
Experimental Plant Biology Program
Doctoral School of Biology
Faculty of Science, Eötvös Loránd University

ANIKÓ MENTES

**Comparative microbiological analysis of shallow lakes
dominated by decomposing plant substances**

-PH.D. THESIS BOOKLET-

Supervisor:
Dr. Tamás FELFÖLDI, PhD
habil. assistant professor

Head of the Doctoral School:
Prof. Anna ERDEI, DSc
university professor

Doctoral Program Leader:
Dr. Gábor KOVÁCS M, PhD
habil. associate professor



Department of Microbiology
Eötvös Loránd University, Institute of Biology
Budapest, Hungary
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Introduction and the aims of the study

Shallow lakes form an individual group of continental water bodies, compared to great lakes and seas, they are more complex and active in almost all cases [1,2]. The nutrient transfer between the sediment and the water column is continuous in these lakes due to their shallowness and wind-induced mixing [3]. Aquatic plants (macrophytes) can reduce the mixing; in this way, they play an important role in the life of shallow lakes [3].

Macrophytes have further direct and indirect negative effect on phytoplankton [4], but in general they have a positive effect on bacterioplankton [5]. Humic substances (also known as coloured dissolved organic substances, CDOM) can be formed during the process of plant detritus humification, and they give the brown colour of the lakes. Despite their persistent nature, CDOM could be the primary energy and carbon source for some bacteria [6]; besides, these compounds are essential regulators of the nutrient cycles in the shallow waters through their direct/indirect impact and their buffering capacity [7].

Understanding the biogeochemical cycles in globally significant shallow lakes, as described above, it includes uncovering of the composition and role of the planktonic microbial communities living there, as well as the environmental factors affecting them. The shallow lakes of Hungary and Romania have been intensively studied in many cases, although the microbiological information is relatively scarce or studies were performed using only low-throughput methods and with methods having low resolution. Most of the studied lakes can be found in a special and protected environment, which carries the significant ecological role of these lakes and the possibility of the presence of unique bacterial communities.

Taking these into consideration, the following points were set as the aims of the PhD work:

1. I looked for a relation between the CDOM content and bacterial production as well as bacterial metabolic potential; I have examined, if there is a continuous change in the taxonomic composition of algal and bacterial communities which can be associated with increasing CDOM content; furthermore, if CDOM could have an effect on the diversity of planktonic algae and bacteria. Does the amount of plant-derived, coloured organic matter affect the composition, diversity, and activity of the planktonic microbial community?

1 Meerhoff and Jeppesen. *Encyclopedia of Inland Waters*. 2009, 645–55.

2 Cooke *et al.* *LakeLine* 2001; Spring:42–6.

3 Scheffer. *Ecology of shallow lakes*. Springer, Dordrecht, 2004.

4 Guo-feng *et al.* *Journal of Pollution Effects & Control* (2000): 1-5.

5 Kalff and Rooney. *Ecosystems* 2003;6:797–807.

6 Moran and Hodson. *Limnol Oceanogr* 1990b;35:1744–56.

7 Steinberg. *The Lakes Handbook*, 2005, 153–96.

2. Is there any horizontal or vertical shift in the composition of the bacterioplankton and phytoplankton communities in the same lake if similar chemical and physical parameters are present (moderate CDOM content)? I looked for correlations between the quality of macrophytes and the composition of the bacterial and algal communities. How does the type of macrophytes affect the composition of the planktonic microbial community?
3. I have aimed the polyphasic description of two new Rhizobiales species, since it was revealed recently that the members of this order are important components of the freshwater planktonic bacterial communities in several cases. Some bacteria belonging to this group were also found in a notable proportion of the studied water samples; therefore, I performed a comparison with the two new species and Rhizobiales members of the studied lakes using phylogenetic methods. I aimed to determine the significance of planktonic Rhizobiales bacteria in shallow lakes, with particular attention to the water bodies dominated by decomposing vegetation.

Materials and methods

My research work relied on the tools of classical and molecular microbiology, and also on limnological methods, as it is usual in the case of microbial ecological studies.

