Microbiological investigations of two thermal baths in Budapest, Hungary. Report: effect of bathing and pool operation type on water quality

Anett Lippai, Rózsa Farkas, Sára Szuróczki, Attila Szabó, Tamás Felföldi, Marwene Toumi and Erika Tóth

ABSTRACT

In Hungary, which is famous for its thermal baths, according to the regulations, waters are investigated in hygienic aspects with standard cultivation methods. In the present study, two thermal baths were investigated (the well and three different pool waters in both) using cultivation methods, taxon-specific polymerase chain reactions (PCRs), multiplex PCRs and next-generation amplicon sequencing. Mainly members of the natural microbial community of the well waters and bacteria originating from the environment were detected but several opportunistic pathogenic taxa, e.g., *Pseudomonas aeruginosa*, *P. stutzeri*, *Acinetobacter johnsoni*, *Acinetobacter baumanni*, *Moraxella osloensis, Microbacterium paraoxydans, Legionella* spp., *Stenotrophomonas maltophilia* and *Staphylococcus aureus* were revealed by the applied methods. Pools with charging-unloading operation had higher microscopic cell counts, colony-forming unit (CFU) counts, number of cocci, *P. aeruginosa* and *S. aureus* compared to the recirculation systems. Bacteria originating from human sources (e.g., skin) were identified in the pool waters with less than 1% relative abundance, and their presence was sporadic in the pools. Comparing the microbiological quality of the pools based on the first sampling time and the following four months' period it was revealed that recirculation operation type has better water quality than the charging-unloading pool operation from a hygienic point of view.

Key words | amplicon sequencing, bacterial diversity, pool operation type, spa, well and pool waters

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HIGHLIGHTS

- The study is a first insight to the microbial community of two thermal baths.
- Standard hygienic investigations completed with special cultivation and molecular methods.
- Investigations of the microbial community of the wells and different pools.
- Operation type of the pools are compared.
- Monitoring of the thermal bath.

INTRODUCTION

The capital of Hungary is famous for one of the greatest natural thermal water systems in Europe, which is used for medical treatments and for leisure purposes (Goldscheider *et al.* 2010). Due to hygienic considerations, disinfection is required to inactivate the microorganisms (Peters 2016). In Hungary, according to the 37/1996 (X.18.) governmental regulation, two types of water operation are allowed in pools: recirculation (with filters and disinfectant) and

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charging-unloading operation type (without disinfectant treatment) (37/1996 (X.18.)). The most commonly used disinfectants are chlorine-based products which have several disadvantages, e.g., the appearance of chlorine-resistant microorganisms or the formation of disinfection by-products (Peters 2016). In Hungary, using disinfectants in thermal pools are permitted only when the disinfectant does not damage those chemical compounds of the water (e.g., bromide, sulfide, iodide) which can be responsible for the 'therapeutic effects' of the water, but most of these beneficial effects have still not been confirmed by laboratory and clinical studies. Therefore, in many thermal pools, the charging-unloading operation type water treatment system is used where disinfectants are not applied, and pools are simply charged with fresh well water before bathing (Vargha *et al.* 2015).

Thermal waters (including the well and pool waters) are monitored by the national authorities using standard cultivation methods with special attention to hygiene. These methods focus on the faecal contamination (e.g., thermotolerant coliform bacteria) and the non-faecally derived (*Legionella* spp., *Pseudomonas* spp., *Staphylococcus aureus*) pathogenic or facultative pathogenic microorganisms (MSZ 13690-3:1989; MSZ 15234:2012; 49/2015 (XI.6.)). The microbial diversity of thermal waters is rarely investigated for their complex diversity, especially not with the combination of cultivation, molecular and microscopic techniques, though it can give an insight into a hidden diversity (Vartoukian *et al.* 2010). Several molecular methods (e.g., polymerase chain reaction (PCR), quantitative PCR, microarrays) have already been used in the detection of pathogens (Deshmukh *et al.* 2016); however these methods focused only on specific pathogenic microorganisms. Next generation amplicon sequencing (NGS) platforms allow the identification and characterization of microbial community members in an ecosystem and also promote the identification of the microorganisms present in low number (Ghilamichael *et al.* 2017).

During our studies two thermal baths were investigated: TB1 and TB2. The well water of the bath TB1 breaks from 372 metre depth with 44 °C temperature and the well water of TB2 breaks from 1,246 metre depth with 76 °C temperature. Both baths have different pools with different temperature, location and operation type. The aim of the present study was to: (1) study the natural bacterial communities of the different well waters; (2) study the possible effect(s) of different pool operation type in the microbial communities; and (3) examine the thermal waters in hygienic aspects and so analyse the bathing effect. For these reasons we used both cultivation and cultivation independent techniques.

METHODS

Sampling

TB1 sampling was carried out from a tap originating from the well (W1) and three thermal pools (PCU38_1, PCU20_1, PCIRC38_1). TB2 sampling was carried out from a tap originating from the well (W2) and three thermal pools (PCU38_2, PCU20_2, PCIRC38_2). The characteristics of the well and pool waters are given in Table 1. After the first sampling the microscopic cell counts were determined four times in the next four months: (1) before the bath's opening and (2–4) after the bath's opening from the well waters and the 38 °C pools in the same day. The number of people observed in the pools at the time of samples' collection was between none and three.

