Article

Testing different membrane filters for 16S rRNA gene-based metabarcoding in karstic springs

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Abstract: (1) Introduction: Karstic springs are used worldwide by rural communities as sources of fresh water for human use and livestock survival. In Romania, 1/3 of the population has no direct access to the public water supply. The present study is part of a country-wide project on developing simple, quick and cheap methods for seasonal environmental and microbiological monitoring of karstic springs used as drinking water supply by rural populations. Critical steps for the monitoring workflow consist in the evaluation of water quality and choosing of suitable membrane filters to efficiently capture environmental DNA for further microbial diversity estimation by 16S rRNA gene-based metabarcoding; (2) Methods: Several commercial membrane filters of different composition and pore sizes were tested on the water sampled from three karstic springs in Romania, followed by water chemistry and whole community 16S rRNA gene-based metabarcoding analysis; (3) Results: We found that the different types of applied membrane filters provide a varying recovery of diversities and abundances of both overall and pathogenic bacteria; and (4) Conclusions: The result of the experiment with different filters shows which are the best for amplicon-based metabarcoding monitoring of karst springs.

Keywords: spring water; karst; 16S rRNA gene; membrane filters; metabarcoding; pathogenic bacteria

1. Introduction

In a recent review [1], the authors considered the main environmental drivers of the global trends in freshwater availability in the 21st century. Changes in freshwater availability are predictive not only for regional food supplies, human and ecosystem health, and energy generation but also the cause for social unrest [1]. Half of the world population use groundwater for domestic needs [2], and groundwater provides 38% of the global consumptive irrigation water demand [3]. Yet, groundwater monitoring and management are challenging tasks for the vastness and unseen distribution of these freshwater sources, mainly found in soluble rocks like limestones and

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dolomites. The high demand for food in conjunction with climate change result in economic and social hardships already reported in many parts of the world, and are foreseen to accelerate the depletion of the available groundwater sources used for drinking and agriculture [4].

The 2006-2017 Eurostat survey (published in 2020, [5]) shows that five out of 12 European countries have less than 90% of households connected to the public water supply with Romania ranked with the lowest percentage of 67.5%. One cause of this shortcoming might be the sparse distribution of houses in most rural areas that makes the access to public water supply very problematic if not impossible. Small rural populations use the local sources of water that are not under the monitoring programs of the water agencies. Usually, such small local sources from porous karstic rocks are prone to periods of low or no flow, or a combination of low flow and concentration of contaminants [6]. The primary sources of contamination in small rural localities are from livestock, septic tanks, mining activities in the area, and the use of chemicals in the agricultural practices [7].

Environmental DNA (eDNA) metabarcoding was proposed as viable alternative for the freshwater quality assessment [8] as it quickly and sensitively revealing the taxonomic inventory of eukaryotic [9, 10] or prokaryotic communities [11]. Recently, eDNA metabarcoding has proved as useful tool to detect waterborne pathogens in rural surface freshwater impacted by faecal pollution [12] and antibiotic-contaminated groundwater [13]. Surprising little information exists on microbiological quality of groundwater sources that are used as drinking water in rural areas worldwide. In most if not all of the cases, detection of *E. coli* as representative for coliform bacteria is the only microbiological test to check the potential for waterborne diseases so far [14]. This approach is however preventing detection of other threatening bacteria including pathogenic or opportunistic species bearing antibiotic resistance traits [15]. In this light, eDNA metabarcoding appears as a novel and feasible approach to assess the microbial diversity of groundwater sources for drinking water in rural areas particularly.

For the rural populations that use spring water as the main source of fresh water, we intended to test and propose tools and protocols for seasonal water quality monitoring [16]. Within the framework of this country-wide project we performed a survey on the physicochemical and microbiological characteristics of selected karst springs to establish a fast and cost-effective monitoring protocol. We aimed at evaluating most suitable membrane filter type and filtration strategy to efficiently recover environmental DNA (eDNA) for further fast and accurate whole community diversity assessment by 16S rRNA gene-based metabarcoding.

