SEASONAL AND SPATIAL CHANGES OF PLANKTONIC BACTERIAL COMMUNITIES INHABITING THE NATURAL THERMAL LAKE HÉVÍZ, HUNGARY

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Lake Hévíz is a unique thermal spa located in Hungary. Owing to the thermal springs nourishing the lake, it has a relatively rapid water turnover. In spring 2011 a comprehensive embankment reconstruction was performed to preserve the water supply of the surrounding wetland habitats. The physical and chemical parameters as well as the planktonic microbial communities were studied with special respect to the effect of the disturbance of the water of Lake Hévíz. According to the abiotic components, both temporal and spatial differences were revealed with the exception of autumn samples. The reconstruction resulted in a short term but dramatic alteration of the total planktonic bacterial and cyanobacterial community structures as revealed by denaturing gradient gel electrophoresis. In addition, greater seasonal than spatial differences of bacterial communities were also observed. Planktonic bacterial community composition of Lake Hévíz included mainly typical freshwater species within phylum Actinobacteria, Chloroflexi, Cyanobacteria and class Alpha-, Beta- and Gamma-proteobacteria. Most of them were aerobic or facultative anaerobic heterotrophic but chemolitotrophic (e.g. *Thiobacillus*) or photolithotrophic (e.g. Cyanobacteria and Chloroflexi) autotrophic microbes were also identified.

Keywords: thermal lake, planktonic bacterial community, 16S rRNA gene, denaturing gradient gel electrophoresis

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Introduction

Thermal springs are distributed worldwide, and are mostly connected to areas with volcanic activities in recent geologic time. They are also common in areas where rocks, regardless of their character and age, have been faulted and intensely folded recently. Therefore, heat of these springs could originate either from volcanic activities or from great depth in areas of granitic or sedimentary rocks [1]. Lake Hévíz functioning as a thermal spa is located in West Hungary (Fig. 1). It is special in respect of its peat bed which is quite uncommon among thermal lakes. The lake is nourished by two thermal springs that arise from upper triassic dolomitic rocks. The springs have different temperatures and runoff into the lake underwater through a spring cave called "Amphora" located 38 m below the water surface. The average water temperature of the spring cave is 39.5 °C, and 90% of the volume comes from the warmer spring (41 °C) and 10% from the cooler spring (26 °C). The temperature of the lake water varies between 33–35 °C in summer and never falls below 22 °C even in winter. Regarding the chemical composition of the water, the major ions are calcium, magnesium and bicarbonate but reduced sulphur compounds are also present in relatively high proportion. Nevertheless, the oligotrophic lake is low in nitrogen forms. Due to the permanent flow from the springs, the lake water is replaced in every 3.5 days giving a "river like" character of the lake and providing habitat for bacteria characteristic to both lakes and rivers [2]. To enhance the water supply of the surrounding wetland habitats, an embankment reconstruction began in the lake in spring 2011. During this work the piles constructing the lake wall curtain were completely removed and replaced. Also, the sloped banks as well as the reed and sedge belt were re-established in order to recover and expand the native habitats and preserve the special wetland flora and fauna. This embankment reconstruction considerably stirred up the sediment of the lake and caused an elevated turbidity influencing the water chemistry as well as the microbial community structure of the lake water. Unfortunately, up to now hardly any information is known about the planktonic microbial communities of Lake Hévíz. Therefore, the aim of the present study was to gain information about the seasonal and spatial changes of the planktonic microbial communities together with the changes in the physical and chemical parameters of the water of Lake Hévíz with special respect on the effect of the disturbance caused by the embankment reconstruction.
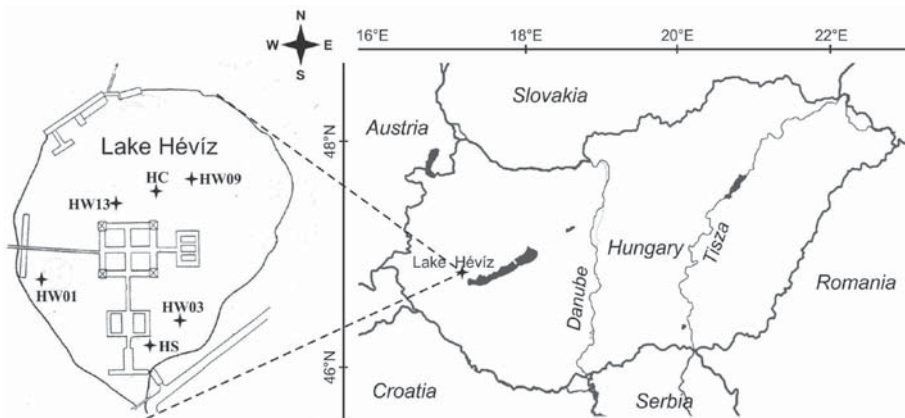
Figure 1. Location of sampling sites in Lake Hévíz. HW abbreviates Hévíz water
and the following number defines the sampling site regarding the samples used for bacterial community
structure investigations. (In case of physical chemical examinations the letter following HW specifies
the location by meaning near the shore (S) or near the crater (C))