The water samples (1 L/sample) were aseptically collected into clean, sterile, glass bottles, supplied with

Table 1 | Characteristics of the samples

Sample code	Temperature (°C)	Location	Operation type	Number of people observed in the pools at time of sample collection
W1	44	NA	NA	NA
PCU38_1	38	Indoor	CU	12
PCU20_1	20	Indoor	CU	1
PCIRC38_1	38	Outdoor	CIRC	8
W2	76	NA	NA	NA
PCU38_2	38	Indoor	CU	1
PCU20_2	20	Indoor	CU	1
PCIRC38_2	38	Outdoor	CIRC	11

W1; W2: well; PCU38_1; PCU20_1; PCIRC38_1; PCU38_2; PCU20_2; PCIRC38_2: pools. NA: not applicable; CU: charging-unloading systems; CIRC: recirculation systems.

sodium-thiosulfate (pentahydrate) as a disinfectant reducing agent. The well waters were taken through a tap and the pool waters were taken from the middle of the pool from 10 to 30 cm subsurface with a sampling pole according to ISO 19458:2006. During transport ice packs were used to cool samples which were transported to the laboratory at 4 °C and processed within 1 hour after sampling. Samples were collected in the middle of the day, and the number of people observed in the pools at the time of samples' collection is shown in Table 1.

Determination of the chemical parameters

To determine the chemical parameters of the well waters, standard tests were used: sodium content and potassium content according to ISO 11885:2007 ISO 11885:2007 calcium content according to MSZ 448-3:1985 magnesium content according to MSZ 448-3:1985 chloride content according to MSZ 448-15:1982 fluoride content according to ISO 10304-1:2007 sulfate content according to MSZ 448-13:1983 and hydrogen-carbonate content according to ISO 9963-2:1994.

Determination of the hygienic microbiological parameters

For this purpose, standard cultivation methods were used: number of faecal coliform bacteria, micrococcus, *S. aureus* and *Pseudomonas aeruginosa* were determined according to MSZ 13690-2:1989; *Legionella* spp. according to ISO 11731-2:2004, colony count according to ISO 6222:1999 using nutrient (DSMZ medium 1, www.dsmz.de) and 10% R2A agar media (DSMZ medium 830).

Determination of microscopic cell counts

Determination of microscopic cell count with DAPI staining was carried out as described by Máthé *et al.* (2014). For the investigations, 10 mL of the pool waters and 200 mL of the well water samples were filtered on sterile polycarbonate filters (Millipore, Billerica, MA, USA) and fixed with 2% paraformaldehyde solution.

Cultivation and taxonomic identification of the isolated bacterial strains

To isolate and maintain bacteria, 10% R2A and minimal synthetic media solidified with agar-agar and with gellan gum were used as described by Szuróczki *et al.* (2016). Instead of distilled water, well water was used for each medium. The plates in the case of the W1 well water sample were incubated at 44 °C for 9 days, in the case of the W2 well water at 55 °C for 7 days. The PCU38_1, PCU38_2, PCIRC38_1, PCIRC38_2 water samples were incubated at 37 °C for 7 days, while the PCU20_1 and PCU20_2 water samples were incubated at 20 °C for 7 days. To enrich bacteria of the well water samples, the PUF (polyurethane foam) technique described in detail by Szuróczki *et al.* (2016) was also used, for this, incubation was carried out at 44 °C for 3 weeks in the case of W1 and at 55 °C in the case of W2 water sample.

DNA was extracted from the isolated bacterial strains as described by Szuróczki *et al.* (2016); the amplification of the 16S rRNA gene was performed as described by Kalwasinska *et al.* (2015). The amplified 16S rRNA gene fragments were digested using *Bsu*RI and *Msp*I enzymes, and bacterial strains were grouped based on their ARDRA profile. Then, group representatives and ungrouped bacterial strains were chosen for 16S rRNA gene sequence analysis. Sequencing and determination of taxonomic identity of bacteria were carried out as described by Szuróczki *et al.* (2016), cut-off value for species level identification was a minimum of 98.65% (Kim *et al.* 2014). GenBank accession numbers of the obtained sequences are MH790296-MH790311, MH915675-MH915682 and MH917333-MH917341 in the case of TB1 bath, MN096612-MN096646 and MN096669-MN096733 in the case of TB2 bath.

Cultivation independent studies

For the molecular investigations, 200 mL of pool water and 500 mL of well water were filtered on sterile cellulose ester filters (Whatman ME 25/21 STL, GE Healthcare Life Sciences, New Jersey, USA).

Taxon-specific PCRs

Taxon-specific PCRs for the following bacterial groups were applied: *P. aeruginosa* according to Lavenir *et al.* (2007),

Legionella spp. according to Cloud *et al.* (2000), *Legionella pneumophila* according to Fiume *et al.* (2005), coliform bacteria according to Bej *et al.* (1990), *Acinetobacter baumannii* according to Tsai *et al.* (2018) and *Stenotrophomonas maltophilia* according to Filho da Silva *et al.* (2004).

