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DIVERSITY OF BACTERIAL COMMUNITIES ADAPTED TO THE SPECIAL HABITAT
CHARACTERISTICS OF LAKE HÉVÍZ

-PhD thesis-

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Introduction and the aims of the study

Freshwater environments provide essential ecosystem services to humanity even though their coverage on the Earth's surface is negligible compared to the area of seas and oceans. Owing to their connective role between atmospheric and terrestrial processes and sensitiveness to environmental changes, they are rather vulnerable which makes them good indicator at local and global scale (Williamson et al., 2008). Some studies showed that biodiversity of these habitats decrease more rapidly than terrestrial or marine ecosystems' and a significant part of the most endangered species are found here (Jenkins, 2003).

Lake Hévíz is a unique natural value of Hungary which provides special habitats for living organisms thanks to its particular physical, chemical and geological characteristics. After the recognition of the mild water temperature and the curative effect of the sediment, the lake was used for bathing since the ancient times, and nowadays it is a popular tourist destination. Nevertheless, the lake preserved its excellent ecological state in spite of the large number of bathers. It can be partly due to the low water retention time but bacterial communities as main drivers of biogeochemical processes of the lake could also play important role in the maintenance of the lake's natural condition. The first recognition of the key role of bacterial communities participating in the biogeochemical cycles of freshwater environments was made by Lindeman in the 1940s (Lindeman 1942) who placed the microorganisms in the centre of the trophic net of a temperate freshwater lake. Since then several studies confirmed the importance of bacteria in the nutrient cycles of freshwater environments (Cotner and Biddanda, 2002). Bacterial communities could also take part in the balneo-therapeutic utilization of Lake Hévíz. Previous studies pointed out that some inhabiting bacteria – e.g. *Streptomyces* species - produce antibiotics while others could convert peat-rich sediment components to biologically active compounds (e.g. estrogens), and an iodine accumulating species (*Microbispora amethystogenes*) was also identified from here (Clemente, 1982).

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In contrast with the previously revealed sediment bacterial communities of Lake Hévíz, diversity, seasonal and spatial heterogeneity of planktonic bacterial communities that could also play important ecological role are hitherto unrevealed, as well as the bacterial community compositions of the hydrothermal springs and biofilms developed in the deep lake water and close to the water surface.

Taking these into consideration the followings were set as the aim of our study:

- Revealing the spatial heterogeneity of planktonic bacterial communities by vertically sampling the water column above the spring cave, and by comparing the community fingerprints of horizontally different sampling points of the lake;
- Monitoring the temporal changes in the genetic diversity of planktonic bacterial communities to disclose seasonal differences;
- Studying the effect of an anthropogenic disturbance caused by lake wall reconstruction work carried out in spring 2011 to enhance the water supply of the surrounding wetland habitats and to restore natural vegetation;
- Molecular biological investigation of the previously unknown bacterial diversity of the spring waters and biofilms formed in the deep and shallow water as well as the sediment;
- Comparing the bacterial community compositions originating from identical sampling points and times using simultaneously cultivation, molecular cloning and pyrosequencing.

Materials and methods

Examinations on the samples from Lake Hévíz were carried out between 2009 and 2015 using microbial ecological techniques.

To study the bacterial communities inhabiting different water depths, samples were taken above the spring crater in every two meters from the water surface to 29 m deep in 2009 November. Bacterial community structures were compared using 16S rRNA gene based Denaturing Gradient Gel Electrophoresis (DGGE). Discrete, intensely stained DGGE bands were excised; DNA was reamplified and sequenced to identify the dominant community members.

In order to study bacterial communities occurring in 1 m water depth, 14 different sampling sites were assigned. Horizontal spatial differences were studied by the determination

of direct cell count values for each point using DAPI staining; bacterial and cyanobacterial community structures were compared also by 16S rRNA gene based DGGE.

To explore the temporal changes in planktonic bacterial communities and the effect of the anthropogenic disturbance caused by the lake wall reconstruction, samples were taken in April, July and October in 2010 and 2011 at the 14 sampling sites assigned for horizontal spatial examinations. Cell count values were determined by DAPI staining; seasonal and annual changes in bacterial and cyanobacterial community structures were investigated by DGGE. Dominant bacterial and cyanobacterial community members were identified by sequencing the DNA from discrete DGGE bands. Detailed diversity analysis of the temporal changes in planktonic bacterial communities was carried out by pyrosequencing.

Cold, warm and mixed spring water, white biofilm formed in 20 m deep lake water and green biofilm originated from 1.5 m water depth, furthermore sediment samples were collected by a diver in June 2015. Diversity of bacterial communities in each sample type were explored by 16S rRNA gene based pyrosequencing.

Cultivation, molecular cloning and pyrosequencing were used simultaneously to explore the bacterial communities and compare the efficiency of cultivation based and independent microbiological methods in spring and autumn 2011. Two types of culture media (Nutrient, PYE) were applied that can be characterized by different organic matter quantity. Bacterial strains and molecular clones were identified by sequencing 16S rRNA gene that was amplified using identical primers. Bacterial strains and molecular clones were grouped by ARDRA (Amplified Ribosomal DNA Restriction Analysis) method based on their restriction pattern, and one representative from each group was sequenced.

