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ORIGINAL PAPER

Insight into the hidden bacterial diversity of Lake Balaton, Hungary

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Abstract
In the present study, the prokaryotic community structure of the water of Lake Balaton was investigated at the littoral region of three different points (Tihany, Balatonmáriafürdő and Keszthely) by cultivation independent methods [next-generation sequencing (NGS), specific PCRs and microscopy cell counting] to check the hidden microbial diversity of the lake. The taxon-specific PCRs did not show pathogenic bacteria but at Keszthely and Máriafürdő sites extended spectrum beta-lactamase-producing microorganisms could be detected. The bacterial as well as archaeal diversity of the water was high even when many taxa are still uncultivable. Based on NGS, the bacterial communities were dominated by Proteobacteria, Bacteroidetes and Actinobacteria, while the most frequent Archaea belonged to Woesearchaeia (Nanoarchaeota). The ratio of the detected taxa differed among the samples. Three different types of phototrophic groups appeared: Cyanobacteria (oxygenic phototrophic organisms), Chloroflexi (anaerobic, organotrophic bacteria) and the aerobic, anoxic photoheterotrophic group (AAPs). Members of Firmicutes appeared only with low abundance, and Enterobacteriales (order within Proteobacteria) were present also only in low numbers in all samples.

Keywords Lake Balaton · Bacterial diversity · Archaea · Bacteria · Next-generation sequencing (NGS)

Introduction

Lake Balaton is the biggest lake of Central Europe, a shallow water ditch, with an average depth of 3.0–3.6 m. The open water of the lake is almost permanently homogenized by wind, resulting concomitant resuspension of the sediment. The lake has a great importance also due to its touristical use. The quality of the water is regularly tested, and monitoring has started already in the 1960s (Szabó 1999; Kiss et al. 2005). In 2006, Hungary joined the water quality assessments in line with the EU Water Framework Directive (EU WFD). In it, the definitions of biological variables were given a much stronger role than before. Moreover, till that time many studies had revealed the state of the water (Istvánovics et al. 2008; Bolla et al. 2010; Hatvani et al. 2014; Maasz et al. 2019).

The first microbiological investigations aimed at studying the prokaryotic diversity of the lake showed that members of the genus *Bacillus* (Firmicutes) are dominant at some regions (Langó 1982). Different Actinobacteria were also isolated from the water of Keszthely Bay, e.g. *Streptomyces* and *Micromonospora* species (Farkas 1982). In 1985, the presence of Enterobacteria (indicating faecal pollution) was found in Keszthely Bay, and *Enterobacter agglomerans, Escherichia coli, Kluyvera cryocrescens and Klebsiella oxytoca* were detected in high number among the cultivated bacterial strains (Bognár, 1990).

Tóth (1995) has studied the bacterial communities of the open water in the Balatonfüred region and found the dominance of many Gram-negative organisms belonging to the genera *Aeromonas*, *Pseudomonas* and *Acinetobacter*. Bacterial partners of *Eudiaptomus gracilis* (important member of the zooplankton of the lake) were also studied (Homonnay et al. 2011) as well as the microbiology of big-headed carps (Borsodi et al. 2017). The first detailed reports about aerobic anoxygenic phototrophs (AAPs) of the lake showed that their abundance as compared to heterotrophic bacteria is between 1 and 7% (Szabó-Tugyi et al. 2019).

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All of these studies are very important, but most of them were based on cultivation techniques, and it is known that less than 1% of bacteria are cultivable from natural aquatic habitats (Amann et al. 1995).

The aim of the present study was to reveal the prokaryotic community structure of the water of the lake at the littoral region (where the water is subjected also to strong human influence due to bathing) by cultivation independent methods [next-generation sequencing (NGS) and microscopic cell counting]. By using specific PCRs, the presence of some hygienically important bacteria was tested. Our results show the hidden prokaryotic diversity of the lake which has never been revealed until now, and provide information about the potential health risks of bathing.