Multiplex PCRs

The presence of extended-spectrum β-lactamases (ESBLs) genes and macrolid-resistance genes ermA, ermB, ermC, msrA and mef were examined in all samples using multiplex PCR, as described by Trung *et al.* (2015) and Zmantar *et al.* (2011).

Next generation amplicon sequencing (NGS)

The 16S rRNA gene amplification, sequencing and identification of bacterial taxa were carried out as described by Szabó *et al.* (2017) except that 27F (Lane 1991) and 534R primers (Muyzer *et al.* 1993) were used for the amplification of the V1–V3 regions. Raw sequence data have been submitted to the NCBI Sequence Read Archive under the BioProject ID PRJNA602207.

RESULTS

Results of chemical investigations

The W1 well water was characterized by 155 mg/mL Na^+, 148 mg/mL Ca^{2+}, 184 mg/mL Cl^-, 600 mg/mL SO_4^{2-}, 145 mg/mL F^- and 380 mg/mL HCO_3^- concentration; the W2 well water was characterized by 175 mg/mL Na^+, 152 mg/mL Ca^{2+}, 195 mg/mL Cl^- and 550 mg/mL HCO_3^- concentration.

Results of the investigations of the total bacterial communities

The microbial communities of the well waters

Based on cultivation, from the 44 °C well of TB1 (W1 sample) the members of Firmicutes were dominant, the class Bacilli appeared in the highest number. Genera *Brevibacillus*, *Micrococcus*, *Brevundimonas* and *Ferrovibrio* were cultivated. In well water of the TB2 bath (76 °C; W2 sample) members of Actinobacteria dominated but the number of isolates of this sample was low (Figure 1). With cultivation the genera *Brevibacillus* and *Micrococcus* were also

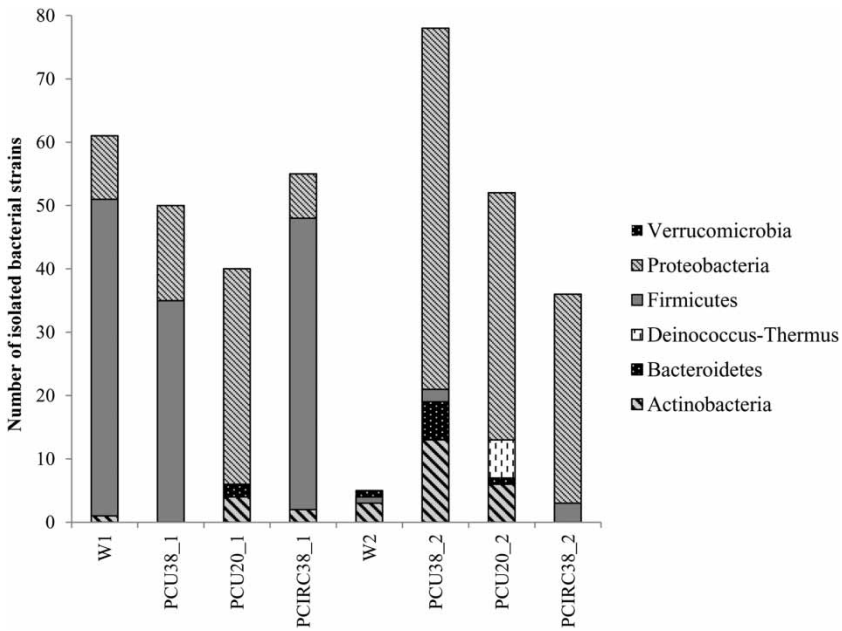

Figure 1 | Phylum-level distribution of bacterial strains detected in the water samples with cultivation. Notation shown in Table 1.

revealed, and as well, *Meiothermus*, *Mycobacterium* and *Roseimicrobium* were detected.

With NGS in TB1 phyla Proteobacteria and Chloroflexi were dominant, and the classes Betaproteobacteria, Gammaproteobacteria and Anaerolineae appeared in high relative abundance. Unclassified *Anaerolineae* (Anaerolineaceae) and unclassified *Zoogloea* (Rhodocyclaceae) were dominant but *Sulfurovum*, *Shewanella*, *Pseudomonas, Actinomyces, Mycobacterium* and *Thiofaba* were also present. In TB2 Proteobacteria was also dominant, and members of Alphaproteobacteria and Gammaproteobacteria were detected (Figure 2). Dominant genera were *Methylobacterium* and *Pseudomonas* but *Sulfurihydrogenibium*, *Shewanella*, *Mycobacterium* and *Actinomyces* were also present.

The microbial communities of the pool waters

With cultivation from TB1 pool waters members of Firmicutes and Proteobacteria were dominant with the classes Bacilli and Betaproteobacteria, except the 20 °C pool (PCU20_1) water sample, where members of Alphaproteobacteria were isolated in the highest number. In TB2 pool waters, Proteobacteria were also dominant with the classes Alpha- and Gammaproteobacteria. In TB1 bath the genera *Brevibacillus*, *Bacillus*, *Hydrogenophaga* and *Rhizobium* and in TB2 bath *Ferrovibrio*, *Pseudomonas*, *Rheinheimera*, *Microbacterium* and *Porphyrobacter* were dominant.