Results and Discussion

Based on the DGGE patterns of different water depths of Lake Hévíz, no clear vertical stratification was observed in the planktonic bacterial communities, only minor spatial differences were observed. This could be explained by the stirring effect caused by the high water flow of the springs which prevents the development and constant presence of physically and chemically different habitats, and consequently the formation of genetically and functionally distinct planktonic bacterial communities. Therefore, minor vertical differences could be considered as momentary state created by the mixing event of crater springs rather than a constant condition evolved by effect of physical and chemical parameters.

Based on the results of Bacteria and Cyanobacteria specific DGGE, no considerable differences were revealed among the planktonic bacterial communities inhabiting the horizontally different sampling points of Lake Hévíz. DAPI staining based direct cell count values were also in 10^6 orders of magnitude per ml at all sampling sites. Regarding the bacterial and cyanobacterial community fingerprints, spatial differences proved to be less significant than seasonal variations probably owing to the low (3.5 days) water retention time which allows only minor differences in physical, chemical properties, and nutrient content between near shore and near crater water habitats. These circumstances could lead to less significant spatial than temporal differences respecting dominant planktonic bacterial community members. Minor spatial changes could be explained by the shallower, slower flowing water, and the presence of vegetation near the shore which could cause locally higher plant and sediment originated organic matter concentrations and lower light intensities due to the shading by plants.

However, temporal changes in planktonic bacterial communities were observed using both DGGE and pyrosequencing based examinations as well. The most distinct group regarding the statistical evaluation of the data comprised samples taken in spring 2011 by both methods. It could be caused by anthropogenic disturbance. Although the lake wall reconstruction did not lead to the change of physical, chemical parameters and microbial cell count values of the water, it caused only elevated turbidity. The influence of the works on planktonic bacterial communities was observed by cultivation and molecular cloning as well. Dominant taxa of the season revealed by pyrosequencing and molecular cloning gave the highest similarity to members of genera *Tabrizicola*, *Thiobacillus* and *Rhodobacter* and class Hydrogenophilaceae. Strains showing 97% sequence similarity to *Rhodobacter blasticus* and 99% to an unclassified *Rhodobacter* previously isolated from sulfidic cave system gave 43% of the strain collection.

Besides, genus *Rhodobacter*, *Cloacibacter rupense* was also revealed exclusively during the reconstruction by cultivation and 96% of the amplicon sequences showing the highest similarity to the species were found then. A major part of the identified taxa was previously described from sediment and biofilm of freshwaters (Hydrogenophilaceae sp., *Thiobacillus* sp., *Cloacibacter rupense*) thus their planktonic presence could be caused by stirring up the sediment. Dominant presence of species *Rhodobacter* during the embankment reconstruction works could also be explained by the elevated turbidity. Previous studies pointed out that these bacteria can be particle associated and could take major part in the degradation and mineralization of vascular plant derived organic matter. Thanks to the peat bed of Lake Hévíz, floating particles consisted mainly plant derived organic matter, that could provide appropriate surfaces and substrates for the *Rhodobacter* species. Regarding the studied two years, the lowest planktonic bacterial diversity was observed during the reconstruction which could also be the result of anthropogenic disturbance. Apart from the effect of the reconstruction works, more considerable seasonal differences were observed in 2011 than in 2010 based on the results of pyrosequencing. The second most distinct group was formed by autumn 2011 sample respecting the results of principal component analysis on OTU (Operational Taxonomic Unit) level (which was assigned on 97% or higher sequence similarity). The most abundant community members taking part in the separation showed the highest sequence identity to MWH-UniP1 aquatic group, *Arcobacter* and *Gemmobacter* related taxa. Planktonic bacterial communities of summers and autumn 2010 samples showed high similarity to each other which was driven by Cyanobacteria, Actinobacteria (*Candidatus Aquiluna*) and Bacteroidetes (NS11-12 marine group, Chitinophagaceae) related OTU-s. The highest planktonic bacterial diversity was determined in spring 2010 which was characterized by unique OTU composition and high ratio (25%) of unclassified Bacteria related sequences. The most abundant OTU (including only 4% of the sequences) showed 92% similarity to an unclassified Phycisphaerae bacterium.

Functionally, planktonic bacterial communities of Lake Hévíz showed similar annual changes during the two studied years. Springs were mainly dominated by sulfur oxidizing bacteria which could be washed in the lake by the uprising spring water in 2010 and also from the stirred-up sediment in 2011. Majority of the most abundant bacterium taxa identified then were not typical planktonic prokaryotes which could be related to the limitation of biological sulfur oxidizing processes to aerobic-anaerobic interfaces. Consequently, these organism can be found in the sediment or in biofilms. The considerable difference between the diversity of planktonic bacterial communities in spring 2010 and 2011 could be caused by the disturbing

effect of the reconstruction works which could provide selective advantage to some taxa (e.g. *Rhodobacter*) while inhibit others. Planktonic bacterial communities of summers could be formed by higher number of autochthonous primary producers (e.g. Cyanobacteria) causing the abundant presence of Actinobacteria (*Candidatus Aquiluna*) and Bacteroidetes (NS11-12 marine group) related heterotrophic bacteria. Majority of dominant bacterium taxa identified in summers showed the highest sequence similarity to typical planktonic prokaryotes. Bacterial community of autumn 2010 was also dominated by epilimnetically occurring heterotrophic organisms, however, the differing community composition indicates that utilization of recalcitrant plant derived organic matter became more dominant in line with the decrease of photosynthetic primary production.