Seven natural water bodies were studied during the doctoral research, which are located at different points in the Carpathian Basin and differ in physical and chemical parameters, moreover varying in their CDOM content, and included the following lakes: the most well-known lake of Central Europe (Lake Balaton), one of the protected freshwater marshes of Hungary (Lake Kolon), soda lakes of the Kiskunság National Park having multiple extreme environmental conditions (Zab-szék, Sós-ér), the largest soda lake in Hungary (Fertő), as well as an acidic peat-bog and a highland freshwater lake in a twin crater of the Romanian Harghita County (Mohos, Lake St. Ana).

The main environmental parameters, the phytoplankton and bacterioplankton communities of these lakes were investigated in a total of 19 water samples collected in the autumn of 2014 (macrophyte sample series) and the summer of 2015 (CDOM sample series).

Within the framework of my doctoral research, in parallel with these studies, two Rhizobiales bacteria were isolated during sampling from Lake St. Ana in 2012, which were examined and described by using polyphasic taxonomy methods.

Determination of environmental variables

In the course of the data analysis, the on-site [temperature, pH, conductivity, dissolved oxygen concentration (DO)] and the laboratory physical and chemical data [nutrient content, e.g. total phosphorus (TP), soluble reactive phosphorus (SRP), total nitrogen (TN), total organic carbon (TOC), dissolved organic carbon (DOC), chlorophyll *a* content (Chl)] of the studied lakes were used as well as the microscopic results [e.g. total bacterial cell count, phytoplankton, algal biomass].

Activity methods

The Biolog EcoPlate™ rapid diagnostic method was used to map the biochemical activity of the bacterial communities. The measurement of bacterial production was achieved by the tritium-labelled leucine uptake-based technique [8,9].

Culture-based techniques

After isolation, the new Rhizobiales species were maintained on a modified R2A medium which contained half of the carbon sources specified in the original description [10], thereafter during the further analyses [e.g. cell and colony morphology, tolerance tests, mobility, enzyme activity, metabolic tests (API), fatty acids and polar lipid composition, genomic DNA G+C content analysis] [11], the SA-276^T strain showed more efficient growth in YMA as well as in *Rhizobium* agar, while the SA-279^T strain was still studied on a modified R2A medium.

DNA-based methods

The entire genomic DNA from the water samples was extracted by using the UltraClean Water DNA Isolation Kit (MoBio), and the V3-V4 region of the 16S rRNA gene was amplified by universal bacterial primers to determine the composition of the bacterial communities

8 Kirchman *et al.* *Appl Environ Microbiol* 1985;49:599-607.

9 Gasol. <http://www.cmima.csic.es/pub/gasol/Manuals/ProdBact/Leucine.htm>; 1999.

10 DSMZ. www.dsmz.de

11 Felföldi *et al.* *Int J Syst Evol Microbiol* 2014;64:1920-1925.

[12,13]. For the CDOM sample series, it was performed by MiSeq (Illumina) Dual Index amplicon sequencing, while for the macrophyte sample series, it was performed by GS Junior (Roche/454 Life Sciences) pyrosequencing. Isolation of DNA from the newly described bacterial strains (SA-276^T and SA-279^T) was performed by alkaline lysis using NaOH solution [14], and the 16S rRNA gene sequence of the strains was determined by Sanger sequencing [15].

Data processing

The next-generation sequencing (NGS) results were processed using mothur, uChime, and the SINA database following the protocol of Szabó *et al.* 2017 [16]. Phylogenetic analysis was performed by using the EzTaxon database, SINA and MEGA 7.0 software. Further statistical analyses were performed with PAST3 and with scripts written in R and Python3 languages, using already existing libraries.

Results and discussion

Some of the studied samples had greater than 100 mg l⁻¹ CDOM content, therefore these sites are considered to be highly coloured lakes [17], while the CDOM content in rest of the samples was considerably below this value. Lake St. Ana, the open-water sites of Fertő and Lake Kolon was characterized by less than 100 mg l⁻¹ of CDOM, which showed that samples from different parts of the same lake might be different.