Results of molecular studies showed that in all pool waters of TB1 and TB2 phylum Proteobacteria was dominant (except in sample PCU38_2), the classes Alpha-, Beta- and Gammaproteobacteria appeared in the highest relative abundance but their ratio was different in the samples. As compared with the results of cultivation, the genera *Hydrogenophaga, Pseudomonas, Rheinheimera, Rhizobium, Flavonacterium, Acidovorax, Brevundimonas, Shingomonas* were also detected with NGS. Only with the latter method were *Sulfurovum*, *Thiofaba*, *Methylobacterium*, *Methyloversatilis*, *Moraxella*, *Cloacibacterium* and *Sulfurihydrogenibium* also revealed as important members of the community.

Results of the hygienic investigations of the pool waters

Results of microscopic cell counts and cultivation-based classical microbiological parameters

The microscopic cell counts as well as CFU numbers were 1–2 magnitude higher in all pools as compared to the well waters. The microscopic cell counts of the well waters were checked after the first sampling: before the bath's opening (without bathers) the cell counts of the pool samples were also 1–2 magnitude higher as compared to the wells. The cell counts of the

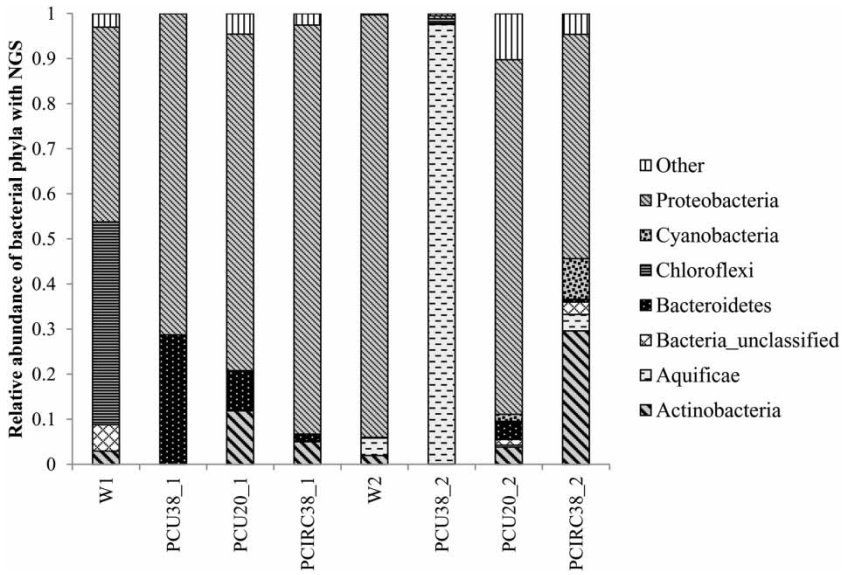

Figure 2 | Phylum-level distribution of amplicon sequencing reads in the water samples detected with NGS. Notation shown in Table 1.

charging-unloading pools were also higher as compared to the recirculation pools (Table 2). CFU values in 10% R2A and CFU in nutrient agar, the number of micrococcus, *S. aureus* and *P. aeruginosa* were higher in all cases in the charging-unloading pools. Faecal coliforms and *Legionella* spp. were not detected in any samples (Tables 3 and 4). According to MSZ 13690-3:1989 MSZ 15234:2012 and 49/2015 (XI.6.) Hungarian regulations, PCU38_1 of TB1 bath exceeded the acceptable limit values regarding the number of micrococcus and *P. aeruginosa*. The value of *P. aeruginosa* was also higher in the PCIRC38_1 (TB1) and PCIRC38_2 (TB2) pools than the limits. None of these bacteria were detected in the well water samples.

Results of taxon-specific PCRs and multiplex PCRs

With taxon-specific PCRs *A. baumannii* and *L. pneumophila* were not detected in any samples. *P. aeruginosa* occurred only in PCU38_2 pool water (with cultivation these bacteria appeared in almost all pool water samples). Coliform bacteria were detected in both 38 C charging-unloading pool water samples (PCU38_1; PCU38_2), *S. maltophilia* occurred in all samples of TB2 and PCU38_2 pool water of TB1 (Table 5).

With multiplex PCRs macrolid-resistance genes were not detected in the samples, ESBL genes were present in the PCU38_1 charging-unloading pool sample.