2. Materials and Methods

2.1. Sampling and samples preparation

Three springs were chosen for their easy access from Cluj-Napoca (Romania), the headquarter of logistics and authors. Banpotoc, Băița (Baita) and Rapolțel (Rapoltel) springs are located in the Apuseni Mountains, north-western Romania (Figure 1). These springs are used by the local populations as drinking water resource for human and livestock consumption and are not included in the national or local water monitoring programs.

In September 2019, 5 L of spring water were collected from each of the three springs in polyethylene bottles. Bottles were rinsed 3 times with the samples waters and kept refrigerated a 4^{o}C until chemical analysis. Physicochemical features (pH, electrical conductivity) were measured

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in situ. Water was sampled and filtered in the same day in all cases for eDNA analysis. Water samples were filtered through the chosen membranes (Table 1) was performed under aseptic conditions using a vacuum pump and a filtering device, composed of a glass filter holder and a funnel, attached to a collecting Kitasato flask.

Nine types of different membrane filters with different pore sizes were tested for their efficiency in retaining biomass that sourced for eDNA from karstic spring water (see Table 1). After filtration, membranes were cut into small pieces and kept at -20°C until further eDNA extraction.

Table 1. The tested membrane filters for metabarcoding in karstic spring water.

Membrane code	Producer	Fabric	Porosity (μm)	Sterile*	Price*
PES	Millipore	polyethersulfone	0.22	sterilized	High
PVDF	Millipore	polyvinylidene fluoride	0.22	sterilized	High
NC	Macherey-Nagel	cellulose nitrate	0.20	sterilized	Low
NYLON	Fioroni	nylon	0.22	sterilized	Low
MCE_a	Fioroni	mixed cellulose ester	0.22	sterile	Low
MCE_b	Whatman	mixed cellulose ester	0.20	sterile	Medium
S-Pak	Millipore	mixed esters of cellulose	0.20	sterile	Low
PSR009	Nahita	cellulose acetate and nitrate	0.22	sterilized	Low
PSR010	Nahita	cellulose acetate and nitrate	0.45	sterilized	Low

* We termed 'sterile' those membrane filters provided sterile by manufacturer and 'sterilized' those membrane filters provided non-sterile and further steam-sterilized at 121°C for 20 minutes.

** We define the prices as low, medium and high, relatively to each other.

2.2. Physicochemical analysis

The pH and electrical conductivity (EC) were measured in situ using a portable multiparameter with a built-in temperature correction (Multi 340i, WTW, Germany). Dissolved organic carbon (DOC) was obtained by subtracting DIC from DC. Dissolved inorganic carbon (DIC) and dissolved carbon (DC) were determined after filtering the samples on 0.45 μm PTFE filters by catalytic combustion and infrared detection of CO_2 using a Multi N/C 2100S Analyzer (Analytik Jena, Germany). Total alkalinity was determined by titration with HCl. The bicarbonate (HCO_3^-) content was calculated by multiplying the alkalinity expressed in mmol/L with 61. Ammonia was determined by UV-Vis spectrometry (Lambda 25, Perkin Elmer, USA) using the salicylate method. Anions were determined by ion chromatography using a 761 Compact IC (Metrohm, Switzerland). The metal concentrations were measured in samples filtered through 0.45 mm cellulose acetate filters and acidulated with 63% HNO_3 by inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 5300 DV, Perkin-Elmer, USA) in case of Na, Mg, Ca and K and inductively coupled plasma mass spectrometry (ICP-MS, ELAN DRC II, Perkin-Elmer, USA) in case of Ba, Sr, Mn, Ni, and Cr.

2.3. 16S rRNA protocol

MiSeq 16S V3-V4 Metagenome Sequencing was performed by a commercial company (Macrogen Europe). The V3-V4 hypervariable regions of the bacterial and archaeal SSU rRNA gene

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were amplified by PCR, using primer 341F (5′-CCTACGGGNGGCWGCAG-3) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) according to Illumina's 16S amplicon-based metagenomic sequencing protocol. The amplicon size achieved was between 400 and 500 bp. The pair-end reads were joined in FLASH (1.2.11) [17]. Pre-processing and clustering were performed in CD-HIT-OTU and rDnaTools [18, 19]. The taxonomy was assigned for the representative OTUs in QIIME against the last version of the RDP – 16Sr DNA database and using a species-level OTU cut-off, 97% sequence identity [20].