Materials and Methods

Sample collection

Water samples from Lake Hévíz were taken using a submersible pump from 1 m below the water surface at 4 different locations (HW01, HW03: near the shore; HW09, HW13: near the crater) in April, July and October in 2010 and 2011 (1004, 1007, 1010 and 1104, 1107, 1110, respectively) (Fig. 1). The geographical coordinates of the center of the lake are 46°47′14″ N, 17°11′35″ E. The approximately 2-2 l of samples were stored at 6–8 °C until laboratory processing within 24 hours. Physical and chemical parameters provided by the Foundation for the Protection of the Underground Catchment Area of the Hévíz Thermal Lake were measured at the same time to the sampling dates. For the measurement of abiotic parameters, one sample from near the shore and one sample from near the crater was taken each occasion which is signed by the last character of the notation (e.g. HW-1004S, HW-1004C). Statistical evaluation of physical and chemical parameters was carried out using Paleontological Statistics software package for education and data analysis (PAST) [3].

DNA extraction and amplification

The water samples were concentrated by filtration through a 0.22 µm pore sized cellulose nitrate filter (Millipore, Billerica, MA, USA) and DNA was isolated from the filter using Ultra Clean Soil DNA (MoBio, Carlsbad, CA, USA) isolation kit according to the manufacturer's instructions. Amplification of partial 16S rRNA gene sequence used for the investigation of bacterial community structure by denaturing gradient gel electrophoresis (DGGE) was performed by two consecutive polymerase chain reaction (PCR) steps. The first was carried out using 27F (AGA GTT TGA TCM TGG CTC AG) [4] and 1401R (CGG TGT GTA CAA GAC CC) [5] primers both for total bacterial and specifically for cyanobacterial community. In the second PCR, 27F-GC and 519R (GWA TTA CCG CGG CKG CTG) [6] primers were applied for total bacterial, and CYA359F-GC (GGG GAA TYT TCC GCA ATG GG) and CYA781R (GAC TAC WGG GGT ATC TAA TCC CWT T) [7] for cyanobacterial communities. A 40 nucleotide GC-rich sequence (5'-CGC CCG CCG CGC CCC GCG CCG GTC CGC CGC CCC CCG CCC G-3') was attached to the 5' end of the forward primers in case of amplifications prior to DGGE. The PCR mixtures with a final volume of 50 µl contained 2 µl of purified genomic DNA, 0.2 mM of each deoxynucleotide, 2 mM MgCl$_2$, 1 U LC *Taq* DNA Polymerase (Fermentas, Vilnius, Lithuania), 1X PCR Buffer (Fermentas, Vilnius, Lithuania) and 0.325 µM of the primers. Temperature profile of the first PCR in both cases included an initial denaturation at 95 °C for 5 min, followed by 32 cycles of denaturation at 94 °C for 30 sec, annealing at 52 °C for 30 sec, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The second PCR was carried out with almost the same temperature profile as the first one, except that the annealing temperature was at 60 °C in the case of Cyanobacteria specific PCR. The PCR products were checked by electrophoresis in ethidium bromide stained 1% agarose gel under UV light.

