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Bacteriome composition analysis of selected mineral water occurrences in Serbia

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Abstract: Bacterial metabarcoding analysis by 16S rDNA of five occurrences of mineral waters in Serbia (Torda, Slankamen Banja, Lomnički Kiseljak, Velika Vrbnica and Obrenovačka Banja) indicated the presence of a high percentage of the Proteobacteria phylum, followed by the Bacteroidetes phylum. The families Rhodobacteraceae, Burkholderiaceae, Pseudomonadaceae, Methylophilaceae and Moraxellaceae were the most dominant in the bacterial flora of the selected occurrences, whereas the most represented genera were *Acinetobacter*, *Pseudorhodobacter*, *Pseudomonas*, *Limnohabitans*, *Massilia*, *Limnobacter* and *Methylotenera*. The presence of coliform bacteria was not detected. Alpha diversity analysis revealed that Slankamen Banja and Lomnički Kiseljak were the richest of the selected occurrences, while the mineral waters of Torda, Velika Vrbnica and Obrenovačka Banja were characterized by similar diversity of bacterial communities determined by beta diversity analysis. Physical-chemical analysis revealed the value of total dissolved solids above 1 g/L, as well as elevated concentrations of some metals and non-metals. The research concluded that specific bacteria contribute to the development of biocorrosion and biofouling processes of water intake facilities. In addition, some of these bacteria might be potential indicators of the organic sources of pollution and/or biotechnological natural remediators in the treatment of contaminated waters.

Keywords: mineral waters; bacterial metabarcoding analysis; 16S rDNA; alpha and beta diversity analysis; physical-chemical analysis.

INTRODUCTION

Microorganisms were long thought to be distributed up to a depth of 100 m in the Earth's crust [1]. Deeper parts of aquifers have been assumed to be isolated from surface effects of contamination because of surface water infiltration through porous layers or due to the existence of roof watertight barriers, thus excluding mineral groundwater (MGW) habitats from the "contamination chain" [2]. However, with the emergence of persistent waterborne infections, previous claims have been shown to be unreliable [3]. MGW are habitats characterized by a unique microbiological diversity, whose number is estimated at 10^3 to 10^8 cells per mL or per gram of sediment, reaching depths of at least 3 km [1,4]. In subterranean ecosystems, microorganisms are found suspended in MGW or attached to aquifer matrix, with benthic organisms being dominant in terms of biomass and biochemical activity [5]. According to some estimates, more than 90% of the microbial biomass in aquifers is attached to appropriate geochemical substrates [6]. Richer microbiological diversity is characteristic of ecotone zones where microorganisms can be rapidly transported along a steady flow of MGWs [7]. In some cases, microorganism transport can be so slow as to cause mineral

groundwater habitats to remain completely isolated from external factors, thus creating favorable conditions for allopatric speciation [8]. Communities of microorganisms in subterranean ecosystems are represented by bacteria and archaea, along with fungi and protozoa [8, 9]. The presence and role of bacteria in MGWs is primarily considered and investigated for practical and economic reasons, since bacteria affect the service life of water intake facilities, inducing the development of biocorrosion and biofouling. Following the succession of bacterial communities, well aging symptoms are observed, which include (i) an increase in bacterial metabolic activity; (ii) decrease in well capacity; (iii) inadequate water quality; (iv) the inability of the wells to distribute the required water quantities [10]. The development of biocorrosion and biofouling processes causes serious financial losses, where bacterial overgrowth represents the 1st or 2nd most expensive factor in groundwater system deterioration in North America [11]. In addition, more than half of the underground installations' corrosive processes are the result of biochemical activity of bacteria [12], while costs caused by iron corrosion can reach several billion dollars [13]. Moreover, some bacteria can metabolize certain corrosion inhibitors, while trivalent iron sediments, in combination with organic matrix (slime), can block drainage and are a special problem that develops during well exploitation [14]. Also, bacteria can affect the qualitative properties of MGWs, influencing the ecological status of water resources including many disease outbreaks. Apart from the negative indications of bacteriological diversity, it is possible to remedy contaminated water by using the metabolic activity of indigenous or allochthonous communities of groundwater bacteria, since subterranean ecosystems are known to create conditions for biological degradation of pollutants [15].

To gain insight into the taxonomic composition of the bacterial community of selected mineral water occurrences, metabarcoding analyses by 16S rDNA were performed. In addition, alpha and beta diversity analyses were undertaken with the intention of comparing the bacterial diversity among the examined mineral waters and within each occurrence individually. Also, physical-chemical analysis of mineral waters served to comprehensively determine the qualitative status of the investigated mineral water occurrences.

MATERIALS AND METHODS

Study areas

The study sites focused on the bacteriological diversity of the mineral waters of Torda (45.5860° N, 20.4581° E), Slankamen Banja (45.1415° N, 20.2586° E), Obrenovačka Banja (44.6570° N, 20.2125° E), Lomnički Kiseljak (43.5110° N, 21.3281° E) and Velika Vrbnica (43.5251° N, 21.1918° E) and included prior summarization of the geological-hydrogeological characteristics of the investigated occurrences, which were sampled for metabarcoding analysis during the spring/summer of 2019. The studied occurrences of mineral waters are located in different parts of the territory of Serbia. Their flow follows the complexity of geological and hydrogeological conditions in the corresponding geotectonic regions, shown in Supplementary Fig. S1 [16,17].