Figure 1. The location (a) of the three tested karstic springs in Romania and photos taken during the sampling campaign (b = Baita, c = Banpotoc, d = Rapoltel).

2.4. Statistical analysis

Principal Component Analysis (PCA) was conducted with XLSTAT 2020.1.3 (Addinsoft, France). PCA is a multivariate data analysis method in which observations (springs) are described by variables (chemical elements).

The diversity indices combine in non-standard way two independent attributes of communities, the number of species and their relative abundances. Chao1 is a diversity index based on the abundance of individuals (OTUs) belonging to a sample. The Shannon diversity index (*H*) is commonly used to characterize species diversity in a community, for both abundance and evenness of the species present.

3. Results

3.1. Chemical analysis

The chemical data quality was assessed by calculating the charge balance between the sum of cations and the sum of anions, expressed in mEq/L. In each case the Charge Balance Error (CBE) was less than 10% (Table 2).

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The Piper diagram (Figure S1) showed that Banpotoc, Baita and Rapoltel springs have a Ca-HCO_3 facies. PCA of the physicochemical variables (Figure 2), separated Baita and Banpotoc along the horizontal axis (F1). F1 axis represented more than 83.6% significance in the analysis. Rapoltel separated from the two other springs along the F2 axis at lower statistical significance (16.4%). The total dissolved solids (TDS) were low in Baita and high in the other two springs, indicating the high mineralization of the latter springs. The Banpotoc and Rapoltel springs were rich in Ca and bicarbonates, and had high alkalinity. Ba, Sr, Mn, and Ni were also high in Banpotoc and Rapoltel. Sulfates were high in Baita spring, while all the other elements were found in low concentrations. DOC was one order of magnitude higher in Banpotoc and Rapoltel than in the Baita spring.

Table 2. The physicochemical characteristics of the three studied karstic springs.

Parameter	Unit	Spring		
		Banpotoc	Baita	Rapoltel
TDS	mg/L	1210	298	987
DOC	mg/L	17.7	1.4	19.3
pH	-	6.3	7.6	6.5
Bicarbonates	mg/L	1122	256	988
Alkalinity	mmol/L	18.4	4.2	16.2
Na	mg/L	28.5	6	8.1
Ca	mg/L	280	72.9	219
Mg	mg/L	37	4.3	33.9
K	mg/L	5.7	0.45	2.4
Ba	µg/L	671	13.6	109
Sr	µg/L	351	92.7	240
Mn	µg/L	74.6	1.5	25.7
Ni	µg/L	13.6	5.7	13.4
Cr	µg/L	1.48	2.61	1.41
NH_4^+	mg/L	0.57	0.12	0.04
Cl^-	mg/L	4.53	2.55	2.2
SO_4^{2-}	mg/L	2.2	19.6	6
NO_3^-	mg/L	<0.2	2.4	<0.2
NO_2^-	mg/L	0.32	<0.05	<0.05