Denaturing gradient gel electrophoresis (DGGE)

Microbial community structures were revealed and compared by DGGE running in 7% (w/v) (for total bacterial community) and 8% (for Cyanobacteria) poliacrylamide gel containing 40 to 60% of denaturant gradient of urea and formamide. The electrophoresis was carried out in 60 °C 1X Tris-acetate-EDTA (TAE) buffer at 120 V for 14.5 hours using an INGENY phorU-2 electrophoresis system (Ingeny International, Goes, Netherlands). The gel was stained with ethidium-bromide, washed in 1X TAE and photographed under UV light. TotalLab (TL 120) version 2006 (Nonlinear Dynamics Inc., Newcastle upon Tyne, UK)

software was used to compare the microbial community structures by dendrograms on the basis of their DGGE patterns.

To identify the dominant members of the community, discrete DGGE bands were excised, and the DNA was extracted by an overnight incubation in 30 µl DEPC-treated water (G-Biosciences, St. Louis, MO, USA). DNA derived from the excised DGGE bands was reamplified and sequenced by Sanger method using 27F (total bacterial community) and CYA359F (cyanobacterial community) primers. Identification of the obtained sequences was carried out using the EzTaxon-e [8] and Basic Local Alignment and Search Tool (BLAST) program [9]. Sequences were submitted to GenBank under the accession numbers LN810094-LN810107.

Results and Discussion

Physical and chemical parameters

At each sampling time in 2010 and 2011, altogether 20 different environmental parameters of the water were measured near the shore and crater of the lake (Supplementary Table I). The water temperature varied between 25.4 °C and 33.3 °C with the minimum in spring and maximum in summer. The water was slightly warmer near the crater in spring and slightly cooler in summer. The amount of dissolved oxygen varied between 2.04 and 5.91 mg l^{-1} with a minimum in autumn in both years. Regarding the sulfur compounds, the concentration of sulphate showed the minimum values (65.2 mg l^{-1} in average) in spring in both years. The amount of sulphide did not show seasonal changes but it was present in higher amount (0.26 mg l^{-1} in average) near the crater in more cases. Lake Hévíz is relatively low in nitrogen forms. The highest nitrate value was 0.4 mg l^{-1}, but mostly it was below 0.3 mg l^{-1}. The amount of nitrite was below 0.01 mg l^{-1} in all cases. Ammonium concentration varied between 0.15 and 0.28 mg l^{-1} and reached its maximum in autumn in both years. Similarly to the forms of nitrogen the lake water is also low in phosphate and considered as oligotrophic, but rich in bicarbonate (378–403 mg l^{-1}), which is – with magnesium (29.1–33.8 mg l^{-1}) and calcium (86.1–96.3 mg l^{-1}) – one of the major chemical constituents of the water.

Following the normalization of the different values of the physical and chemical parameters, a principal component analysis (PCA) was carried out. The objects of the analysis were the different locations of different sampling times while physical and chemical parameters were used as variables. The first two components explained 59% of the total variances. The obtained biplot (Fig. 2)

Table I. Physical and chemical parameters of the water of Lake Hévíz in respect of sampling times and locations