Bacterial communities of spring waters obtained by pyrosequencing showed different composition even on higher taxonomic levels. Based on Shannon and Simpson indices, the highest diversity was revealed from the mixed spring water, while the lowest was determined from the warm spring water. The most abundant taxa of the bacterial communities of warm spring water were *Arcobacter* (Epsilonproteobacteria), *Thiovirga* and *Acinetobacter* (Gammaproteobacteria) related of which the members of former two genera could take part in biological sulfur oxidization processes in the spring cave. *Aeromonas* related sequences were the most dominant member of the bacterial community of cold spring water. Community composition of mixed spring water showed notable similarities to bacterial communities of lake water suggesting that many taxa revealed from the lake water could originate from the spring cave. Most abundant taxa revealed from mixed spring water showed the highest similarity to members of phylum Bacteroidetes (Chitinophagaceae) and class Alpha- (*Gemmobacter*, *Tabrizicola*) and Betaproteobacteria (*Polynucleobacter*).

Shannon and Simpson indices, based on the distribution of amplicon sequences, obtained from the biofilm samples among OTU-s indicated higher diversity of the green biofilm sample. Most abundant OTU-s of the less complex white biofilm showed the highest similarity to taxa within class Beta- (Hydrogenofilaceae) and Gammaproteobacteria (*Thiovirga*) which could generate energy by the oxidization of reduced sulfuric compounds provided by the springs and could contribute to primary production in the deep water by fixing carbon dioxide. Most dominant members of bacterial communities of the green biofilm were related to members of Alpha- (*Methylobacterium*), Beta- (Hydrogenophilaceae), Gamma- (*Rheinheimera*, *Thiovirga*) and Deltaproteobacteria (Desulfobacterales, *Dissulfurimicrobium*). Bacterial diversity of the green biofilm implies its highly complex structure including aerobic and

anaerobic microhabitats, and is dominated by bacteria involved in aerobic sulfur oxidization and anaerobic sulfate reduction processes. High metabolic diversity was also observed regarding the conversion processes of carbon compounds. Autotrophic carbon dioxide fixing bacteria (*Thiovirga*), one-carbon compound utilizers (*Methylobacterium*) and taxa taking part in mineralization of complex organic matters (*Rheinheimera*) were also identified which indicates the functional heterogeneity of the biofilms evolved in shallow water exposed to sunlight.

Sediment bacterial community of Lake Hévíz differed greatly from those revealed from other freshwater lake sediments. The most dominant taxa obtained from here belonged to phyla Cyanobacteria and Chloroflexi and comprised the half of the identified sequences. The most abundant OTU-s of the mentioned phyla showed the highest sequence similarity to an unclassified Anaerolineaceae bacterium and an unclassified cyanobacterium. Significant presence of Cyanobacteria and Chloroflexi related prokaryotes is typical to hot springs, consequently, microbial mat similar to those found in the hot springs could evolve on the sediment surface of Lake Hévíz. Survival and reproduction of the phototrophic taxa could be supported by the transparent, oligotrophic water which allows the penetration of photosynthetically active radiation mostly even to the sediment surface.

In accordance with the results of earlier investigations, significant differences were found between the community structures on phylum and order level obtained by cultivation based and cultivation independent techniques due mostly to the selectivity of the cultivation circumstances and the used media. Cultivation parameters applied in present study facilitated the growth of aerobic and facultative anaerobic heterotrophic bacteria having better adaptation to high organic matter containing media, and did not allow the multiplication of obligate anaerobic and phototrophic prokaryotes and chemolithotrophic taxa requiring special nutrients and physical chemical circumstances. Among these, particularly bacteria characterized by the latter two types of metabolism were identified using cultivation independent methods in high proportions which contributed to different taxon composition. Cell wall structure related differences could also explain the distinct community structures, since Gram-positive bacteria were observed in higher numbers by cultivation than molecular cloning or pyrosequencing at both sampling times. The more resistant cell wall - which is due to the thick murein layer - provides better tolerance against the cultivation conditions, on the other hand disruption of these bacterial cells is more difficult during DNA extraction procedure which leads to underrepresentation by cultivation independent approaches. Bacterial community compositions

revealed by molecular cloning and pyrosequencing showed high similarity to each other owing probably to the use of identical 16S rRNA gene region (V1-V3) for identification by both approaches, and the alignment and identification of clone and amplicon sequences under the same conditions. Minor differences could be explained by dissimilar throughputs of the methods which often lead to the detection of the least abundant taxa only by pyrosequencing.

PUBLICATIONS IN CONNECTION WITH THE SUBJECT OF THE THESIS

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