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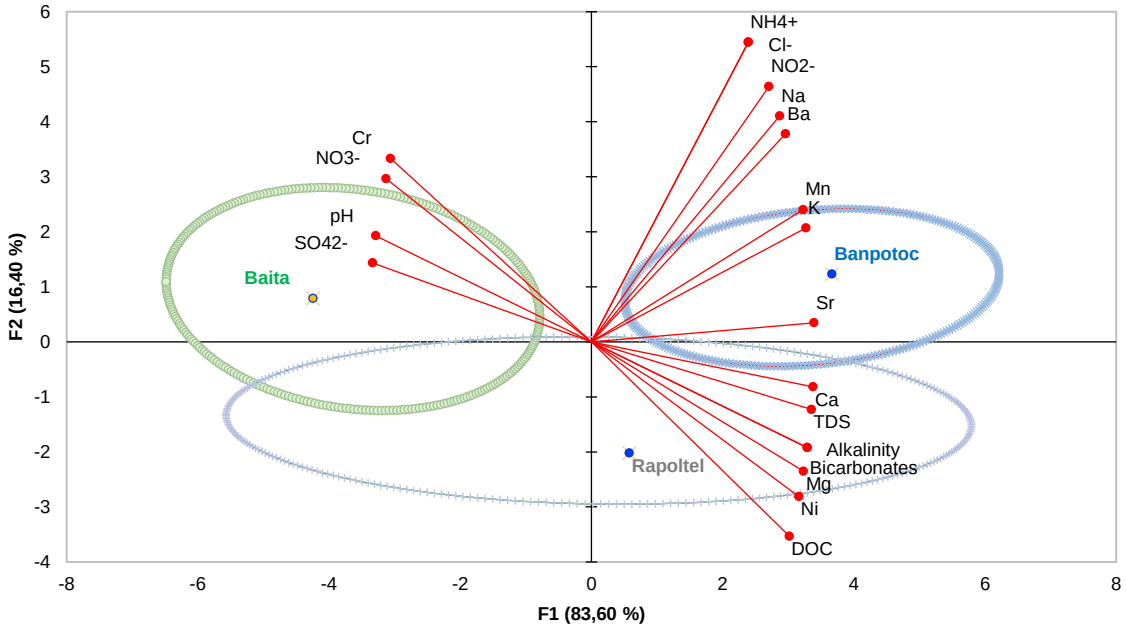

Figure 2. PCA of the relationship between the three springs and their physicochemical characteristics (see also Table 2).

In the Romanian legislation regulating the quality of the drinking water [21], the Maximum Allowable Concentration (MAC) for ammonia is 0.5 mg/L. This value that was slightly exceeded in Banpotoc, possibly indicating the leaching of nitrates from agricultural land use.

3.2. Microbial diversity inferred by 16S rRNA gene-based metabarcoding

After quality-filtering and operational taxonomic unit (OTU)-clustering at the 97% sequence identity, we obtained 1,025,064 good-quality reads grouped into 1,106 OTUs following singleton removal. OTUs pertaining to Archaea and Bacteria were identified, with Bacteria-related sequences dominating in all samples.

The identified taxa showed high variations of diversity and abundances between the 28 analyzed filter samples (Table 3). The highest diversity was in Baita samples followed by Rapoltel and Banpotoc samples. E23 (Nylon) and E4 (PSR009) from Banpotoc, E13 and E16 from Baita (MCE_b and Nylon, respectively), and E33 (MCE_b) from Rapoltel were the most diverse.

Table 3. Collected samples in the three karstic springs with their corresponding diversity indices.

Spring	Code	Membrane code	OTUs	Diversity indices	
				Chao1	Shannon
Banpotoc	E20	PES	186	188.6250	2.8280
	E21	PVDF	226	264.2778	2.5738
	E22	NC	228	306.9643	2.6665
	E23	NYLON	264	294.3571	2.7375
	E24	MCE_a	251	318.0000	2.7688
	E25	MCE_b	241	257.5000	2.8318
	E26	S-Pak	261	320.2941	2.9322

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	E4	PSR009	263	267.5000	4.9879
	E27	PSR010	115	126.5500	1.2992
Baita	E11	PES	1280	1699.2250	6.4304
	E12	PVDF	1335	1679.3142	6.8048
	E18	NC	1368	1652.6980	6.6819
	E16	NYLON	1538	1803.8583	7.1422
	E15	MCE_a	1271	1569.4933	7.0039
	E13	MCE_b	1566	1734.7447	8.5057
	E19	S-Pak	1455	1752.9286	7.9813
	E17	PSR009	794	1009.5301	4.9305
	E14	PSR010	718	915.3356	4.3866
Rapoltel	E29	PES	200	202.0000	4.8509
	E31	PVDF	221	224.7500	5.1441
	E32	NC	235	242.2000	4.4465
	E30	NYLON	174	174.6000	3.9005
	E34	MCE_a	214	217.3333	4.1399
	E33	MCE_b	358	360.5000	5.0208
	E2	S-Pak	310	312.5000	5.7927
	E28	PSR009	260	265.1429	5.1996
	E35	PSR009	255	255.4286	5.0427
	E3	PSR010	168	169.5000	3,8749

Most Archaea-related OTUs (16 in total) belonged to Euryarchaeota, with the following most frequently encountered species, *Methanolobus taylorii*, *Methanobacterium aggregans* and *M. palustre*. Crenarchaeota and Thaumarchaeota were also represented in one to maximum four samples. Sample E25, Bantopotoc (MCE_b) was the richest in Archaea-related OTUs.