Materials and methods

Sampling

Sampling was carried out in 30. 08. 2018 at three points of the lake: Tihany (65.91900 N; 17.88903 E), Keszthely (46.76014 N; 17.25448 E) and Balatonmáriafürdő (46.70725 N; 17.38276 E) (later: Máriafürdő), from the littoral water region. The water samples (1–1 L) were aseptically collected into sterilized screw-capped flasks from 10 cm subsurface according to ISO 19458:2006. Samples were transported to the laboratory in a cooler bag (at 4 °C) and processed within 4 h after sampling.

Analysis of physical and chemical parameters

To measure the physical and chemical parameters, the following standards were used: MSZ 448-32:1977 (conductivity), MSZ ISO 5813:1992 (dissolved oxygen, DOC), MSZ 1484-13:2009 (nitrate concentration), MSZ 448-13-1983 (sulphate concentration), MSZ ISO 10260:1993 (chlorophyll *a*). The measures were done by the Central Transdanubian Water Authority, and we got it via the National Directorate of Water Management (OVF); data were obtained from the office except values of water temperature and pH which were measured on site.

Determination of microscopy cell counts

Determination of bacterioplankton abundance (microscopy cell counts) was carried out as described by Kéki et al. (2019) using Nikon80i epifluorescent microscopy and NisElements program package. For the investigations, 10 mL water samples were filtered on sterile polycarbonate filters (Millipore, Billerica, MA, USA) and fixed with 2% paraformaldehyde solution.

DNA extraction from the water samples

For DNA extraction, 0.25L water samples were filtered through 0.22 µm pore size, mixed cellulose filter (type GSWP; Millipore, Billerica, MA, USA) using the Ultraclean® PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions.

Specific PCRs

Taxon-specific PCRs were applied for the following bacterial groups: *Pseudomonas* spp. and *Pseudomonas aeruginosa* according to Lavenir et al. (2007), *Legionella* spp. according to Cloud et al. (2000), *Legionella pneumophila* according to Fiume et al. (2005), *Acinetobacter baumannii* according to Tsai et al. (2018) and *Stenotrophomonas maltophilia* according to Filho et al. (2004).

At the sampling sites there were no people at the time of sampling (it was late pm), but the sites were subjected to bathing in the previous days, therefore, the bacterial community was tested also for extended spectrum beta-lactamase (ESBL)-producing and macrolide-resistant bacteria.

The presence of ESBL genes was tested by multiplex PCRs in the water samples according to Trung et al. (2015) and macrolide-resistance genes following the protocol described by Zmantar et al. (2011), testing five genes (ermA, ermB, ermC, msrA and mefA) simultaneously.

Next-generation DNA sequencing and data analysis

For the identification of bacterial and archaeal taxa, the 16S rRNA gene of the DNA samples was amplified with PCRs in separate, triplicate reactions using primers with the following target-specific sequences: Bact_341F (5′-CCT ACG GGN GGC WGC AG-3′) and modified Bact_805R (5′-GAC TAC NVG GGT ATC TAA TCC-3′) for bacteria (Herlemann et al. 2011), and A519F (5′ -CAG CMG CCG CGG TAA-3′) and Arch855R (5′ -TCC CCC GCC AAT TCC TTT AA-3′) for Archaea (Klindworth et al. 2013). DNA sequencing was performed on an Illumina MiSeq platform using MiSeq standard v2 chemistry by the Genomics Core Facility RTSF, Michigan State University, USA. Sequencing and the description of bioinformatic analysis were done according to Benedek et al. (2019). OTUs were determined at the species-level delineation based on Tindall et al. (2010). Sequence reads were deposited in the NCBI SRA database and are accessible through the BioProject PRJNA628507, Biosample ID SAMN14732963 (Tihany), SAMN14732959 (Máriafürdő) and SAMN14732964 (Keszthely). Diversity indices were calculated using mothur (Schloss et al. 2009),

and reads were subsampled to the read number of the sample having the lowest sequence count.