|	2010						2011					
	April		July		October		April		July		October	
	HW-S	HW-C	HW-S	HW-C	HW-S	HW-C	HW-S	HW-C	HW-S	HW-C	HW-S	HW-C
Conductivity (μS cm^{-1})	748	746	740	740	741	740	736	741	677	667	748	744
Temperature (°C)	25.4	26	31.2	32.1	30.2	30.3	26.2	27.5	33.3	33.1	28.4	29.3
pH	7.2	7	6.8	6.8	7.43	6.81	7.38	7.23	7.19	7.25	7.22	7.06
Dissolved O$_2$ (mg l^{-1})	4.15	3.39	4.2	3.1	2.9	2.7	5.91	4.5	5.46	4.62	2.26	2.04
Reduction potential (mV)	174	−180	206	−216	−17	−201	31	−150	39	−82	108	−26
S^{2-} (mg l^{-1})	<0.02	0.14	<0.02	0.98	<0.02	<0.02	<0.03	<0.03	<0.03	0.26	<0.03	0.14
SO$_4^{2-}$ (mg l^{-1})	69	65	75	74	74	89	66	61	78	93	73	78
NH$_4^+$ (mg l^{-1})	0.18	0.18	0.15	0.17	0.22	0.21	0.15	0.16	0.18	0.2	0.28	0.28
NO$_2^-$ (mg l^{-1})	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
NO$_3^-$ (mg l^{-1})	0.3	<0.3	<0.3	<0.3	0.3	<0.3	<0.3	<0.3	0.4	0.3	<0.3	<0.3
Cl$^-$ (mg l^{-1})	20	21	20	20	20	20	20	20	20	20	20	20
COD (mg O$_2$ l^{-1})	0.85	1.25	1.03	2.9	1.21	0.54	1.14	1.06	0.99	0.77	0.6	0.39
PO$_4^{3-}$ (mg l^{-1})	<0.05	<0.05	<0.05	<0.05	<0.05	0.2	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Fe^{2+} (mg l^{-1})	1.01	1.05	0.29	0.34	0.53	0.52	0.58	0.61	0.41	0.43	0.38	0.46
Na$^+$ (mg l^{-1})	19	18.1	16.2	15.8	22.1	21.8	19.6	20.9	21.5	21.4	21.2	22.6
Mn^{2+} (mg l^{-1})	0.06	0.05	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Mg^{2+} (mg l^{-1})	30.4	29.2	29.1	29.2	32.3	32.8	32.7	32.9	32.9	33.8	31.7	32.2
K$^+$ (mg l^{-1})	7.46	7.15	6.39	5.99	6.73	6.54	6.46	6.83	6.58	6.61	7.31	7.86
Ca^{2+} (mg l^{-1})	89.5	92	92.9	96.3	88.4	86.1	89.9	90.2	88.8	90.2	89.4	88.5
HCO$_3^-$ (mg l^{-1})	378	378	384	384	378	378	384	403	403	390	390	390
CO$_3^{2-}$ (mg l^{-1})	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
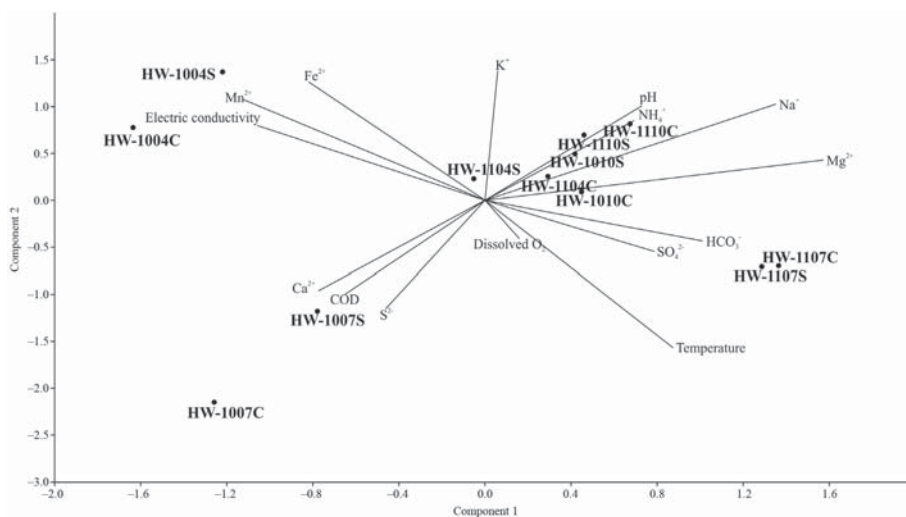
Figure 2. Two-dimensional principal components biplot describing the changes in physical and chemical parameters of Lake Hévíz. Component 1 and Component 2 explains 32%, and 27% of total variances, respectively. (The number following HW defines the year and the month of sampling, respectively. The letter following the date numbers specifies the location by meaning near the shore (S) or near the crater (C))

showed that except for autumn, samples from the two years greatly differed from each other. Regarding spring and summer samples, seasonal differences proved to be more significant than the spatial in both years. Before the reconstruction in 2010, spring and summer samples were separated according to the sampling sites along the component 1 and according to the sampling times along the component 2 (Fig. 2). However, the spatial differences disappeared at the beginning of the reconstruction (in spring 2011) which could be explained by homogenization of the water caused by the operations. Samples taken in spring grouped together with autumn samples in 2011 (Fig. 2). The characteristic seasonal separation of the summer samples from 2011 can also be observed but their spatial similarity reflected the disturbing effects of the reconstruction.