The water samples also differed in their trophic state (from oligotrophic to hypertrophic) according to the modified OECD classification system, which takes into account the TN, TP, Chl, TOC and Secchi depth [18]. The two sample series were comparable through the water samples of Lake Kolon. The summer samples of Lake Kolon had higher amount of DO, TP, and algal biomass, but they had notably lower CDOM content than the autumn samples.

In the case of both sample series, the phytoplankton results confirmed that open water sites are distinct from the sites with remarkable macrophyte stands in many ways. In the

12 Klindworth *et al. Nucleic Acids Res* 2013;41:e1.

13 Apprill *et al. Aquat Microb Ecol* 2015;75:129-137.

14 Birboim and Doly. *Nucleic Acids Res* 1979;7:1513-23.

15 Máthé *et al. Extremophiles* 2014;18:501-514.

16 Szabó *et al. Extremophiles* 2017;21:639-649.

17 Hessen and Tranvik. *Ecological Studies* 1998;333-42.

18 Grochowska *et al. J Element.* 2016.

presence of extreme environmental factors, the phytoplankton community was characterized by the dominance of a single algal species, such as the exclusive dominance of chrysoflagellates in the anoxic bladderwort-dominated sample of Lake Kolon, and the *Anabaena spiroides* which dominated in the Sós-ér sample. Members of the phytoplankton belonged mainly to the habitat types group which was typical for the studied water sample [19] but the Reynolds classification of the phytoplankton has to be handled with restrictions [20], since the occurrence of a functional group can be influenced by several physicochemical factors and the interactions between the organisms [21].

Positive significant correlations were found between the bacterial production rate and TN, TP, SRP, TOC, DOC, CDOM, and Chl. These correlations have complemented previous observations that bacterial production may be affected by algal biomass [22,23], nutrients (phosphorus, nitrogen forms) and salt concentration [24,25]. However, there was no significant correlation among the value of the average well colour development from the bacterial substrate utilization method, the number of utilized carbon sources and the CDOM content of the lakes.

The bacterial communities of the studied lakes utilized the five types of carbon sources in approximately equal rate, except the bacteria in the open-water sample of Fertő, where the utilization of polymers was preferred. During the summer period, prolonged sunlight results in a relatively high availability of organic acids through the photodegradation of humic matter [26]. Probably this is why heterotrophic bacteria utilizing small organic molecules were common in the samples. However, the carbon source utilization profile is an *in vitro* technique that reveals the inherent metabolic characteristics of the environment only partially. Many carbon sources of EcoPlate™ might be absent from the natural environment, or some natural carbon sources might be missing from EcoPlate™. Therefore, the metabolic processes which occur in the environment and the properties revealed by EcoPlate™ are only partially overlapping [27].

The composition of the bacterial communities in the CDOM sample series was different among the samples, both at phylum and genus level, however, in the macrophyte sample series, the bacterioplankton was similar in water samples which were dominated by the same type of macrophyte. All samples were dominated by members of phyla Proteobacteria, Actinobacteria