Results

Physico-chemical and biological parameters

The physico-chemical parameters together with the cell count values of the samples of the Lake Balaton are given in Table 1. Data of the three sampling sites showed similarities though some differences could be detected: the quantity of total organic carbon (TOC), total organic nitrogen (TON), total organic phosphorous (TOP) as well as chlorophyll-*a* concentration were the lowest in the Tihany region. Most TOC appeared in dissolved form (DOC), and the water was aerated at all sites.

Specific PCRs

The taxon-specific PCRs showed (Table 2) that *Pseudomonas* spp. and *Legionella* spp. were present at all sites, but pathogenic *L. pneumophila* and *P. aeruginosa* as well as *Acinetobacter baumannii* could not be detected. At Keszthely and Máriafürdő sites ESBL-producing bacteria could also be detected.

Amplicon sequencing

NGS results showed that diversity indices of Keszthely and Máriafürdő samples were similar in case of all types of indices, while Tihany sample (despite the fact that cell counts were high even at that site) produced much lower values. The Chao indices (abundance-based estimator of species richness) were 747.5 in case of Tihany, 1433.4 in case of Keszthely and 1509 in case of Máriafürdő sample, respectively.

The distribution of the different bacterial phyla and dominant classes among the samples is given in Fig. 1.

Results show that on the level of phyla the samples are similar: Proteobacteria, Bacteroidetes and Actinobacteria were the most abundant members of the communities at each site and many unclassified bacteria: Planctomycetes, Cyanobacteria and Patescibacteria also occurred. Abundance of Firmicutes was relatively low in case of each sample. On the other hand, the ratio and composition of the different lower-level taxa differed among the samples. Most abundant orders among Alphaproteobacteria were Rhizobiales, Azospirillales, Rhodobacterales, Rhodospirillales and members of SAR11 clade (Azospirillales and Rhodospirillales were present only at Tihany region), among Deltaproteobacteria, Bdellovibrionales, Myxococcales and many sulphate-reducing bacteria (SRB) appeared, while Gammaproteobacteria

Table 1 Results of the determination of physical and chemical parameters and cell count values of Lake Balaton in the summer of 2018

Sampling site	T °C	Conductivity (µS/cm)	pH	Dissolved oxygen (mg/L)	TOC (mg/L)	DOC (mg/L)	TON (mg/L)	TOP (µg/L)	Chlorophyll a (mg/m^3)	NO_3^- (mg/l)	SO_4^{2-} (mg/l	Cell count/mL
Tihany	22	680	8.6	9.14	7.7	7.6	0.646	39	5.33	0.1	119	6.34E+06
Máriafürdő	22.3	610	8.5	9.52	9.6	8.5	0.956	66	21.3	0.1	94	6.13E+06
Keszthely	23.4	600	8.4	10.01	9	8.7	0.766	65	7.7	0.1	91	2.75E+06

Table 2 Results of the taxon-specific, ESBL-producing and macrolide resistance genes by PCRs

Sampling site	*Pseudomonas* spp.	*P. aeruginosa*	*Legionella* spp.	*L. pneumophila*	*Stenotrophomonas maltophilia*	*Acinetobacter baumannii*	ESBL-production	Macrolide resistance
Tihany	+	−	+	−	+	−	−	−
Máriafürdő	+	−	+	−	+	−	+	−
Keszthely	+	−	+	−	+	−	+	−