Table 2 | Microscopic cell counts of the samples during the four months' period

Sample code	Microscopic cell count before opening, Day 1 (cell*mL^{-1})	Microscopic cell count after opening, Day 2 (cell*mL^{-1})	Microscopic cell count after opening, Day 3 (cell*mL^{-1})	Microscopic cell count after opening, Day 4 (cell*mL^{-1})
W1	1.5×10^{4}	1.1×10^{4}	5.9×10^{4}	2.7×10^{4}
PCU38_1	5.9×10^{5}	3.9×10^{6}	5.1×10^{5}	$2.4\times$
PCIRC38_1	2.0×10^{5}	2.8×10^{5}	8.6×10^{4}	3.8×10^{5}
W2	4.8×10^{3}	5.5×10^{4}	6.2×10^{3}	3.9×10^{3}
PCU38_2	1.9×10^{6}	2.9×10^{6}	9.8×10^{5}	1.9×10^{6}
PCIRC38_3	2.7×10^{4}	2.0×10^{6}	4.5×10^{5}	7.5×10^{5}

Notation is shown in Table 1.

Table 3 | Hygienic parameters of the water samples

Sample code	Microscopic cell count (cell/mL)	10% R2A medium (CFU/mL)	Nutrient medium (CFU/mL)	Number of faecal coliform bacteria (CFU/100 mL)	Number of micrococcus (CFU/100 mL)	Number of *Staphylococcus aureus* (CFU/100 mL)	Number of *Pseudomonas aeruginosa* (CFU/100 mL)	Number of *Legionella* spp. (CFU/100 mL)
W1	1.4×10^{4}	1.7×10^{2}	2.0×10^{1}	0	0	0	0	0
PCU38_1	4.9×10^{6}	4.0×10^{5}	2.0×10^{4}	0	4,020*	18	80*	0
PCU20_1	2.6×10^{6}	1.7×10^{5}	2.3×10^{3}	0	2,000	0	50	0
PCIRC38_1	7.8×10^{5}	1.3×10^{5}	1.6×10^{3}*	0	76	0	15*	0
W2	6.1×10^{3}	1.2×10^{2}	0	0	0	0	0	0
PCU38_2	1.4×10^{6}	6.4×10^{4}	1.7×10^{3}	0	2,000	10	50	0
PCU20_2	3.7×10^{5}	8.2×10^{3}	6.5×10^{3}	0	400	0	0	0
PCIRC38_3	9.0×10^{5}	2.2×10^{3}	1.5×10^{2}	0	50	0	10*	0

*Exceeded the standard limit values.
NA: not applicable.
Notation is shown in Table 1.

Table 4 | Standard limit values due to Hungarian regulation according to MSZ 13690-3:1989; MSZ 15234:2012 and 49/2015 (XI. 6.)

	Sandard limit values				
Operation type	***E. coli* (CFU/100 mL)**	**Micrococcus (CFU/100 mL)**	***S. aureus* (CFU/100 mL)**	***P. aeruginosa* (CFU/100 mL)**	***Legionella* (CFU/1,000 mL)**
Charging-unloading	100	2,500	20	50	100
Recirculation	1	250	1	2	100

Notation is shown in Table 1.

Table 5 | Potentially pathogenic bacteria identified from the two thermal baths in Budapest

Genus	Bath	Detection technique	Species	Risk group	Possible diseases	Pool type
Acinetobacter	TB1, TB2	NGS and cultivation	*johnsonii*	2	Pneumonia	CU
			baumanni	2	Pneumonia, blood infection, meningitis, urinary tract infection	CU
Moraxella	TB1, TB2	NGS and cultivation	*osloensis*	2	Conjunctivitis	CU and CIRC
Cloacibacterium	TB1, TB2	NGS	–	1	Urinary tract infection	CU and CIRC
Stenotrophomonas	TB1, TB2	Taxon-specific PCR	*maltophila*	2	Pneumonia, endocarditis	CU and CIRC
Pseudomonas	TB1, TB2	NGS, taxon-specific PCR and cultivation	*aeruginosa*	2	Skin and soft tissue, urinary tract, gastrointestinal diseases	CU and CIRC
			alcaligenes	2	Blood infection, endocarditis	CU and CIRC
			stutzeri	1	Blood, respiratory tract, urinary tract infection	CU
Legionella	TB1, TB2	NGS	–	2	Legionnaires' disease, Pontiac fever	CU and CIRC
Staphylococcus	TB1, TB2	NGS and cultivation	*aureus*	2	Dermal, respiratory, nervous system infection	CU and CIRC
Microbacterium	TB1	Cultivation	*paraoxydans*	1	Peritonitis, catheter-related infections	CIRC
Brevundimonas	TB1, TB2	NGS and cultivation	*nasdae*	1	Pneumothorax, empyema	CU

CU: charging-unloading systems; CIRC: recirculation systems.
Notation is shown in Table 1.

Results of cultivation and NGS

Most of the potentially pathogenic taxa detected by cultivation were revealed also by NGS (Table 5), although obviously more taxa could be detected by the molecular method. On the other hand, the resolution of the applied NGS method allows reliable taxonomic assignment at the genus level, but with the 16S rRNA gene sequencing of the cultivated bacteria, species level identification was also possible.