Microbial community structures

Denaturing gradient gel electrophoresis is a frequently used, cost-effective fingerprint method for investigating the spatial and temporal changes of microbial communities. This method provides the possibility to identify the community members by reamplifying and sequencing of the excised bands. Previously,

DGGE technique was successfully applied to reveal the alterations of bacterial or total microbial communities in freshwater and seawater habitats influenced by different environmental factors [10, 11, 12, 13, 14].

According to the UPGMA dendrogram based on the DGGE patterns, significant seasonal changes with less spatial differences were found among the total planktonic bacterial communities of Lake Hévíz (Fig. 3). On the dendrogram, the first branching separated the samples taken in spring 2011. This separation could be explained by the disturbance of the embankment reconstruction that began at that time and probably played important role in the development of special microbial community structures due to the stirring of the sediment. Further branches on the dendrogram correlated firstly to annual and then seasonal changes indicating that the influence of the reconstruction on planktonic bacterial communities was limited to spring 2011. It is interesting to note that the same seasons of different years showed distinct bacterial community structures, although no significant differences were observed between the numbers of dominant taxa regarding the different seasons and years.

Regarding the cyanobacterial communities (Figure 4), the first significantly separable group on the dendrogram included the samples taken in spring 2011 as a result of the reconstruction, similarly to that of total bacterial communities. Except this separation, summer and autumn samples from 2011 showed high similarity to each other and formed a distinct group on the dendrogram. In 2010 greater differences were detected among the cyanobacterial communities than in 2011. Contrary to the differences observed in the structures of cyanobacterial communities in the studied two years, summer samples were highly similar to each other. Spatial differences were even less significant than it was in the case of the total bacterial communities (Fig. 4). In tempered (and also in subtropical) regions planktonic communities (e.g. microalgae, cyanobacteria) are often characterized by a seasonal succession regarding the species compositions and abundances [15, 16], however owing probably to the relatively consistent temperature and nutrient access of Lake Hévíz the effect of the seasonal changes proved to be less significant on cyanobacterial communities.

Microbial community compositions

Dominant members of the bacterial communities were identified by excision of DGGE bands and sequence analysis of the reamplified PCR products (Fig. 3 and 4). Representatives of phylum Actinobacteria, Chloroflexi, Cyanobacteria and classis Alpha-, Beta- and Gammaproteobacteria were identified.
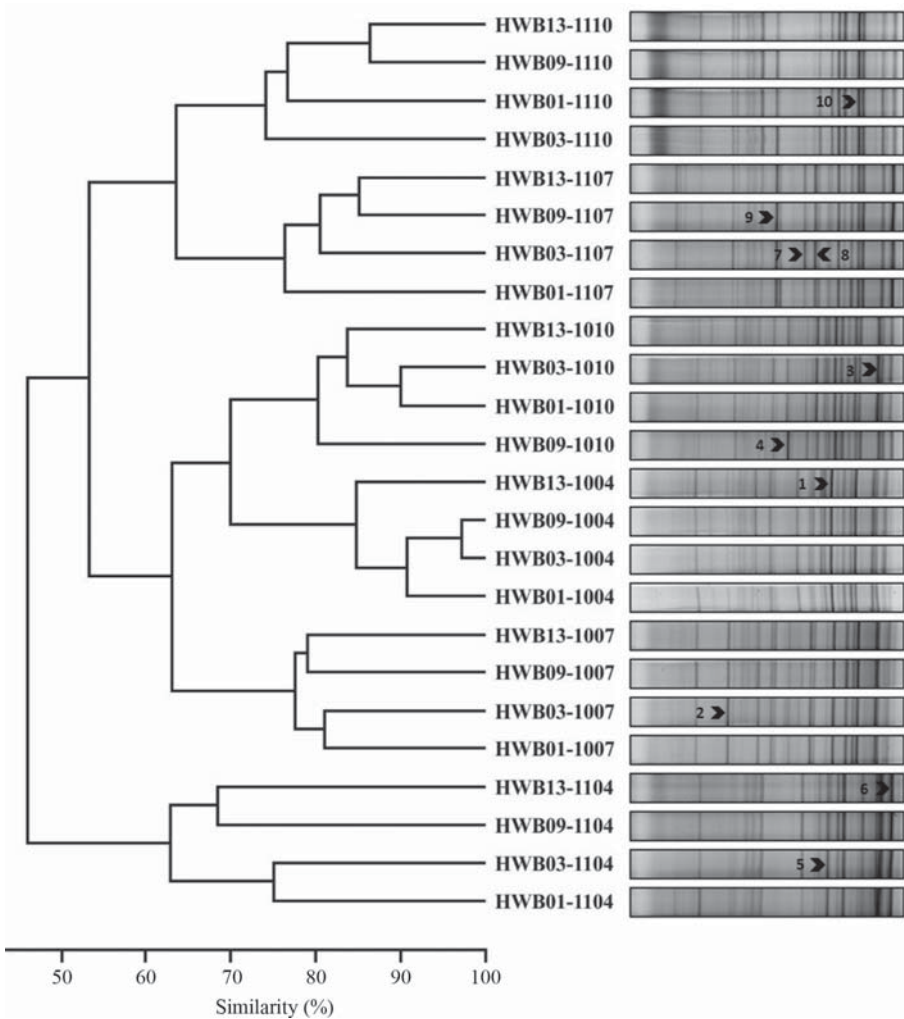
Figure 3. UPGMA dendrogram constructed on the basis of DGGE patterns
of total bacterial communities revealed from Lake Hévíz. The marked bands were excised
and identified by sequencing of the 16S rRNA gene

