-depleted conditions using different strategies. 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. *Prostheco bacter* (Gonzalez-Martinez et al. 2016). *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 H₂ during their metabolic processes.

III.3. Polyphasic approach to study new bacterial strain isolated from the water sample of Szentendre

A Gram-stain-positive, non-motile, oxidase- and catalase-negative, rod shaped, bacterial strain SG_E_30_P1T with lightly yellow-colored colonies was isolated from the groundwater sample of Szentendre, Hungary. Based on 16S rRNA phylogenetic and phylogenomic analyses, the strain forms a distinct lineage within the family *Microbacteriaceae*. Its closest relatives based on near full-length 16S rRNA gene sequences are *Salinibacterium hongtaonis* MH299814 (97.72 % sequence similarity) and *Leifsonia psychrotolerans* GQ406810 (97.57%). The novel strain grows optimally at 20 to 28 °C, at neutral pH with the presence of NaCl (1-2 w/v%). Strain SG_E_30_P1T contains MK 7 and B-Type peptidoglycan with diaminobutyrate (DAB) as the diagnostic amino acid. The major cellular fatty acids are anteiso-C15:0, iso-C16:0 and iso-C14:0, the polar lipid profile is composed of diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG) as well as of an unidentified aminoglycolipid (AGL), aminophospholipid (APL) and some unidentified phospholipids (PL).

The assembled draft genome is contig with a total length of 2,897,968 bp and a DNA G+C content of 65.55 %. An amino acid identity (AAI) of <62.54 to its closest relatives with sequenced genomes and other genome distance measures indicate that this bacterium represents a novel genus within the family *Microbacteriaceae*. We suggest SG_E_30_P1T (=DSM 111415=NCAIM B.02656) represents the type strain of a novel genus and species for which the name *Antiaquibacter oligotrophicus* gen. nov., sp. nov. is proposed.

IV. Thesis points

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,

Berzsényi, Taploca, Polányi kút and Kossuth Lajos spring. Dandár and Ciprián water samples made distinct points compared to all the others.

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.

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.

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.

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.

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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