19 Reynolds *et al.* *J Plankton Res* 2002;24:417–428.

20 Padišák *et al.* *Hydrobiologia* 2008;621:1–19.

21 Maileht *et al.* *Hydrobiologia* 2012;704:115–26.

22 Erikson *et al.* *Hydrobiologia* 1998;382:27–39.

23 Eronen-Rasimus *et al.* *ISME J* 2017;11:2345–55.

24 Soares *et al.* *Microb Ecol* 2017;76:144–55.

25 Maresca *et al.* *Appl Environ Microbiol* 2018;84.

26 Bertilsson and Stefan. *Limnol Oceanogr* 1998;43:885–95.

27 Comta and del Giorgio. *Environ Microbiol* 2009;11:1704–16.

and Bacteroidetes. Only two Proteobacteria classes (alpha and gamma) were present in the bacterioplankton of the CDOM sample series, but four Proteobacteria classes (alpha, beta, gamma, delta) were found in the macrophyte sample series, showing the difference in their abundance among the sampling sites. Some samples were characterized by the presence of ubiquitous marine bacteria, which may even occur in freshwaters, such as Actinobacteria from hgcI clade [28] and CL500-29 [29]. We observed that some detected bacterial taxa were characteristic for a particular type of environment. The acidophilic Acetobacteraceae and Acidimicrobiia taxa were common in the acidic peat bog, as well as alkalophilic bacteria (e.g. Nitriliruptoraceae, *Roseinatrobacter*) had high relative abundance in soda lakes. Various methanotrophic/methylotrophic microorganisms were found in Lake Kolon (e.g. *Methyloparacoccus*, *Crenothrix*, *Methylobacter*, *Methylocaldum*). In the water samples, several bacterial taxa were detected that could use decomposing plant substances or CDOM for their metabolic processes (e.g. *Mucilagnibacter* [30]; Phycisphaeraceae [31]). For example, the members of the *Polynucleobacter* genus can utilize the photodegradation products of CDOM in humic-rich habitats [32], while Flavobacteria are capable of degrading certain macromolecules [33].

Based on the principal component analysis of the CDOM sample series, community separation could not be explained by the CDOM content of the samples, but with differences in the pH and nutrient content, which suggested that the composition of the bacterioplankton and phytoplankton could be strongly influenced by the regional characteristics, physical and chemical parameters of the studied lakes. The effect of CDOM was negligible on microbial community composition, although it was significant on bacterial production, despite of the mentioned differences in the physical and chemical parameters of the water bodies.

Based on the principal component analysis and the cluster analysis of the macrophyte sample series, differences in bacterial communities suggested that, macrophytes determined the composition of the bacterial communities living there. Despite similar environmental parameters, the horizontal differences observed in the bacterioplankton of Lake Kolon could be explained by the presence of different type of macrophyte stand. The nutrient content of the dominant species of the studied macrophyte stands also differed significantly: the bladderwort has the lowest C/N and C/P ratio of the prevailing aquatic plants. On this basis, it can be

28 Tang *et al.* Can J Microbiol 2015;61:22–31.

29 Liu *et al.* Front Microbiol 2015;6:64.

30 Pankratov *et al.* Int J Syst Evol Microbiol 2007;57:2349–54.

31 Ntougias *et al.* Microbes Environ 2016;31:401–9.

32 Jezberová *et al.* Env Microbiol 2010;12: 658–669.

33 Krieg *et al.* Bergey's Manual of Systematic Bacteriology, Springer Science & Business Media, 2011.

assumed that the bladderwort has a higher decay ratio than the other two dominant aquatic plants (water lily and reed) [34]. The presence and the absence of macrophytes is also an essential factor since macrophytes have a significant direct and indirect effect on the local aquatic ecosystem [35,36,37,38,39]. The anoxic sample of the bladderwort-dominated site was segregated regarding its phytoplankton and bacterioplankton composition from the other Lake Kolon sample due to the vertical difference caused by DO and light intensity within the sampling site.

In the case of the CDOM sample series, the open-water sites of the studied lakes were characterized by higher species richness, and in the case of the macrophyte sample series, the diversity of the open-water site of the studied lake was also higher. These can be explained by the lack of macrophytes since there are more wind-induced turbulent flow and sediment resuspension in the open-water site of the shallow lakes [40]. Thus, the resuspended sediment particles often contribute to high spatial and chemical heterogeneity [41]. Generally, the open-water sites were characterized by higher species number and diversity than macrophyte-dominated sites. In humic ponds, the presence of different carbon sources (e.g. CDOM) can increase [42], while the higher phosphorus content can reduce bacterial diversity [43], which was well reflected by the low diversity values observed in the case of the Sós-ér sample. The very high or low CDOM content of the samples similarly negatively affected the bacterioplankton and phytoplankton.

In the water samples, many genera contributed with less than 5% relative abundance to the community composition, which refers to high bacterial diversity. For the CDOM sample series, 30 taxa in the bacterioplankton community had more than 5% ratio individually, from which 18 taxa did not have any cultivated representative yet. While in the macrophyte sample series, 18 taxa contributed with at least 5% to the bacterioplankton community and half of them was uncultivated. It has shown that several important but still uncultivated bacterial taxa were found in the studied water samples with lacking information about their metabolism.