The identified Bacteria-related OTUs were classified within 34 phyla, where the most abundant were Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes (Figure 3). Other 11.54% of the total OTUs belonged to non-identified phyla. Proteobacteria dominated in all the samples. In Banpotoc, Actinobacteria was the next diverse phylum, while Bacteroidetes dominated Baita samples. Baita had also the highest diversity in phyla. Rapoltel samples had no other dominant group except for Proteobacteria by far the most diverse in this spring. Only E28 had high OTUs abundance belonging to Firmicutes.

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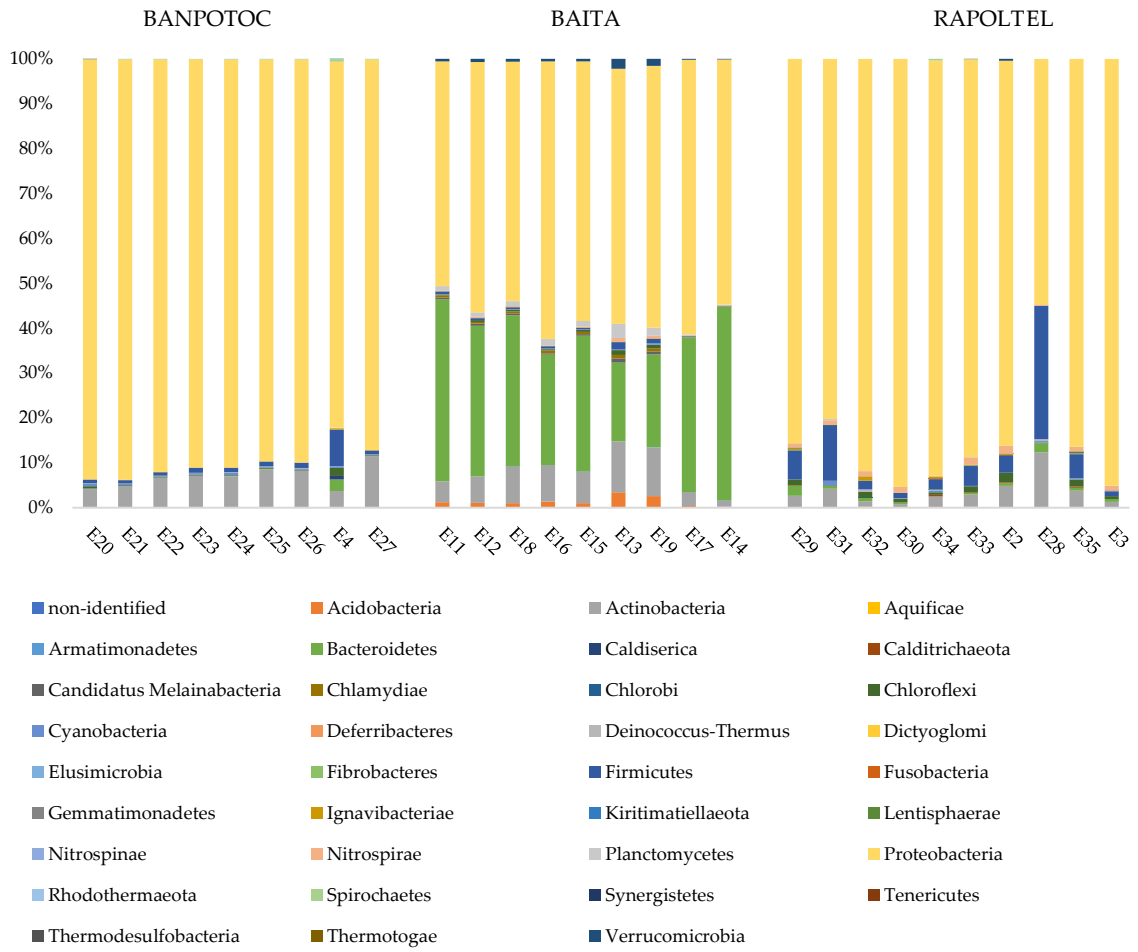

Figure 3. The relative abundance of the Bacteria phyla in the analyzed membranes, showing in yellow the dominant Proteobacteria.