DGGE bands of bacterial communities that were present in every sampling sites and times were related to uncultured bacteria within *Burkholderiales, Thiobacillus* sp. (Betaproteobacteria) and *Prochlorothrix* (Cyanobacteria) as well as 'Candidatus Aquiluna rubra' (Actinobacteria). Further two taxa, an uncultured *Synechococcus* (Cyanobacteria) and *Gemmobacter lanyuensis* (Alphaproteobac-
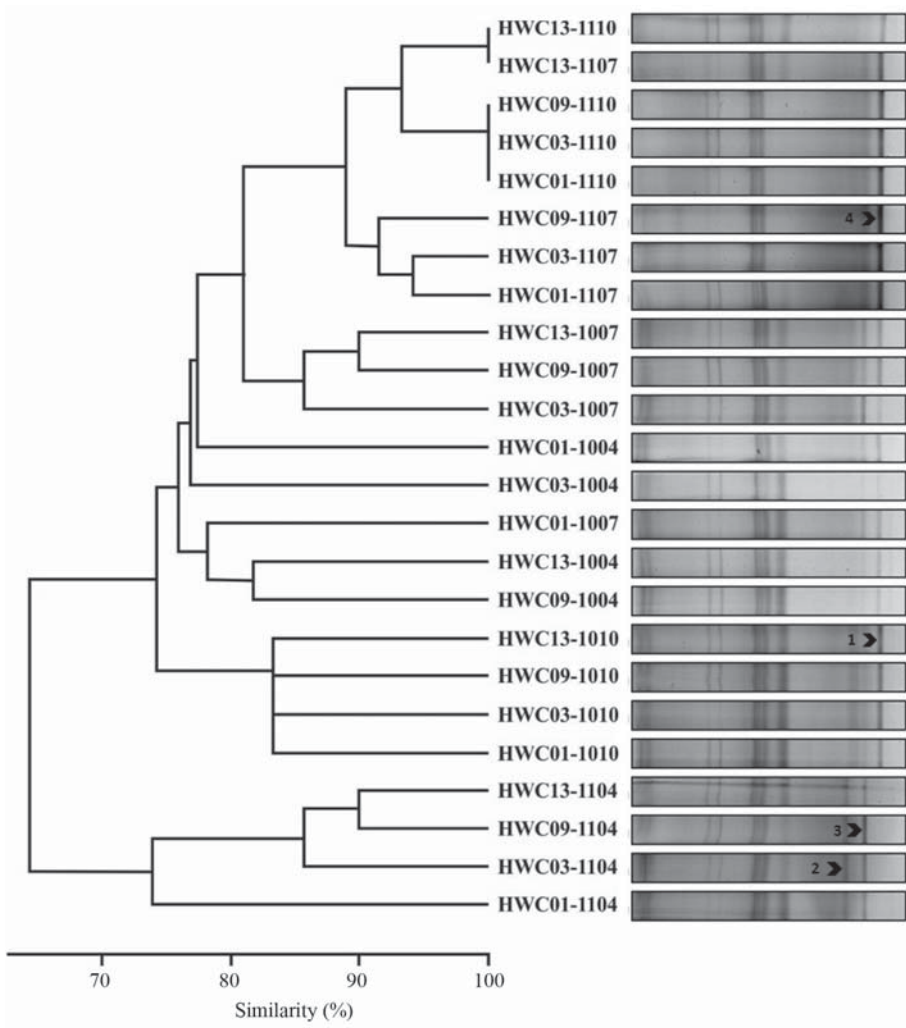
Figure 4. UPGMA dendrogram constructed on the basis of DGGE patterns
of cyanobacterial communities revealed from Lake Hévíz. The marked bands were excised
and identified by sequencing of the 16S rRNA gene