Bacteria originating from human sources (e.g., skin) was detected from the two thermal baths, although not any obligatory pathogenic bacteria had been isolated and only a few opportunistic pathogenic bacteria were identified in the pool waters with cultivation, e.g., *Pseudomonas stutzeri* (PCU38_1), *Acinetobacter johnsonii* (PCU20_1; PCU20_2), *A. baumanni* (PCU20_2), *Brevundimonas nasdae* (PCU20_1; PCU38_2), *Pseudomonas alcaligenes* (PCIRC38_1; PCU38_2; PCU20_2), *Microbacterium paraoxydans* (PCIRC38_1; PCU38_2), *P. aeruginosa* (all pools of TB2) and *Moraxella osloensis* (PCU38_2; PCU20_2) (Table 5). The appearance of these opportunistic pathogens was higher in those pools where charging-unloading operation type was used without disinfectants (Table 5). Based on the amplicon sequencing the relative abundance of these genera, except *Pseudomonas*, was low in all samples.

Moraxella and *Cloacibacterium* were detected only from pool waters by NGS. With cultivation *M. osloensis* was detected only in the charging-unloading pools of TB2, and this bacterium was missing from all recirculation pool samples. The genus *Cloacibacterium* was detected only with NGS and was identified both from charging-unloading and recirculation pools.

To investigate the bathing effect and the hygienic status of the pool waters in more detail using NGS, taxa under 1% relative abundance were also examined. In that case, several bacteria were detected, which could have originated from human sources (e.g., *Dermabacter*, *Dermacoccus*, *Mobilicoccus*, *Kytococcus, Enterococcus, Staphylococcus*) (Figure 3). These bacteria occurred sporadically in the charging-unloading 20 °C pools and recirculation 38 °C pool samples. The genus *Acinetobacter* was detected both with molecular methods and with cultivation, *A. johnsonii* was observed in the charging-unloading 20 °C pools (PCU20_1; PCU20_2) and *A. baumanni* in the charging-unloading pool of TB2. *Microbacterium* was also detected (this geneus also has opportunistic pathogenic members), *M. paraoxydans* was cultivated from the charging-unloading pool of TB2 (PCU38_2) and in the recirculation pool of TB1 (PCIRC38_1) (Table 5).

DISCUSSION

Bathing is a popular activity for people who use thermal baths for recreation, education, exercise and rehabilitation therapy (Neumann *et al.* 2001; Rapolienė *et al.* 2015). Microbiological investigations of thermal waters play an important role in public sanitation, as due to the shared use of pools several microorganisms can be introduced into the water