Sampling, DNA extraction, library preparation and NGS sequencing

The mineral waters of the selected occurrences were sampled using deep sampling bottles from a depth of 50 cm below the water surface. The samples were then collected and transported in accordance with SRPS EN ISO – 19458:2009 standards in sterile glass bottles. Samples were collected from all types of mineral water in 3 replicates and DNA extraction was performed separately for each of them. For each sample, 1000 mL of water was filtered with Isopore™ membrane filters (Merck Millipore Ltd., Dublin, Ireland). The DNA was extracted from 0.22-μm polycarbonate Isopore™ filters for each replicate using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions. The total DNA from each replicate was pooled into 1 sample for each sampling site. The DNA yield of the analyzed samples was measured using Qubit Fluorometric Quantitation (Qubit 4 Fluorometer, Invitrogen™, Carlsbad, CA, USA). The amplicons were amplified following the Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A). The gene-specific sequences used in this protocol target the 16S rDNA V3 and V4 region with defined forward (5'-CCTACGGGNGGCWGCAG-3') and reverse (5'-GACTACHVGGGTATCTAATCC-3')

primers. Illumina adapter overhang nucleotide sequences were added to the gene-specific sequences. The bacterial genomic DNA (5 ng/μL in 10 mM Tris pH 8.5) was used to initiate the protocol. After 16S rDNA amplification, the multiplexing step was performed using a Nextera XT Index Kit (FC-131-1096). One μL of the PCR product was run on a Bioanalyzer DNA 1000 chip to verify the size (~550 bp). The libraries were sequenced using a 2×300 bp paired-end run (MiSeq Reagent kit [MS-102-3001]) on a MiSeq Sequencer according to manufacturer's instructions (Illumina, San Diego, CA, USA).

Sequence data processing, taxonomy annotation and bioinformatic analysis

Quality assessment was performed using the Prinseq-lite program [18], applying the following parameters: min_length_50; trim_qual_right_30; trim_qual_type (mean), and trim_qual_window_20. The sequence data were analyzed using the QIIME 2 pipeline. Denoising, paired-end joining and chimera depletion was performed starting from paired-end reads using the DADA2 pipeline [19]. Taxonomic affiliations were assigned using the Naive Bayesian classifier integrated in QIIME 2 with a taxonomic assignment based on the SILVA_release_132 database [20,21]. Statistical analysis was performed using an ad hoc pipeline written in the RStatistics environment with the Vegan and Biostrings packages [22]. All statistical representations were obtained with the dataset after removing the taxa represented by less than 3 sequences. Alpha diversity was determined by sampling-based analysis of operational taxonomic units (OTUs) and shown through estimator indices (Shannon, Simpson, invSimpson and Fisher Alpha). Observed and estimated richness was determined according to the following estimators: number of observations (OBS), Chao1 and ACE. The data obtained were subjected to analysis of variance (ANOVA). The mean separation was accomplished by the Duncan post hoc multiple comparison test. Significance was evaluated at $P<0.05$ for all tests. Statistical analyses were conducted by the general procedures of IBM SPSS Statistics v.20 (SPSS, Inc.). Additionally, rarefaction analysis was performed to allow an estimation of the overall diversity covered by the obtained sequences. Beta diversity was determined using principal coordinates analysis (PCoA) based on the Bray-Curtis dissimilarity index, which allows visualizing the information in a data set containing individuals/observations described by multiple intercorrelated quantitative variables [23], as well as through hierarchical clustering analysis. Hierarchical clustering analysis was performed using a distance correlation coefficient and the UPGMA method for phyla, families and genera. A thousand bootstraps were applied to obtain statistics for the hierarchical clustering analysis.

Physical-chemical analysis and inductively coupled plasma, optical emission spectroscopy analysis

Mineral waters of selected occurrences were sampled in 5-L volume from wells using deep sampling bottles. The sterile plastic bottles with caps were in accordance with international standards ISO 9001, ISO 14001 and OHSAS 18001. Before use, the packaging for physical-chemical analysis was treated with 1% HCl, rinsed with tap water, then with distilled water, and finally dried. After sampling, the samples were delivered within the standard time limit to the accredited laboratory of the City Institute of Public Health in Belgrade (Serbia), where all necessary parameters defining the physical-chemical composition of mineral waters (total dissolved solids, electrical conductivity, color, odor, oxidizability, Na^+, K^+, Ca^{2+}, Cl^-, SO_4^{2-}, NO_2^-, NO_3^-, NH_3, CO_2, etc.) were analyzed.

The metal contents of the mineral water samples were examined according to the US EPA Method 3051a. Before microwave digestion, the samples were dried at 40°C for 12 h, homogenized and sieved through a 100-mesh sieve. About 500 mg of sample was weighed in closed PFA digestion vessels and 9 mL of concentrated HNO_3 and 3 mL of concentrated HCl were added. A Jupiter-A microwave oven was used for digestion with the following time procedure: 7 min with temperature rising to 175°C, followed by 4 min of temperature maintained at 175°C. After digestion, the vessels were allowed to cool down at room temperature. Then, digests were transferred to volumetric flasks and diluted to 50 mL with deionized water. These solutions were stored in polyethylene flasks until analysis. All prepared samples were analyzed using the Thermo Scientific™ iCAP™ 7400 inductively coupled plasma, optical emission spectroscopy (ICP-OES) analyzer.