Two new Rhizobiales (Alphaproteobacteria) species were described by polyphasic taxonomic methods in parallel with the sample series studies: *Siculibacillus lacustris* gen. nov.,

34 Enríquez *et al.* *Oecologia* 1993;94:457–71.

35 Huss and Wehr. *Microb Ecol* 2004;47:305–15.

36 Wu *et al.* *Environ Microbiol* 2007;9:2765–74.

37 Hempel *et al.* *Aquat Microb Ecol* 2009;58:79–94.

38 Zeng *et al.* *Appl Environ Microbiol* 2012;78:177–84.

39 Zhao *et al.* *Can J Microbiol* 2013;59(4):237–244.

40 Jeppesen *et al.* The structuring role of submerged macrophytes in lakes. *USA, Germany: Springer Science & Business Media*, 2012.

41 Simon *et al.* *Aquat Microbiol Ecol* 2002;28:175–211.

42 Sigeo. *Fresh water microbiology. John Wiley & Sons*, 2004.

43 Margalef. *Temporal succession and spatial heterogeneity in phytoplankton. University of California press*, 1958.

sp. nov. and *Rhizobium aquaticum* sp. nov. The Rhizobiales order contains more than 15 families, many well-known genera are pathogenic to humans and animals (e.g. *Bartonella*, *Brucella*), or they are capable of symbiosis with plants (e.g. *Rhizobium*) or they live in soil (e.g. *Nitrobacter*) and some of them could be also found in wastewater treatment bioreactors (e.g. *Chelatococcus*). However, it has recently unravelled that some taxa of Rhizobiales can play a remarkable role in the bacterioplankton in freshwater and marine environments [44,45].

The NGS results showed that in some of the studied water samples, the Rhizobiales bacteria contributed significantly to the composition of the planktonic bacterial community. Rhizobiales OTUs were found in almost all studied water samples, and compared to the newly described species, OTUs from the macrophyte sample series showed distant relatedness with *Rhizobium aquaticum* (family Rhizobiaceae), while OTUs from the CDOM sample series showed a distant relationship with *Siculibacillus lacustris* (family Ancalomicrobiaceae). Based on the phylogenetic analysis, OTU representatives of the two sample series classified into 12 cultivated Rhizobiales families, but many type species were not linked to a family with a valid name yet. Most of the OTUs from the two sample series were related to members of the Methylocystaceae and Beijerinckiaceae families, while most of the OTUs originate the Beijerinckiaceae and the Hyphomicrobiaceae family. According to our results, there were several Rhizobiales bacteria present in the bacterioplankton of the studied lakes, which can be classified into a new family which is not cultivated yet. Based on these findings, Rhizobiales bacteria represent a diverse but lesser-known component of the planktonic microbial communities in shallow lakes.

44 Liu *et al.* *Int J Syst Evol Microbiol* 2015;65:4449–54.

45 Sheu *et al.* *Int J Syst Evol Microbiol* 2015;65:472–8.

Theses of the PhD dissertation

The knowledge is scarce about the composition of the bacterioplankton community in the Hungarian shallow lakes and the environmental factors which affect them. According to the literature, the CDOM content and the macrophytes of the shallow lakes can be important factors for these microbial communities. Our conclusions based on our results presented in the dissertation could be summarized in the following thesis points:

1. there is a linear relationship between the bacterial production and the nutrient, DOC, CDOM content of the shallow lakes;
2. there is not any significant correlation between the metabolic potential of the bacterioplankton communities and the CDOM content of the shallow lakes;
3. some physical and chemical parameters of the shallow lakes have a more significant effect than CDOM on the composition of bacterioplankton and phytoplankton communities;
4. the composition of bacterioplankton in shallow lakes is determined by the type of macrophyte when a remarkable amount of macrophyte stands is present;
5. Rhizobiales taxa are important, but a little-known component of the bacterial diversity in the planktonic communities of shallow lakes;
6. two new Rhizobiales species and a new genus have been described using polyphasic taxonomy: *Siculibacillus lacustris* gen. nov., sp. nov. and *Rhizobium aquaticum* sp. nov.