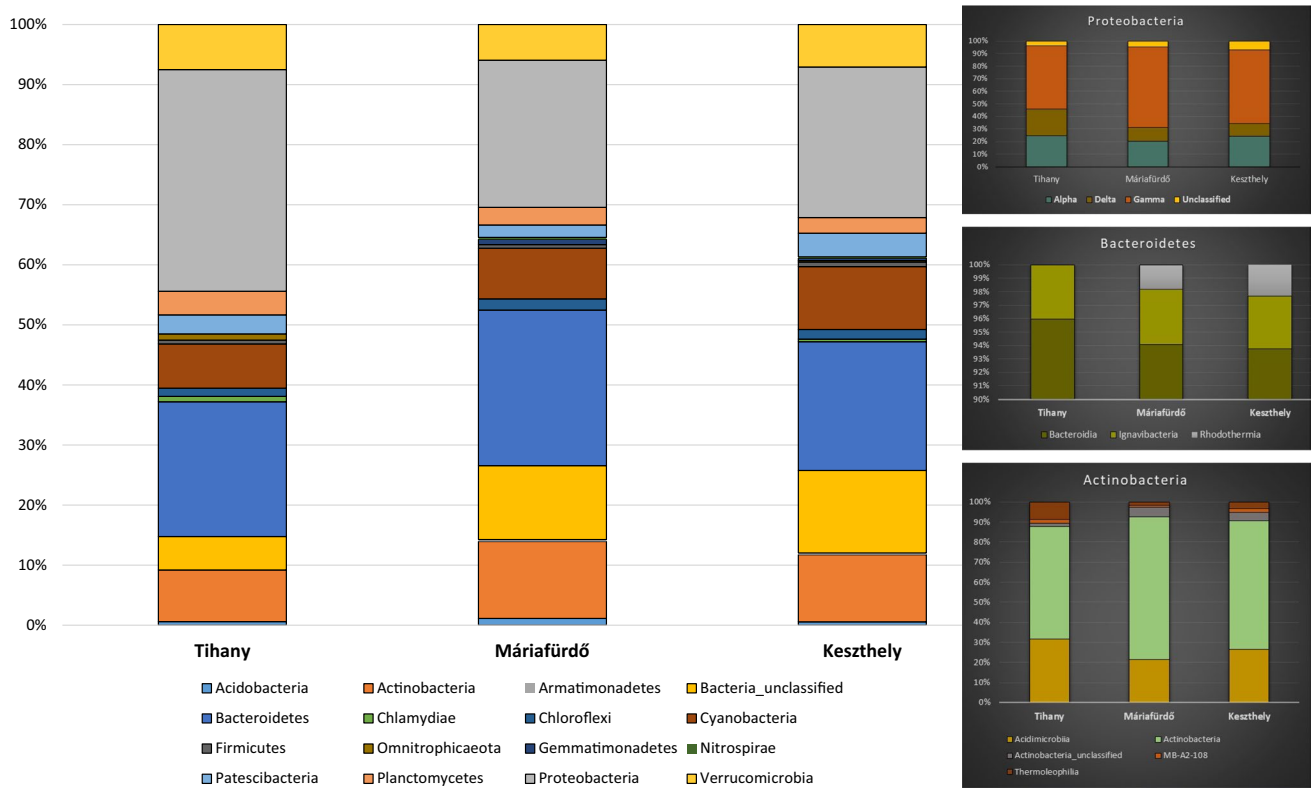

Fig. 1 The distribution of the different bacterial phyla and the ratio of dominant classes among the samples of Lake Balaton

(giving more than 60% of Proteobacteria at each site) were represented by the abundance of unclassified Burkholderiaceae, Methylococcales (was not present at Tihany sample), Betaproteobacteriales, Pseudomonadales and Xanthomonadales. Enterobacteriales were present only in low numbers in all samples.

From the phylum Actinobacteria members of Microtrichales, Frankiales and Micrococcales appeared in high number, their ratio was higher at Máriafürdő and at Keszthely. Among Bacteroidetes, the orders Chitinophagales, Cytophagales, Flavobacteriales and Sphingobacteriales were abundant, at Tihany sample, less orders were identified than in the other two samples.

Three different types of phototrophic metabolisms appeared in the samples: Cyanobacteria are oxygenic phototrophic organisms, Chloroflexi are anaerobic, organotrophic bacteria and the aerobic, anoxic photoheterotrophic group (AAPs). Their distribution among the samples and the ratio of different Cyanobacteria are displayed in Fig. 2.