In a PCA of diversity (Figure 4), the samples from the three springs were significantly separated along the F1 axis (90.85% significance) for Banpotoc and Baita, while Banpotoc separated less significantly along the F2 axis (9.05% significance) from the Rapoltel samples. Banpotoc membranes are more similar to each other and so the Rapoltel membranes, except for E3 (PSR010). The Baita membranes are more different from each other. Along the F1 axis, E17 (PSR009) and E14 (PSR010) have a relatively separate position from the other membranes of Baita, position explained by the analysis at species level (see below).

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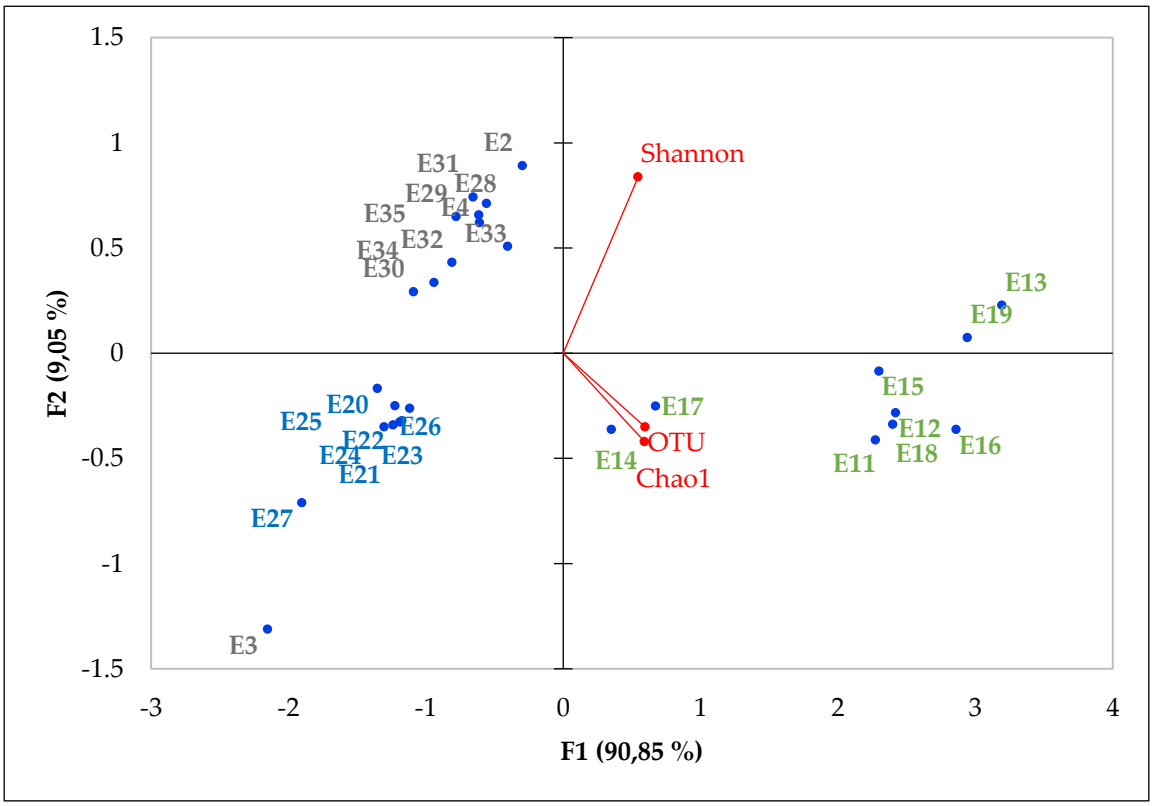

Figure 4. PCA of the samples in the three studied springs separated according to the related OTUs and the diversity indices, Chao1 and Shannon.