RESULTS

Bacterial community composition

To identify bacterial taxa in the selected mineral water occurrences, 16S rDNA sequences were classified according to the SILVA reference database. Results on the bacterial and archaeal phyla and families are summarized in Fig. 1. The most dominant phylum in all mineral water samples was Proteobacteria (Fig. 1A). The largest number of phyla was recorded in the mineral waters of Slankamen Banja with 14 identified phyla. At the family level, Burkholderiaceae was detected in all examined mineral water occurrences (Fig. 1B).

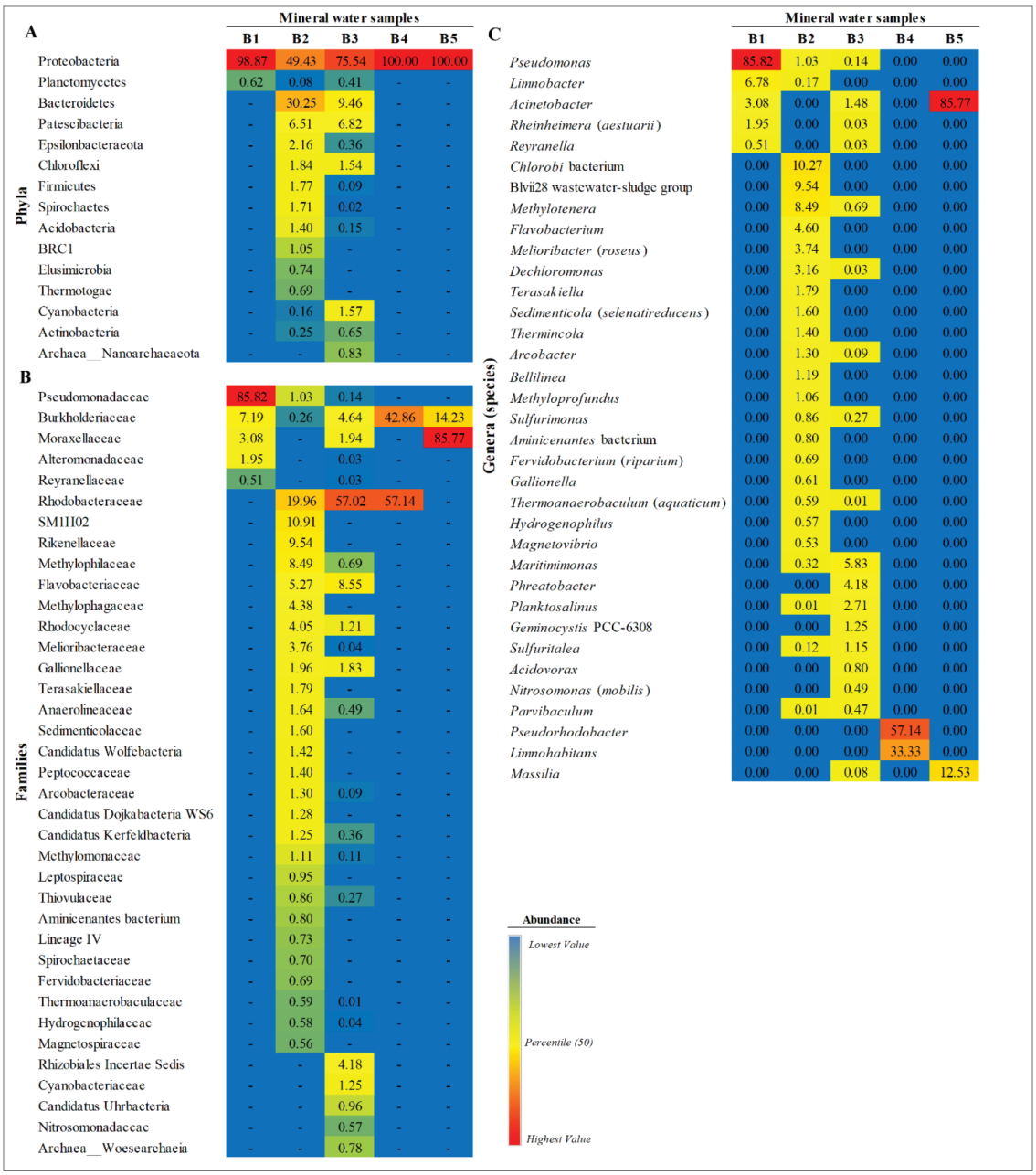

Fig. 1. Relative abundance of bacterial and archaeal taxa as assessed by 16S rDNA sequences at phylum (**A**), family (**B**) and genus (species) (**C**) levels. Torda mineral water – sample B1; Slankamen Banja mineral water – sample B2; Lomnički Kiseljak mineral water – sample B3; Velika Vrbnica mineral water – sample B4; and Obrenovačka Banja mineral water - sample B5. Only taxa with total percentage abundance above 0.5% for all samples were included.