Among Cyanobacteria, ratio of Nostocales was high at Keszthely and Máriafürdő samples, while members of Synechococcales were more abundant in the Tihany region, and they are represented by the dominance of Cyanobium (picocyanobacterium). Among Chloroflexi, members of the order Anaerolineales were the most abundant, but they did not appear in Tihany sample.

In case of Archaea, the diversity indices showed similar tendencies than in case of bacteria, Chao indices were 108.27 in case of Tihany, 178.9 in case of Keszthely and 119.02 in case of Máriafürdő sample, respectively, and values were much lower than in case of bacteria.

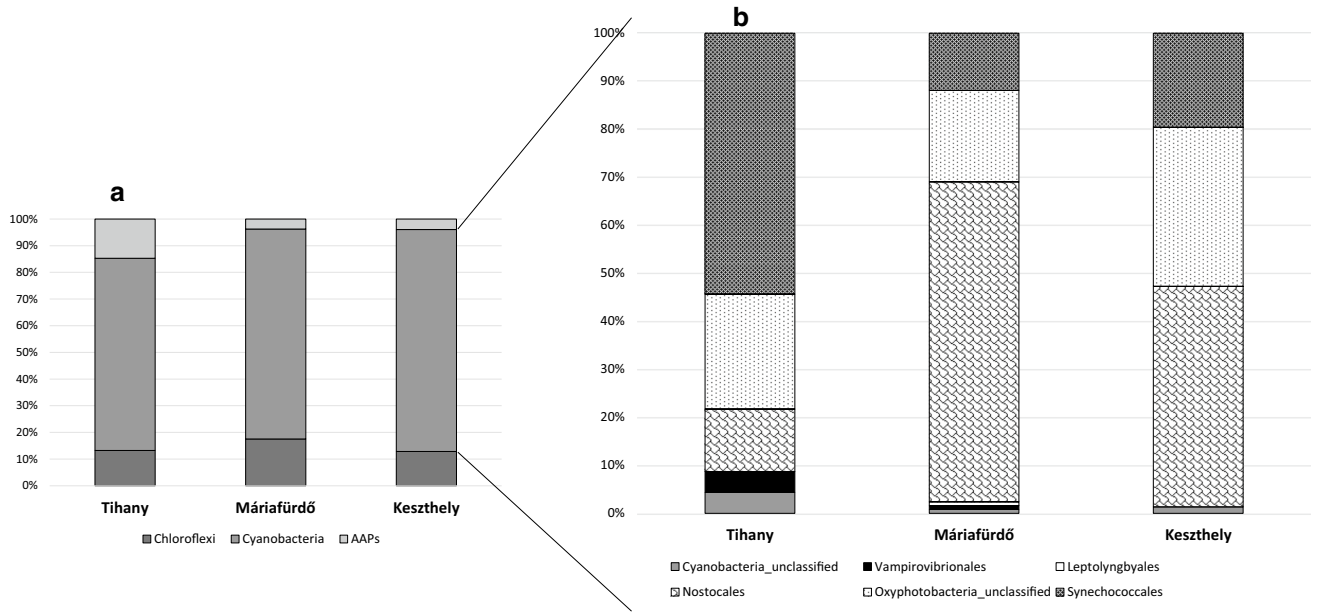

Fig. 2 **a** Distribution of phototrophic bacteria among the samples. **b** Ratio of the different Cyanobacteria (Nostocales, Synechococcales, Vampirovibrionales, Leptolyngbiales and unclassified Cyanobacteria) among the samples of Lake Balaton