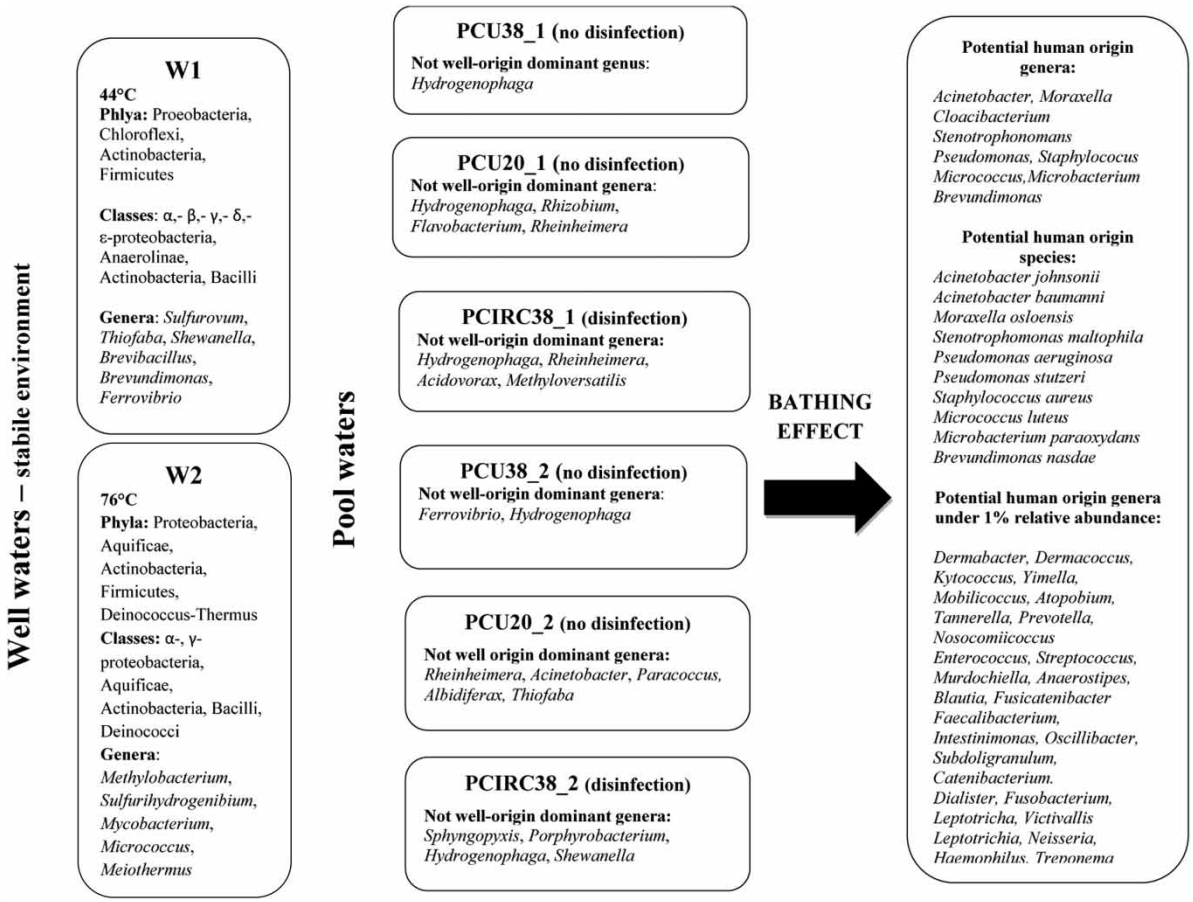

Figure 3 | Changes in the bacterial community structures from the wells to the pools highlighting the bathing effect. Notation shown in Table 1.

during bathing (Vargha *et al.* 2015). Swimming pools are known as transmission vehicles for infection diseases all over the word (bacterial diseases, parasitic diseases, viral diseases) (Rabi *et al.* 2007), although the number of waterborne diseases are usually under-reported and only a few known reports exist which focus on the epidemiology of waterborne microorganisms (Dale *et al.* 2010). In Hungary, we have only limited information about waterborne diseases, the regulations focus on the bacterial contamination, and there are only a few investigations about the viral contamination of thermal baths (Vargha *et al.* 2015).

In the present study, well and pool waters of two thermal baths were investigated with different microbiological methods. Based on our results, the detected taxa were mainly the members of the natural microbial communities which have important roles in aquatic ecosystems. The metabolic properties of these microorganisms have a significant role on water quality, thus the knowledge on microbial taxa provides information about relationships among prokaryotes and environmental conditions (Valeriani *et al.* 2018).

As the wells are not exposed to external disturbance, a stabile bacterial community could be present there. Most probably due to the SO_4^{2-} content of the well water, several detected bacteria in W1 could have important roles in the sulfur cycle (*Sulfurovum*, *Thiofaba*) – hot springs with a constant supply of sulfide are major sites of sulfur-oxidizing bacteria, often developing large biomasses (e.g., microbial mats). The presence of sulfur-oxidizing bacteria is crucial, since they have a significant influence on carbonate equilibrium and promoting carbonate dissolution (Valeriani *et al.* 2018). The genus *Sulfurovum* was also detected in the well water of TB2; the strictly chemolitoautotrophic and thermophilic *Sulfurihydrogenibium* presented only in the 76 °C W2 water (Figure 3). Most probably due to the lack of disinfectants the relative abundance of this genus was higher in the charging-unloading pools.

Both with cultivation and NGS, bacteria of the natural aquatic sources were detected from the samples, i.e., genera often described from aquatic habitats, e.g., *Mycobacterium*, *Flavobacterium*, *Acidovorax*, *Methyloversatilis*, *Rheinheimera*, *Hydrogenophaga*, *Rhizobium*, *Brevundimonas*, *Sphingomonas*, *Pseudomonas* (Sikorski *et al.* 2002; Busse *et al.* 2003; Kumar *et al.* 2014; Szuróczki *et al.* 2016) (Figure 3). *Pseudomonas* was also detected in South African thermal springs using molecular approaches where other members of Proteobacteria (*Hydrogenophaga, Mycobacterium*) were also present (Tekere *et al.* 2015) as in our study. In several cases many sequence reads were only distantly related to any of the known species, therefore, the present study indicates the possibility of undescribed microbes in the investigated thermal baths.

Bacteria from the well water flow into a new environment (to the pools), where the anthropogenic effect and the water treatment influence bacterial growth. Applying cultivation methods, certain heterotrophic microbes can repress the growth of others by their quicker multiplication.

Most probably as a result of the lack of disinfectant water treatment in some pools, the total microscopic cell counts, CFU values on 10% R2A and nutrient agar were higher in the charging-unloading pools than in the recirculation pools which are treated also by chlorine, and of course, all these values were higher than that of the wells. The microscopic cell counts of the charging-unloading pools were also higher before the bath's opening as compared to the recirculation pools. It should be considered that from the wall of the pools, biofilms can get into the water which increase the values in those pools where disinfectant is not applied.

It should be noted that the number of people bathing, the size and temperature of the pools and the outdoor environmental factors should also be considered when the effect of water usage is considered.

In our study only a few opportunistic pathogenic bacteria were detected by the applied methods. Based on previous laboratory investigations in the charging-unloading pools, it varied which bacteria exceeded the standard limit values prescribed by the government regulation, but in many cases, *Escherichia coli* was present which indicates faecal contamination (Vargha *et al.* 2015). In public sanitation the presence of faecal contamination can be distressing due to the other microorganisms that can get into the pool waters. Micrococcus and *Staphylococcus* represent the overload of the pools while *Pseudomonas* and *Legionella* most probably indicate the presence of biofilms within the systems (Vargha *et al.* 2015). From TB1 and TB2 with standard methods faecal coliform bacteria and *Legionella* spp. were not detected in any samples but the

number of micrococcus and the number of *P. aeruginosa* and *S. aureus* were higher in the charging-unloading pools than in the recirculation pools, most probably due to the application of chlorine in the recirculation systems. The quality of the waters (except the PCU38_1, PCIRC38_1 and PCIRC38_2 pools) was good and complied with the standard values recommended by the regulations. Most probably due to the charging-unloading operation type and the overload of the PCU38_1, the number of micrococcus exceeded the standard limit values. The higher number of *P. aeruginosa* in PCU38_1 and in the recirculation 38 °C pools most probably referred to the presence of biofilms in the pools. During sampling biofilms of the pool surfaces were not sampled, as the investigation's initial aim was into the microbial community of the thermal pools as compared to the original source (well) water.