Table 1. Biodiversity measures of obtained taxa trough microbial richness and alpha diversity indices

Occurrence of mineral waters	Shannon	Simpson	InvSimpson	Fisher Alpha	**OBS***	CHAO1	ACE
Torda	$0.44^{b} \pm 0.2$	$0.18^{c} \pm 0.1$	$1.24^{b} \pm 0.1$	$1.40^{b} \pm 0.4$	$9.00^{b} \pm 2.5$	$9.00^{b} \pm 2.5$	$9.25^{b} \pm 2.6$
Slankamen Banja	**$2.72^{a} \pm 0.6$**	**$0.83^{a} \pm 0.1$**	**$9.57^{a} \pm 3.3$**	**$21.79^{a} \pm 9.4$**	**$174.00^{a} \pm 70.7$**	**$174.00^{a} \pm 70.7$**	**$174.00^{a} \pm 70.7$**
Lomnički Kiseljak	**$1.89^{a} \pm 0.4$**	$0.58^{ab} \pm 0.1$	$2.56^{b} \pm 0.4$	**$23.04^{a} \pm 9.4$**	**$162.00^{a} \pm 66.6$**	**$162.00^{a} \pm 66.6$**	**$162.00^{a} \pm 66.6$**
Velika Vrbnica	$0.53^{b} \pm 0.3$	$0.35^{bc} \pm 0.2$	$1.73^{b} \pm 0.4$	$0.45^{b} \pm 0.2$	$2.00^{b} \pm 0.6$	$2.00^{b} \pm 0.6$	$1.00^{b} \pm 1.0$
Obrenovačka Banja	$0.29^{b} \pm 0.1$	$0.16^{c} \pm 0.1$	$1.22^{b} \pm 0.1$	$0.27^{b} \pm 0.1$	$2.00^{b} \pm 0.6$	$2.00^{b} \pm 0.6$	$1.00^{b} \pm 1.0$

*OBS – observed species richness; values followed by the same letter for each estimator are not statistically significant according to the Duncan post hoc multiple comparison test

In the mineral waters of Torda, Pseudomonadaceae was the most prevalent. The family Moraxellaceae dominated with 85.77% relative abundance in the mineral waters of Obrenovačka Banja (Fig. 1B). The family Rhodobacteraceae was dominant in the mineral waters of Velika Vrbnica, Slankamen Banja and Lomnički Kiseljak. The richest diversity characterized the mineral waters of Slankamen Banja with 29 families identified.

Different genera dominated in the mineral water communities of Serbia (Fig. 1C). These results are positively correlated with the previously observed spatial dynamics of phyla and families. In the sample of mineral waters from Torda, *Pseudomonas* was the most represented genus. *Acinetobacter* sp. was dominant in the mineral water sample of Obrenovačka Banja, while *Massilia* was the codominant genus. The mineral water of Lomnički kiseljak was characterized by the dominant presence of unidentified genera in the family Rhodobacteraceae. The genera *Pseudorhodobacter* and *Limnohabitans* were the main constituents of the mineral water of Velika Vrbnica. In the sample of mineral waters from Slankamen Banja, with the dominant presence of an unidentified genus from the family Rhodobacteraceae, *Chlorobi* bacterium and bacteria Blvii28 from the wastewater sludge group from the family Rikenellaceae were recorded at a higher percentage. Also, in the Slankamen Banja sample, compared to the other examined occurrences, the presence of a larger number of genera was recorded at percentages greater than 1%, including representatives of the genera *Methylotenera, Flavobacterium, Melioribacter* (with identified species *M. roseus*), *Dechloromonas, Terasakiella, Sedimenticola* (with identified species *S. selenatireducens*), *Thermincola, Arcobacter, Bellilinea, Methyloprofundus* and *Pseudomonas*.

Diversity of bacterial communities

Investigation of the bacterial communities' dynamics and their abundance in five different samples by metabarcoding analysis was performed. The bacterial richness and alpha diversity indices for each sample are presented in Table 1. Alpha diversity at all observed taxonomic levels indicated significantly high bacterial diversity in 2 samples, with the mineral waters of Slankamen Banja characterized by richer alpha diversity according to the Simpson and InvSimpson diversity indices as compared to the mineral waters of the Lomnički Kiseljak. According to the other two indices (Shannon and Fisher Alpha), there was no statistical difference between them. However, the occurrence of mineral waters in Torda, Velika Vrbnica and Obrenovačka Banja that were characterized by a very scarce alpha diversity were not statistically different, particularly according to the Shannon and Fisher Alpha indices (Table 1). The observed richness according to OBS revealed significantly the highest bacterial richness for mineral waters from the Slankamen Banja and Lomnički Kiseljak. According to estimates from the Chao1 and ACE indices, there is a positive correlation between the observed and estimated richness. In general, both diversity and richness can be arranged in the following order: Slankamen Banja > Lomnički Kiseljak > Torda = Velika Vrbnica = Obrenovačka Banja, which is in accordance with all used indices.