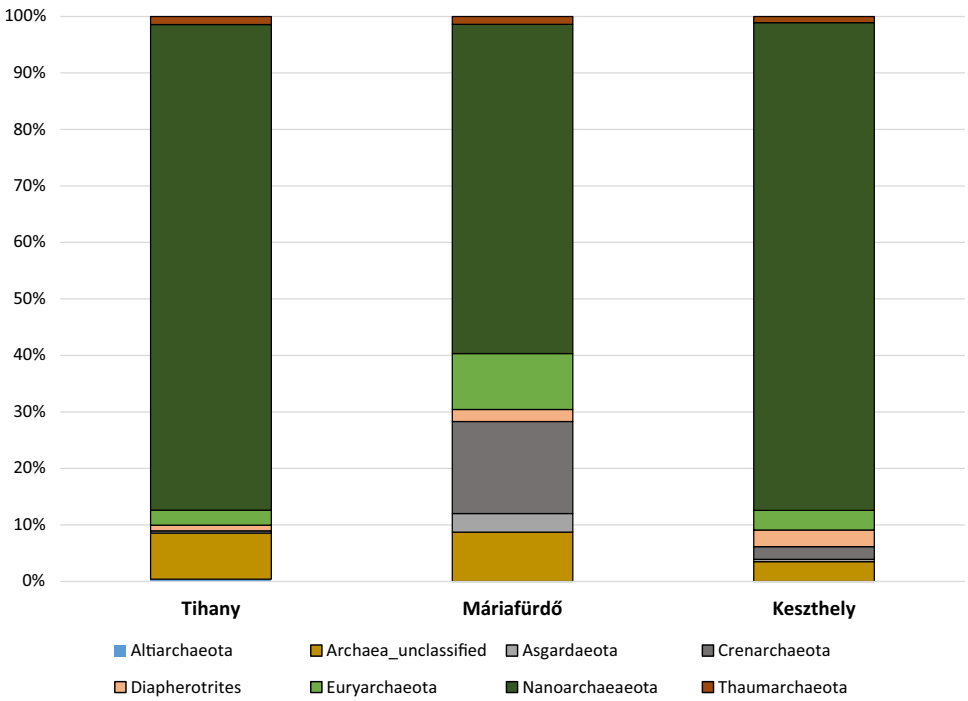

Fig. 3 Distribution of archaeal phyla among samples of Lake Balaton

The archaeal phyla appearing at the samples are shown in Fig. 3.

The absolute dominance of Nanoarchaeota was obvious in all samples, and Woesearchaeia dominated each sample among them: representing more than 65% of each community. Crenarchaeota (e.g. Bathyarchaeia) and Euryarchaeota (e.g. Methanobacteria and Thermoplasmata) were very less abundant in Tihany sample, and Asgardeota did not even appear there. Unclassified Archaea were present in all of the three samples.

Discussion

The quality of the surface waters (e.g. lakes, rivers, streams) is very important, they often serve as basis for drinking waters or act as touristic sites, also for bathing purposes. In the nature, complex systems exist, where the characteristics of the given habitat are determined by inside and outside effects, influenced by interacting microbial populations as well as anthropogenic effects (e.g. bathing or any contamination). Studies using next-generation sequencing (NGS) technologies are providing new insights into the ecology of microbially mediated processes that influence freshwater quality.

Among the three sampling sites at Lake Balaton, only small differences could be revealed in the composition of bacterial communities, and all can probably be explained, e.g. by the small shifts and differences in the organic material (TOC, TON, TOP) content or the light access of the different sampling sites. Though the water body is aerobic (the dissolved oxygen content was always high), being a shallow lake, the wind can easily stir the whole water column, so anaerobic microbes can originate also from the sediment. Moreover, microbes can live in microhabitats where the oxygen concentration can differ strongly that of the water column.

In case of Lake Balaton, based on the chemical and biological parameters all the three sampling sites are low nutrient content habitat, seemed to be oligotrophic (https://www.vizugy.hu/index.php?module=vizstrat&programelemid=149; Borics and Kiss 2015) at time of samplings, though to state it, more investigations should be done (e.g. checking macrozoobenthos, macrophyton, etc.). But our result shows that even at the end of a summer period, when anthropogenic effect is higher due to bathing, the self-purification of the lake is adequate. Studying the seasonal dynamics of the phytoplankton, Somogyi et al. (2016) revealed a west-east trophic gradient in the longitudinal axis in the lake, similar to the previous years.