There are several reports on the microbiological quality of swimming pools when pools were contaminated with *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *Klebsiella pneumoniae*, *Enterococcus faecalis* and the number of micrococcus exceeded the limit values using standard cultivation methods (Papadopoulou *et al.* 2008; Agbagwa & Young-Harry 2012; Amala & Aleru 2016). In our study, these bacteria were also detected in the pool waters (*P. aeruginosa*, coliforms and *S. aureus*). The genus *Micrococcus* has several opportunistic pathogenic members, e.g., *M. luteus* was detected with oligotrophic cultivation which can cause nosocomial infections (Yang *et al.* 2001).

There is only limited information about the investigations of spa waters with molecular methods regarding hygienic aspects, although in Eritrea five hot springs which are used for recreational purposes were studied with NGS. Members of Proteobacteria and Firmicutes, the genera *Pseudomonas*, *Bacillus*, *Legionella*, *Acinetobacter*, *Moraxella* occurred in these hot springs, too. With NGS the genus *Moraxella* was also detected in all pool water samples in this study but not from the well waters, indicating that these bacteria can get into the pools from human sources (Maruyama *et al.* 2018). Furthermore, *M. osloensis* was successfully cultivated, which can cause bacteraemia, and previously was described from clinical specimens (Maruyama *et al.* 2018; Shah *et al.* 2019). The genus *Acinetobacter*, which has several opportunistic pathogenic members, was detected with NGS and also with cultivation. The presence of two species within this genus was confirmed: *A. johnsonii* which is commensal on human skin and can be the causative agent of bacteraemia in immunocompromised patients (Seifert *et al.* 1993) and *A. baumanni* which can colonize the respiratory tract, skin, urinary system and gastrointestinal system, and can be responsible for nosocomial infections (Hakyemez *et al.* 2013). The abundance of these bacteria was low, and only one strain was cultivated from the 20 °C charging-unloading pool of TB2 (PCU20_2).

The cultivated *Microbacterium paraoxydans* has never been isolated from environmental sources and can cause nosocomial infections in immunocompromised patients (Laffineur *et al.* 2003; Chorost *et al.* 2018).

Most detected opportunistic pathogenic bacteria are classified as Risk 2 group members and occurred mainly in the charging-unloading pool waters but several bacteria are also classified as Risk 1 group members (Table 5). The presence of extended-spectrum β-lactamases (ESBLs) genes in the charging-unloading pool also suggests the presence of microbes of human origin, even that of pathogenic or facultatively pathogenic bacteria. It suggests that in spite of the medical effect of the waters, the charging-unloading system is not adequate for eliminating all hazardous microorganisms from the water body.

Traditional disinfectants are mainly chlorine-based products, but there are other chemicals which can promote adequate microbial quality of pool waters, e.g., bromide, ozone, UV radiation, hydrogen-peroxide, but they are not applied widely because of the costs and special terms of usage, therefore most of them can be used as an additional treatment (Kruithof *et al.* 2007; Holmgreen 2012). In Hungary, there are several studies with alternative water treatments, but at present, integrated regulation does not exist. Therefore, water treatment has an important role in water quality, and with adequate operation the public sanitation risks can be minimized (Vargha *et al.* 2015).

To maintain the appropriate microbiological quality of pool waters, the operators should follow the recreational water guidelines for proper management of (swimming) pools; bathers should adhere to good sanitary practices and the public health authorities should monitor swimming pools. Proper sanitation is needed to maintain the visual clarity of water and to prevent the transmission of infectious diseases. Sanitation methods include filtration to remove

pollutants, disinfection to kill infectious microorganisms and swimmer hygiene to minimize the introduction of contaminants into pool water.

CONCLUSIONS

In the present study, the microbial communities of two thermal baths were investigated by cultivation and molecular techniques from ecological as well as from hygienic aspects. Results revealed that mainly the members of the natural microbial community of the well waters and bacteria originating from the environment occur in the pools but bacteria of human sources can also appear. The operation type of the pools strongly influences the water quality: from a hygienic point of view the recirculation operation type is much more effective in eliminating opportunistic pathogens than the charging unloading type. Therefore, careful consideration must be adopted with regard to spa pools and ensuring a balance between microbiological risk, disinfectant use and the medical effect(s) of the waters.

The study was a first insight into the composition of the microbial community of thermal baths, considering the effect of bathing and water treatment. The investigation of biofilms remains a further step of the research.

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