The Bray-Curtis dissimilarity index revealed compositional differences among samples (Fig. 2). Where relative distances are small this implies that two communities are compositionally similar and share a common evolutionary history. A scatter plot revealed that mineral water samples in Torda, Velika

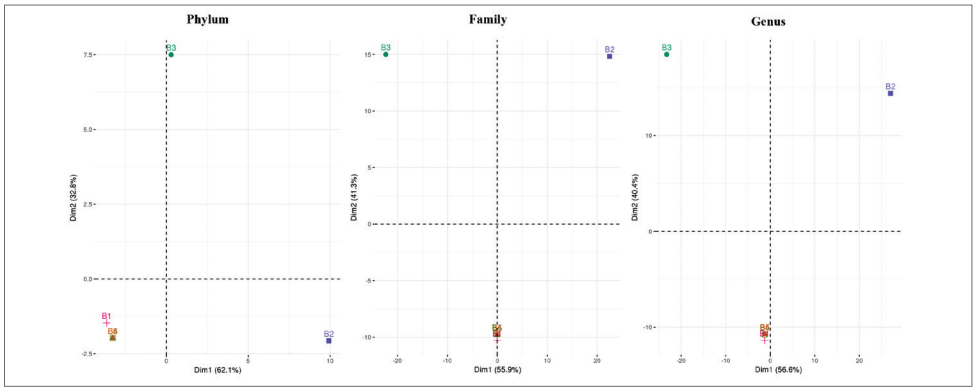

Fig. 2. Principal component analysis of bacterial compositions in five different samples at phylum, family and genus taxonomic levels. Torda mineral water – sample B1; Slankamen Banja mineral water – sample B2; Lomnički Kiseljak mineral water – sample B3; Velika Vrbnica mineral water – sample B4; and Obrenovačka Banja mineral water – sample B5. The individual samples are color-coded to indicate differences between them.

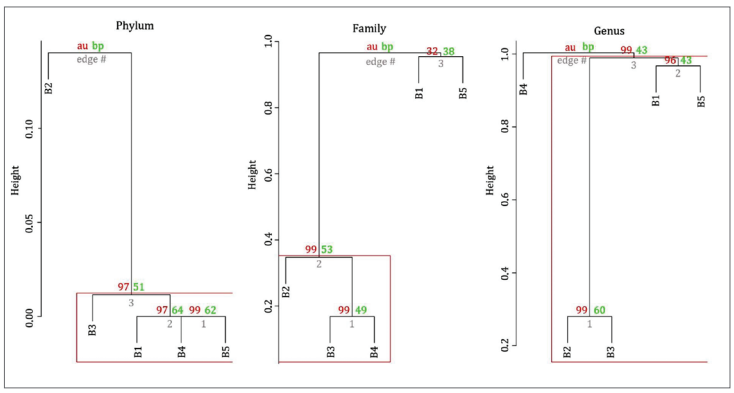

Fig. 3. Cluster analyses of bacterial compositions in five different samples at phylum, family and genus taxonomic levels. Torda mineral water – sample B1; Slankamen Banja mineral water – sample B2; Lomnički Kiseljak mineral water – sample B3; Velika Vrbnica mineral water – sample B4; and Obrenovačka Banja mineral water – sample B5. Dendrogram reports of AU and BP scores (P-values). AU P-values (red) are an "approximately unbiased" P-value, calculated by multi-scale bootstrap resampling. BP values (green) are "bootstrap probability" values, which are less accurate than AU values. Clusters (edges) with high AU values (≥95%) are strongly supported by the data. Red frames report approximately unbiased P-values higher than 95%.

Vrbnica and Obrenovačka Banja have a very similar biodiversity of bacterial communities. Compared to them, the bacterial diversity in samples of mineral waters of Slankamen Banja and Lomnički Kiseljak was different at all taxonomic levels (with higher values for Dim1). According to the values of Dim2, the samples Slankamen Banja and Lomnički Kiseljak displayed less dissimilarity in the bacterial communities at the family and genus levels. However, hierarchical clustering analysis based on the abundances of specific taxa at the phylum level showed that the mineral waters of Torda, Velika Vrbnica and Obrenovačka Banja show similarity of bacterial communities and less dissimilarity than the samples of mineral waters from Lomnički Kiseljak, while the mineral waters of Slankamen Banja were the most distant. At the family level, clustering was found between the samples of mineral waters of Lomnički Kiseljak, Velika Vrbnica and Slankamen Banja, while the mineral waters of Torda and Obrenovačka Banja were the most distant. However, at the genus level, the greatest similarity was confirmed between the mineral waters of Slankamen Banja and Lomnički Kiseljak, as well as Torda and Obrenovačka Banja, while the mineral waters of Velika Vrbnica differ from the remaining four samples. However, the height also indicated that they diverge and differ between each other (Fig. 3).

Physical-chemical analysis of the investigated occurrences

According to the classification of medicinal mineral waters by temperature and pH values, the occurrence of mineral waters in Obrenovačka Banja was determined as a warm (subthermal) water, while the mineral waters of Velika Vrbnica, Lomnički kiseljak, Slankamen Banja and Torda belong to cold waters [24, 25]. Weakly acidic mineral waters are found in the occurrence of Velika Vrbnica, neutral occurrences are in Lomnički kiseljak, while weakly alkaline mineral waters are found in the occurrences of Slankamen Banja, Obrenovačka Banja and Torda (Table 2). Based on total dissolved solids, all investigated occurrences of mineral waters can be classified as brackish waters. The investigated occurrences of mineral waters differed in the aspect of characteristic

Table 2. Applied standard for determining the value of tested physical and chemical parameters of mineral waters and results of physical-chemical analysis of the investigated occurrences of mineral waters