teria) were revealed in every locations and times except for spring 2011. The
dominant members of the bacterial communities in spring 2011 showed the high-
est sequence similarity to the '*Candidatus* Aquiluna rubra', an uncultured *Thio-
bacillus* sp. and an uncultured cyanobacterium within Oscillatoriales.

Every dominant member of the bacterial communities showed the highest sequence similarity to bacteria previously detected in freshwater environments (Table I). Some of these bacteria were revealed mainly from still water (lakes, ponds, reservoirs), e.g. *Aquiluna rubra, Rheinheimera aquatica, Polynucleobacter cosmopolitanus, Polynucleobacter acidiphobus* from which 'Candidatus Aquiluna rubra' and both *Polynucleobacter* species are typical planktonic freshwater microbes [14, 17, 18, 19]. Others, e.g. *Gemmobacter lanyuensis* and an uncultured *Thiobacillus* species were identified from freshwater springs [20, 21]. Members of the latter genus are often isolated from thermophilic environments likewise the uncultured cyanobacterium within Oscillatoriales related to our cyanobacterial DGGE band which was firstly revealed from a Turkish hotspring. Species of the genus *Synechococcus* are widespread in marine environments, but they are also common in freshwater [22] taking part in the primary production in both ecosystems. Sequences obtained from the cyanobacterial community of Lake Hévíz showed the highest similarity to an uncultured member of the non-marine group of the genus [23].

Most of the identified sequences (e.g. *Aquiluna rubra, Rheinheimera aquatica, Polynucleobacter cosmopolitanus, Polynucleobacter acidiphobus, Gemmobacter lanyuensis*) showed the greatest similarity to aerobic or facultative anaerobic heterotrophic bacteria, however presence of chemolitotrophic autotrophic (e.g. *Thiobacillus*) or photolithotrophic autotrophic (e.g. Cyanobacteria and Chloroflexi) microbes were also revealed. Permanent presence of the litoautototrophic bacteria in the water of Lake Hévíz indicates the importance of phototrophic and sulphur based chemotrophic primary production.

To explain the observed distribution of aquatic microorganisms it is necessary to find the environmental parameters that affect the bacterial community structures. Ionic composition appears to have a significant influence on microbial community composition that is confirmed, e.g. by the relative lack of Betaproteobacteria related species in open ocean environment, however members of the class are often dominant in freshwater habitats [24]. Particle associating ability, depth (which defines a wide range of physical and chemical parameters), pH, dispersion opportunities, resource availability, disturbance and physical chemical heterogeneity of the environment also play decisive role in forming the community structures [25]. Lew et al. [27] found large seasonal changes of bacterial community composition and Proteobacteria and Actinobacteria dominance during the microbial investigation of two peat bog lakes. The study also pointed out the influence of organic matter originated from peat moss on forming the microbial communities. A previous study on the microbiota of 15 freshwater lakes using reverse line blot hybridization based on 16S rRNA gene showed similar

Table I. Phylogenetic affiliation of 16S rRNA gene sequences from excised DGGE bands regarding sampling sites and dates of Lake Héviz.