In 2018, the tendency is similar, though the cell counts of bacteria were in the same magnitude of all sampling sites. At the same time, the diversity indices (based on amplicon sequencing) showed the bacterial communities are less diverse in the Tihany region. It is also true that the DOC values as well as TON and TOP were the less at that site: if the amount of the available substrates is lower, most probably their concentration is limited, and these circumstances favour the reproduction of fewer types of organisms (Howarth and Marino 2006).

Taxon-specific PCRs did not show pathogenic microbes among the studied ones, but the presence of ESBL-producing bacteria at Keszthely and Máriafürdő may indicate anthropogenic effect or other faecal contamination (De Boeck et al. 2012; Blaak et al. 2014), since these regions are strongly affected by bathers.

If we check the composition of bacterial communities, we can immediately see that the diversity of the lake is much higher than we have ever thought. NGS results have not only shown the presence of many non-cultivable taxa but indicated also that those bacteria which previously were thought to be dominant, occur in the lake only with low frequency. Since, that our results are based on molecular data comparisons with previous studies is not really applicable, it is worth mentioning: members of Firmicutes appeared only with low abundance in the water of the lake at each sampling sites (e.g. *Bacillus*, *Exiguobacterium* species) though they were frequently isolated earlier (Langó 1982; Homonnay et al. 2011).

Among Proteobacteria, mainly different *Pseudomonas*, *Aeromonas* and *Acinetobacter* species were detected previously (Tóth, 1995), their seasonal abundance was also remarkable.

The present studies showed that in the water there are several bacteria which can be responsible for N_2 fixation (Rhizobiales, Azospirillales, etc.) which phenomenon can be very important in low nutrient content environments, where the nitrogen is often in limited concentration (Howarth and Marino 2006). Nitrogen fixation was documented also in case of Lake Balaton earlier (Kovács et al. 2012; Borics et al. 2016), authors revealed that during eutrophication processes nitrogen-fixing, filamentous cyanobacteria became frequent in the western part of the lake. This time also the heterotrophic, free living nitrogen-fixing bacteria were identified.

Based on literature data, bacteria belonging to SAR11 have an oligotrophic lifestyle with slow but efficient nutrient uptake at low substrate concentrations (Salcher et al. 2011). The concentration of TOC was low at each sampling site, which could have a positive effect on the abundance of subgroup III of SAR11.

The presence of Bdellovibrionales and Myxococcales (and even that of Vampirovibrionales) shows that even predator/parasite prokaryotes are present in the water body (Li et al. 2014).

Actinobacteria were also detected earlier in Lake Balaton (Szabó, 1982; Farkas, 1982; Tóth, 1995), they are often described in freshwaters and sediments of lakes. Holmfeldt et al. (2009) demonstrated that the prevalence of the clades of different Actinobacteria has changed in relation to total phosphorus (TOP) and Chl A, respectively. In our studies, several actinobacterial taxa were detected, among them Frankiales with the abundance of the hgcI clade. Though the exact role of these bacteria in Lake Balaton has to be defined by later studies, it is known that they can play a dominant role in degradation processes. Members of hgcI clade are common and abundant in a wide range

of freshwater habitats (Warnecke et al. 2004); they have also the potential to utilize sunlight via actinorhodopsin which might promote anaplerotic carbon fixation (Ghylin et al. 2014). Ghai et al. (2014) studying a freshwater lake in Spain by metagenomic analysis showed a remarkable potential of their genomes to transform recalcitrant plant detrital material (e.g. lignin-derived compounds). Moreover, they detected that the abundances of Actinobacteria correlate inversely to those of Cyanobacteria that can be responsible for prolonged damage to freshwater ecosystems.