Tested parameters	Standard	Torda	Slankamen Banja	Obrenovačka Banja	Lomnički Kiseljak	Velika Vrbnica
BASIC PHYSICAL-CHEMICAL PROPERTIES						
T (°C)*	*	11.0	18.4	30.6	13.0	12.0
TDS at 105°C (mg/L)	SMEWW 19th	2207	7440	1730	4700	1530
pH	SRPS ENISO	7.9	7.6	7.9	6.8	6.5
Color (°Co-Pt scale)	US EPA	<5	<5	60	<5	<5
Apparent color (°Co-Pt scale)	US EPA	10	10	80	5	5
Odor	US EPA	Rotten cabbage	Oil	Ammonia	Oil	No odor
Oxidizability (mg/L)	PRI	1.8	22.6	/	0.9	1.3
EC (µS/cm)	SRPS EN	4550	11020	2290	6400	2050
MACROCOMPONENTS (mg/L)						
Cations						
Na^+	US EPA	984	2452	492	1712	132
K^+	US EPA	0.1	30.4	22.4	117.0	17.0
Mg^{2+}	US EPA	169.0	107.0	8.4	109.0	137.0
Ca^{2+}	US EPA	46.1	198.0	10.1	163.0	331.0
Anions						
HCO_3^-	SRPS ENISO	629.9	272.7	1476.2	4648.2	1714.1
SO_4^{2-}	US EPA	1380.3	3.5	1.2	217.6	66.8
Cl^-	US EPA	255.6	4615.0	74.2	220.1	85.2
NO_3^-	SMEWW 19th	39.4	13.4	3.8	6.8	12.8
MICROCOMPONENTS (mg/L)						
Li	US EPA	0.037	0.808	0.689	5.200	0.292
Sr	US EPA	0.86	13.10	1.47	2.18	1.37
Se	US EPA	<0.001	/	/	/	/
Mn	US EPA	<0.001	0.028	0.032	0.278	0.005
Fe	US EPA	0.01	<0.01	1.17	0.03	<0.01
B	US EPA	2.36	9.83	2.87	19.30	0.74
F^-	US EPA	0.423	0.025	1.680	0.352	0.210
I^-	WA 1988	<2.0	5.7	0.5	<2.0	<2.0
NO_2^-	SRPS EN	0.012	0.036	<0.007	<0.007	<0.007
NH_3	PRI	<0.05	83.80	24.00	<0.05	0.14
GASES (mg/L)						
Free CO_2	SMEWW 19th	<0.5	9.4	<0.5	786.6	46.2
H_2S	ISO	<0.04**	<0.04**	<0.04	<0.04**	<0.04**

*T – Temperature: Torda [24], Slankamen Banja, Obrenovačka Banja, Lomnički kiseljak, Velika Vrbnica [25]; TDS – Total dissolved solids; EC - Electrolytic conductivity; **Hydrogen sulfide soluble as S^{-2}; / – Parameter value not specified.

ionic composition and in ions exhibiting the highest concentrations (Table 2). The chemical composition of the investigated occurrences, indicating basic ionic types of mineral waters, is also presented in the Piper diagram (Fig. 4). Hence, HCO_3 – Na mineral waters were found in the occurrences of Obrenovačka Banja and Lomnički Kiseljak, HCO_3 – Mg, Ca, Na in the occurrence of Velika Vrbnica, Cl – Na in the occurrence in Slankamen Banja, while SO_4, HCO_3 – Na, Mg were found in the mineral water occurrence in Torda. Regarding metals, increased Li and B concentrations were observed in the mineral waters of Lomnički Kiseljak, and an elevated Sr concentration was recorded in Slankamen Banja. A Fe content above 1 mg/L was recorded in Obrenovačka Banja. A low Mn content characterized all the investigated

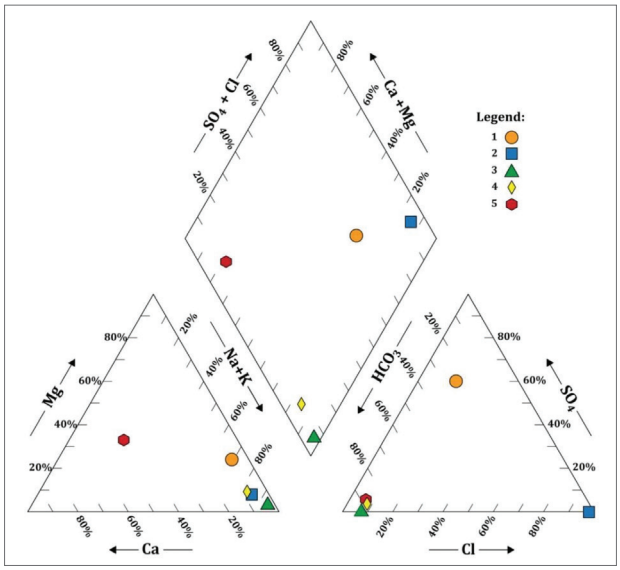

Fig. 4. Piper diagram of the chemical composition of the studied occurrences of mineral waters. Torda mineral water – sample 1; Slankamen Banja mineral water – sample 2; Obrenovačka Banja mineral water – sample 3; Lomnički Kiseljak mineral water – sample 4; and Velika Vrbnica mineral water – sample 5.

occurrences of mineral waters. High F and NH_3 contents characterized the mineral waters in Obrenovačka Banja, while increased I and NH_3 contents characterized the mineral waters in Slankamen Banja. As for gas composition, an extremely high content of free CO_2 was recorded in the mineral waters of Lomnički Kiseljak. A low H_2S content characterized all studied occurrences of mineral waters.