Band	Nr.	Nearest relative	Similarity	2010			2011		
				April	July	October	April	Juli	October
HWB_1	Bacterium clone DP10.1.39 (FJ612332)	99% (429/434)	Betaproteobacteria, Burkholderiales	+	+	+	+	+	+
HWB_2	Chloroplast *Chlorella sorokiniana* (X65689)	99% (345/350)	Eukarya	+	+		+	+	+
HWB_3	*Aquiluna rubra* (AJ565416)	97% (413/424)	Actinobacteria	+	+	+	+	+	+
HWB_4	*Rheinheimera aquatica* (GQ168584)	99% (410/415)	Gammaproteobacteria			+			
HWB_5	Bacterium clone H-6 (HQ661222)	97% (370/381)	Chloroflexi, Chloroflexales	+		+	+	+	
HWB_6	Uncultured Thiobacillus sp. (AM167962)	96% (370/383)	Betaproteobacteria, Thiobacillus f.	+	+	+	+	+	+
HWB_7	*Polynucleobacter cosmopolitanus* (AJ550672)	99% (404/407)	Betaproteobacteria	+	+		+	+	
HWB_8	*Polynucleobacter acidiphobus* (FM208180)	100% (419/419)	Betaproteobacteria	+	+			+	
HWB_9	Uncultured bacterium LEGE 07320 (HM217051)	99% (369/372)	Cyanobacteria, Synechococcus	+	+	+		+	+
HWB_10	*Gemmobacter lanyuensis* (JN104393)	98% (376/385)	Alphaproteobacteria	+	+	+		+	+
HWC_1	Uncultured bacterium LEGE 07320 (HM217051)	99% (341/345)	Cyanobacteria, Synechococcus	+	+	+		+	+
HWC_2	*Pseudoscillatoria* sp. ZDb (HQ916861)	98% (366/374)	Cyanobacteria, Oscillatoriales				+		
HWC_3	Clone 28bk46 (FJ262743)	99% (370/375)	Cyanobacteria, Oscillatoriales, Prochlorothrix	+	+		+		
HWC_4	Uncultured bacterium LEGE 07320 (HM217051)	99% (366/370)	Cyanobacteria, Synechococcus	+	+	+		+	+

results to present investigation regarding the identified phyla and classes of Proteobacteria. However, representatives of phylum Verrucomicrobia, those were also found then and are often revealed form freshwater [27], was not identified from the water of Lake Hévíz. Furthermore, the study specified three parameters such as pH, hydrological retention time and temperature as the strongest environmental factors influencing the microbial communities. Another investigation on bacterioplankton of freshwater mesocosm revealed that bacterioplankton communities were influenced by environmental physicochemical variables linked to the increasing level of eutrophication moreover nitrogen concentration correlated directly with the bacterioplankton composition [28]. Occurrence of Verrucomicrobia related sequences were correlated with more euthrophic circumstances while members of phylum Actinobacteria were linked to less eutrophic conditions, which could explain the absence of former taxon in the oligotrophic water of Lake Hévíz. However, it was found there in the deep (10 cm) sediment layers previously using molecular cloning. At that time representatives of 10 phyla (Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, Cyanobacteria, Chlorobi, Chloroflexi, Deferribacteres, Nitrospirae, Spirochaetes and Verrucomicrobia) were identified by cloning and 4 phyla (Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes) by cultivation, nevertheless the overlap between the sediment and planktonic bacterial community compositions were negligible regarding the lower taxonomic (e.g. genus) levels [29]. Other microbial investigations on aquatic environments also confirmed that bacterial communities associated with surfaces clearly differed from free-living communities [30, 31]. Due to decomposition processes taking place in freshwater sediments, their carbon content and the available electron acceptors may generally differ from those of the overlaying water layers which may contribute to the development of distinct microbial communities [32].

Conclusions

Planktonic bacterial communities and abiotic components of Lake Hévíz were studied in two consecutive years including undisturbed and disturbed periods. Previous to an embankment reconstruction, significant seasonal differences were observed between spring and summer water samples according to the both total bacterial community composition and physical-chemical parameters. Due to the relatively low retention time of the lake water, the impact of disturbance limited to the intervention period which was reflected in the water chemistry and in the altered bacterial community structure. From the water of Lake Hévíz besides the aerobic or facultative anaerobic heterotrophic bacteria (e.g. *Aquiluna*

rubra, Rheinheimera aquatic, Polynucleobacter cosmopolitanus, Polynucleo-bacter acidiphobus, Gemmobacter lanyuensis), dominant and permanent presence of chemolitotrophic (e.g. *Thiobacillus*) and photolithotrophic (e.g. Cyanobacteria and Chloroflexi) autotrophic bacteria were revealed.

Acknowledgement

Conflict of Interest

The authors declare that they have no conflict of interests.

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