Our results connected to other phototrophic bacteria showed that their abundance is high in the water of Lake Balaton, which is not new. Kovács et al. (2012) described the effect of irradiance on the germination of N_2-fixing, filamentous cyanobacteria in the sediment of Lake Balaton with varying phosphorous supply. They focused their studies on the most invasive Cyanobacteria of the lake (*Cylindrospermopsis raciborskii* and *Aphanizomenon flos-aquae*). Somogyi et al. (2016) detected the dominance of picocyanobacteria in the summer and dominance of picoeukaryotes in the winter period. Cyanobia (Synechococcales) which are picosized cyanobacteria were observed also in the open water of Lake Fertő (Somogyi et al. 2010); we showed they are dominant in the Tihany region in the summer of 2018, and the abundance of these bacteria is not surprising. Felföldi et al. (2011) demonstrated also the diversity and seasonal dynamics of photoautotrophic picoplankton in Lake Balaton.

The members of Anaerolineales (Chloroflexi) are strictly anaerobic, organotrophic organisms, they are often found in freshwater sediments (Wise et al. 1997) and they can be responsible for metabolizing even different organic substrates.

Aerobic anoxic photoheterotrophic bacteria (e.g. Rhodobacterales) are a diverse group of bacteria that produce bacteriochlorophyll a in the presence of oxygen (Ruiz-González et al. 2020). They are facultative photoheterotrophic bacteria being able to gain energy also from the utilization of light, which gives them an advantage over obligate heterotrophic bacteria (Szabó-Tugyi et al. 2019).

Cytophaga–Flavobacterium group and Chitinophagales are Gram-negative, chemoheterotrophic organisms (Kirchman, 2002), often able to degrade polymer substrates as cellulose, and chitin may even degrade recalcitrant organic substrates (Berg et al. 2009).

In case of Archaea, our knowledge is still less connected to their possible role as most of the novelty discovered phyla do not have any cultured representatives (Castelle and Banfield 2018). On the basis of 16S rDNA sequence comparisons, the phylum "Nanoarchaeota" represents a deep lineage within the Archaea. The predominant archaeal group of our water samples belonged to Woesearchaeia; they are widely distributed in nature, anaerobic, fermentative, syntrophic bacteria that contribute significantly to the biogeochemical cycles of iron and methane (Gayner 2018).

Members of Bathyarchaeia (phylum Crenarchaeota) are generalists, which appear in various environments. They are incorporated in Feammox processes-anaerobic ammonium oxidation coupled to Fe(III) reduction (Rios-Del Toro et al. 2018). Some of them are involved in anaerobic methane oxidation. Evans et al. (2015) presumed also the syntrophy between Bathyarchaeota and sulphate-reducing bacteria (SRB) towards anaerobic oxidation of methane.

Conclusions for future biology

As a conclusion, we can state that the bacterial as well as archaeal diversity of the water of Lake Balaton is high and even when many taxa are uncultivable, the novel molecular studies can reveal their hidden diversity. Prokaryotes are organized in complex communities, they can be involved in many different metabolic processes, chemotrophic as well as phototrophic organisms were also detected, just as bacteria involving many oxidation/reduction reactions, etc. The microbial communities we have revealed can be responsible for many steps in the biogeochemical cycles of carbon, nitrogen, sulphur but also that of phosphorous or iron: to discover it in details, further investigations are necessary. It is true that the diversity and abundance of bacterioplankton depend on the phytoplanktonic primary production, but heterotrophic bacteria most probably are involved also in degradation of the residuals of different chemical contaminations which have been detected in the lake (Simon-Delso et al. 2015; Avar et al. 2015; Maasz et al. 2019), these can be removed by the help of these complex bacterial communities. Though the self-purification of the water is strong, humans must consider keeping the water quality safe for further use.

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Data accessibility Sequence reads were deposited in the NCBI SRA database and are accessible through the BioProject ID SAMN14732963 (Tihany), SAMN14732959 (Máriafürdő) and SAMN14732964 (Keszthely). Data of physical and chemical analysis were collected from the official database of National Directorate of Water Management.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest connected to data of the paper.

Ethical statement Our paper does not contain any experiments connected to humans or animals.

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