DISCUSSION

To acquire a better description of the bacterial diversity in analyzed mineral waters which are used for drinking (Torda, Lomnički kiseljak and Velika Vrbnica) and bathing (Slankamen Banja and Obrenovačka Banja), we performed metabarcoding analysis. The observed diversity of bacterial phyla and their representatives revealed their association with the aquatic environment. The selected mineral water occurrences were classified after considering the data on the richness of microbiological diversity by the observed dominant phyla and families of bacteria. Thus, the mineral waters of Torda, Velika Vrbnica, Lomnički Kiseljak and Obrenovačka Banja can be designated as Proteobacteria mineral water, while the occurrence in Slankamen Banja can be designated as Proteobacteria-Bacteroidetes mineral water. The Pseudomonadaceae mineral waters (Torda), Rhodobacteraceae mineral waters (Slankamen Banja and Lomnički Kiseljak), Rhodobacteraceae-Burkholderiaceae mineral waters (Velika Vrbnica) and Moraxellaceae mineral waters (Obrenovačka Banja) were designated as Proteobacteria mineral waters. An earlier study documented the dominant presence of representatives of the Proteobacteria phylum in biofilm communities [26] that were associated with the development of biocorrosive processes [27]. Also, it is known that Proteobacteria representatives are the most common groups of bacteria in drinking water, and their physiological diversity and ubiquity could have potential implications regarding the sanitary and hygienic safety of the water resources [28]. Thus, the correlation between contamination of recreational and drinking water and *Pseudomonas* bacteria [29] and the development of biocorrosion and bacterial overgrowth (biofilm formation) has already been established [30]. Besides biofouling, representatives of the Gallionellaceae family have biotechnological significance as their metabolic activity could be used to remove divalent iron from groundwater [31]. Also, representatives of the genus *Acinetobacter* from the family Moraxellaceae possess biotechnological potential in the remediation treatment of contaminated water, and it is known that they can induce the development of mucus [32]. The *Sedimenticola* genus was shown to participate in sulfur compound oxidation, while in anoxic conditions and in the absence of light it can use nitrogen compounds [33]. On the other hand, the role of the Proteobacteria phylum in biofouling processes is suggested by representatives of the Hydrogenophilaceae family that were previously isolated from aerobic digesters in sludge treatment [34], as well as representatives of the Rhodobacteraceae family whose presence was recorded in mineral water biofilm deposits with sulfur and carbon monoxide oxidation isolates [35, 36]. In the Burkholderiaceae family, the genera *Acidovorax, Limnobacter, Massilia* and *Limnohabitans* were identified. Species of the *Limnobacter* genus have been found in volcanic deposits, seawater, etc., and can participate in sulfur oxidation, oxidative phosphorylation, ethanol fermentation, etc. [37]. The *Massilia* genus is present in freshwater habitats, with some representatives isolated

from drinking water and heavy-metal-contaminated soil [38], while the *Limnohabitans* genus is a characteristic inhabitant of acidic and alkaline habitats [39]. The *Pseudorhodobacter* genus was isolated from sandy sediments [40], which is in accordance with its occurrence in the studied mineral waters. From the Flavobacteriaceae family, the *Maritimimonas, Planktosalinus* and *Flavobacterium* genera were detected, and it was shown earlier that they can contribute to the mineralization of organic substances [41].

In addition to the presence of the Proteobacteria phylum in biofilm communities, the Bacteroidetes phylum with the species *Melioribacter roseus* was also detected. Previously it was shown that this species is found in biofilms of thermal water drainage zones with indications of biofouling of water intake facilities [42, 43]. Also, the presence of Bacteroidetes and Firmicutes phyla were documented in rust samples, pointing to the risk of developing biocorrosive processes [44]. From the Rikenellaceae family, the uncultivated genus from the Blvii28 wastewater-sludge group was found in the Slankamen Banja sample, as well as in anaerobic wastewater treatment systems and freshwater habitats, with the ability to ferment carbohydrates and generate hydrogen [45]. The genus *Thermincola*, which was found in Slankamen Banja, includes anaerobic thermophilic species that perform anaerobic oxidation of carbon monoxide [46], pointing to a possible role in biogeochemical transformations of chemical elements in the ecosystem of mineral waters. Besides Proteobacteria and Bacteroidetes, the presence of other phyla might also indicate biofouling processes. The ability of the Patescibacteria phylum to survive in groundwater habitats is conditioned by genome size, ultra-small cell size and the ability to avoid phage invasion [47]. The Chloroflexi phylum can participate in the processes of transformation of organic substances [48], while the Acidobacteria phylum was established in biofilm samples of drinking water treatment plants [49]. The presence of Acidobacteria could be the result of vertical seepage of water from the upper layers of the soil, considering that representatives of Acidobacteria are especially widespread in the soil [50]. It was previously observed that seasonal hydrological conditions could have an impact on the bacteriological diversity of subterranean ecosystems because they affect the transport of bacteria from one environment to another [51]. The presence of the Spirochaetes phylum could be an indication for the presence of contaminants of organic origin such as hydrocarbons in mineral waters, while some of its representatives possess a pathogenic character, indicating a health risk in using such mineral water [52].