Publications

The dissertation is based on my results published in these papers

- Felföldi T, Márton Zs, Szabó A, **Mentes A**, Bóka K, Márialigeti K, Máthé I, Koncz M, Schumann P, Tóth E. *Siculibacillus lacustris* gen. nov., sp. nov., a new rosette-forming bacterium isolated from a freshwater crater lake (Lake St. Ana, Romania). *Int J Syst Evol Microbiol* 2019 (in press). doi: 10.1099/ijsem.0.003385 (IF 2017: 1.932)
- Máthé I, Tóth E, **Mentes A**, Szabó A, Márialigeti K, Schumann P, Felföldi T. A new *Rhizobium* species isolated from the water of a crater lake, description of *Rhizobium aquaticum* sp. nov. *Antonie van Leeuwenhoek Int J Gen Mol Microbiol* 2018;111: 2175–2183. doi: 10.1007/s10482-018-1110-0 (IF 2017: 1.588)
- Mentes A**, Szabó A, Somogyi B, Vajna B, Tugyi N, Csitári B, Vörös L, Felföldi T. Differences in planktonic microbial communities associated with three types of macrophyte stands in a shallow lake. *FEMS Microbiol Ecol* 2018;94:fix164. doi: 10.1093/femsec/fix164 (IF 2017: 3.495)
- Mentes A**, Szabó A, Jurecska L, Tugyi N, Somogyi B, Csitári B, Vörös L, Boros E, Felföldi T. Az Izsáki Kolon-tó mikrobiológiai felmérése. = Microbiological survey of Lake Kolon (Izsák, Hungary). *Hidrol Közl* 2016;96:59-63. (in Hungarian)
- Mentes A**, Tóth E, Kéki Zs, Kosztik J, Márialigeti K, Máthé I, Felföldi T. Vizes környezetekből izolált potenciálisan új baktériumfajok taxonómiai jellemzése. = Taxonomic characterization of potentially new bacterial species isolated from aquatic environments. *Hidrol Közl* 2015;95:56-58. (in Hungarian)

Other publications

- Magyar Cs*, **Mentes A***, Fichó E, Cserző M, Simon I. Physical background of the disordered nature of “Mutual Synergetic Folding” proteins. *Int J Mol Sci* 2018;19:3340. doi:10.3390/ijms19113340 (IF 2017: 3.687)
- Felföldi T, Fikó DR, **Mentes A**, Kovács E, Máthé I, Schumann P, Tóth E. *Quisquiliibacterium transsilvanicum* gen. nov., sp. nov., a novel betaproteobacterium isolated from a waste-treating bioreactor. *Int J Syst Evol Microbiol* 2017;67:4742-4746. doi: 10.1099/ijsem.0.002368 (IF: 1.932)
- Felföldi T, Schumann P, **Mentes A**, Kéki Zs, Máthé I, Tóth E. *Caenimicrobium hargitense* gen. nov., sp. nov., a new member of the family Alcaligenaceae (Betaproteobacteria) isolated from activated sludge. *Int J Syst Evol Microbiol* 2017;67:627-632. doi: 10.1099/ijsem.0.001674 (IF: 1.932)
- Felföldi T, **Mentes A**, Schumann P, Kéki Zs, Máthé I, Márialigeti K, Tóth E. *Rufibacter quisquiliarum* sp. nov., a new member of the phylum Bacteroidetes isolated from a bioreactor treating landfill leachate. *Int J Syst Evol Microbiol* 2016;66:5150-5154. doi: 10.1099/ijsem.0.001488 (IF: 2.134)
- Pálffy K, Felföldi T, **Mentes A**, Horváth H, Márialigeti K, Boros E, Vörös L, Somogyi B. Unique picoeukaryotic algal community under multiple environmental stress conditions in a shallow, alkaline pan. *Extremophiles* 2014;18:111-119. doi: 10.1007/s00792-013-0602-0 (IF: 2.306)