Furthermore, the hydraulic connection between mineral and surface waters affects the more intensive development of groundwater bacteria, which indirectly accounts for the greatest richness of alpha diversity, especially in the tested samples of Slankamen Banja and Lomnički Kiseljak, which was confirmed by metabarcoding analysis, particularly when bearing in mind the character of the mineral waters of Slankamen Banja and Lomnički Kiseljak and their vicinity to the Danube and Lomnica Rivers, respectively.

Physical-chemical analysis revealed high contents of macrocomponents in samples originating from different types of mineral water occurrences. The total dissolved solids of the investigated phenomena indicated elevated concentrations of basic ions as the result of solubilization of inorganic salts in the mineral waters of an appropriate mineralogical-petrographic type. Also, the higher levels of total dissolved solids indicate elevated conductivity values [53]. After analyzing the content of microcomponents, B and NH_3 had the highest values. As an element, B regularly participates in the composition of minerals of the Earth's crust, thus its presence in mineral waters is often reported [54]. Elevated concentrations of B could also be the result of anthropogenic pollution [55], i.e., an indication of the connection between the mineral waters of Lomnički Kiseljak and contaminants of organic origin. The extremely elevated NH_3 concentrations recorded in the mineral waters of Slankamen Banja and Obrenovačka Banja are an indicator for the presence of contaminants of organic origin, resulting from seepage from industrial plants, animal compost, various fertilizers, etc., given that elevated concentrations of NH_3 of natural origin in hydrological systems have not been fully demonstrated [56].

According to the Rulebook on the quality and other requirements for natural mineral, spring and table waters of Serbia [57], the results of physical-chemical analysis of the examined mineral waters of Torda, Slankamen Banja, Lomnički Kiseljak and Velika Vrbnica corresponded with the range of tested

parameters. However, considering the Rulebook on the hygienic safety of drinking water of Serbia [58], the mineral water of Obrenovačka Banja is not chemically suitable due to changed organoleptic properties, increased consumption of $KMnO_4$ and elevated concentrations of NH_3, F, B, Fe, K and Na. Also, according to the same Rulebook [58], the values for B measured in the occurrences of Torda and Lomnički Kiseljak, as well as for Mn in all occurrences, are not allowed in drinking and bottled natural waters. Coliform bacteria, as one of the most common causes of bacteriological contamination of drinking water samples [58], were not detected in the analyzed samples. The presence of the genus *Pseudomonas* in the mineral waters of Torda, Slankamen Banja and Lomnički Kiseljak is not allowed according to the Rulebook from 2005 and 2013 [57], which primarily addresses the presence of *P. aeruginosa*, and we cannot confirm the existence of the typical species. In addition, according to the World Health Organizations (WHO) guidelines for drinking-water quality, there are no criteria regarding *Pseudomonas* species since it was not proven that water resources containing these species are sources of infection for the human population [59]. Similarly, the United States Environmental Protection Agency has stated that the presence of *Pseudomonas* species is not mandatory for monitoring primary drinking-water national regulations [60]. Furthermore, according to the WHO [59], the potentially pathogenic bacteria from the genus *Acinetobacter* that were detected in the mineral waters of Torda, Lomnički Kiseljak and Obrenovačka Banja do not represent a health risk to the general human population consuming the water.

CONCLUSIONS

A detailed insight into the bacterial flora and physical-chemical properties of mineral waters was obtained. All investigated occurrences were characterized by elevated values of total dissolved solids and basic ionic components, while in some occurrences extremely elevated concentrations of chemical compounds were recorded. Bacteriological diversity with different physiological groups of bacteria that differed among the examined occurrences was found. The research concludes that many representatives of identified bacteria can contribute to the development of biocorrosion and biofouling processes of water intake facilities. In addition, the presence of bacteria with a biotechnological potential for use in the remediation of contaminated water, and indicators of organic pollution are reported. Under conditions of multipurpose use of mineral waters, it is necessary to apply appropriate treatments for the revitalization of water intake facilities and/or remediation of mineral waters, while ensuring continuous monitoring of the selected occurrences. In this way, the water intake facilities would be maintained in a state of maximum functionality, without any negative effects of bacteriological diversity on the service life of wells that could continuously provide safe mineral water resources.

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Conflict of interest disclosure: None to declare.

Data availability: The metagenomics datasets are deposited in the BioProject database (NCBI) as PRJNA627735 available at https://www.ncbi.nlm.nih.gov/sra/%20PRJNA627735 (under the following accessions: [Torda mineral water – sample B1 (SAMN14682966), Slankamen Banja mineral water – sample B2 (SAMN14682967), Lomnički kiseljak mineral water – sample B3 (SAMN14682968), Velika Vrbnica mineral water – sample B4 (SAMN14682969), Obrenovačka Banja mineral water – sample B5 (SAMN14682970)].

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Supplementary Material

The Supplementary Material is available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Saraba%20et%20al_7354_Supplementary%20Material.pdf