To infer the efficiency of different filtrating membranes the results were further analyzed separately for each spring at species level. Differences were even more evident at species level, both between springs and between membranes for each spring. Relative abundances of the dominant species are represented on Figure S2. The dominant species in Banpotoc samples were *Sulfurovum lithotriphicum* and *Sulfurimonas autotrophica*, both involved in the redox sulfur cycle (Figure 5). They represented 68.5% of the identified Banpotoc total OTUs total. *Flavobacterium succinicans* and *Rhodoferax saidenbachensis* represented more than 34% of the identified OTUs of Baita samples. *Sideroxydans lithotrophicus* was by far the most abundant species in all Rapoltel samples, representing more than 43% of the total of identified OTUs. The E4 (PSR009) and E27 (PSR010) samples provided different results than the other membranes of Banpotoc, all abundant in *Sulfurimonas autotrophica* and *Sulphurovum lithotrophicum*. E4 membrane had *Acinetobacter junii* as the dominant taxa, while E27 was almost entirely represented by *Sulphurovum lithotrophicum*. In Baita, two species were dominant, *Flavobacterium succinicans* and *Rhodoferax saidenbachensis*, except for E13 (MCE_b) and E19 (S-Pak) where no species was particularly dominant. All Rapoltel samples except for E28 (PSR009), were dominated by *Sideroxydans lithotrophicus*. E28 was dominated by *Staphylococcus epidermis*.

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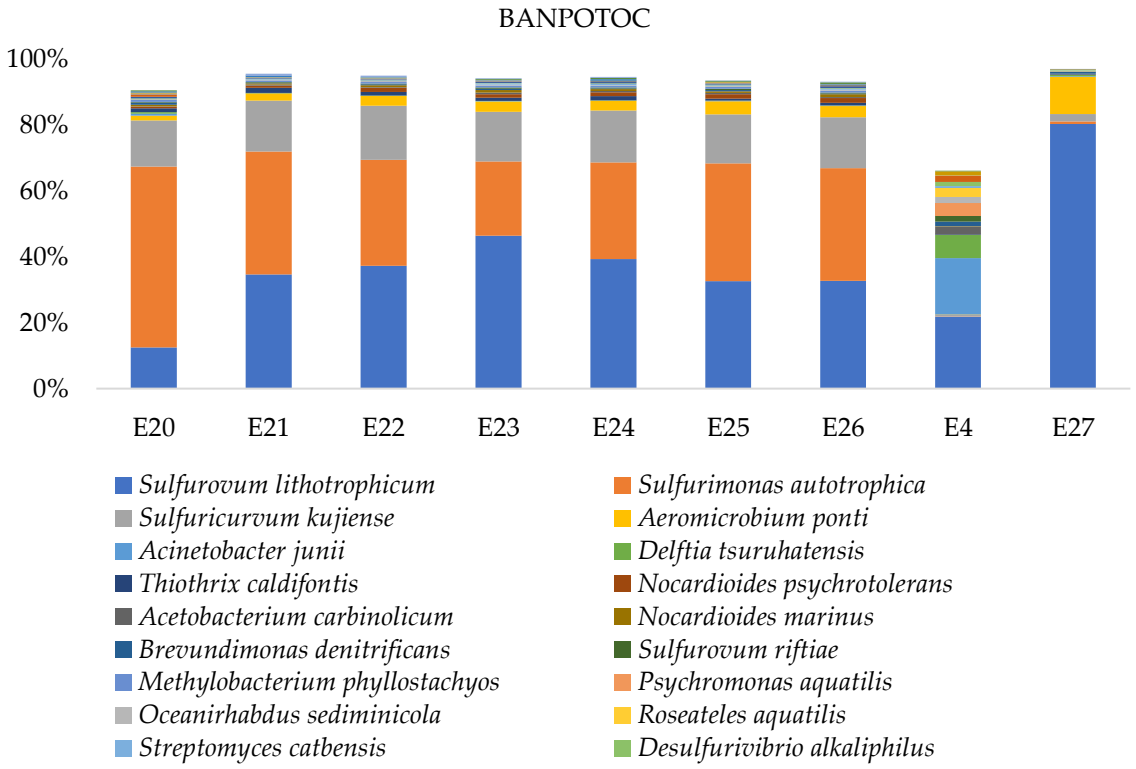

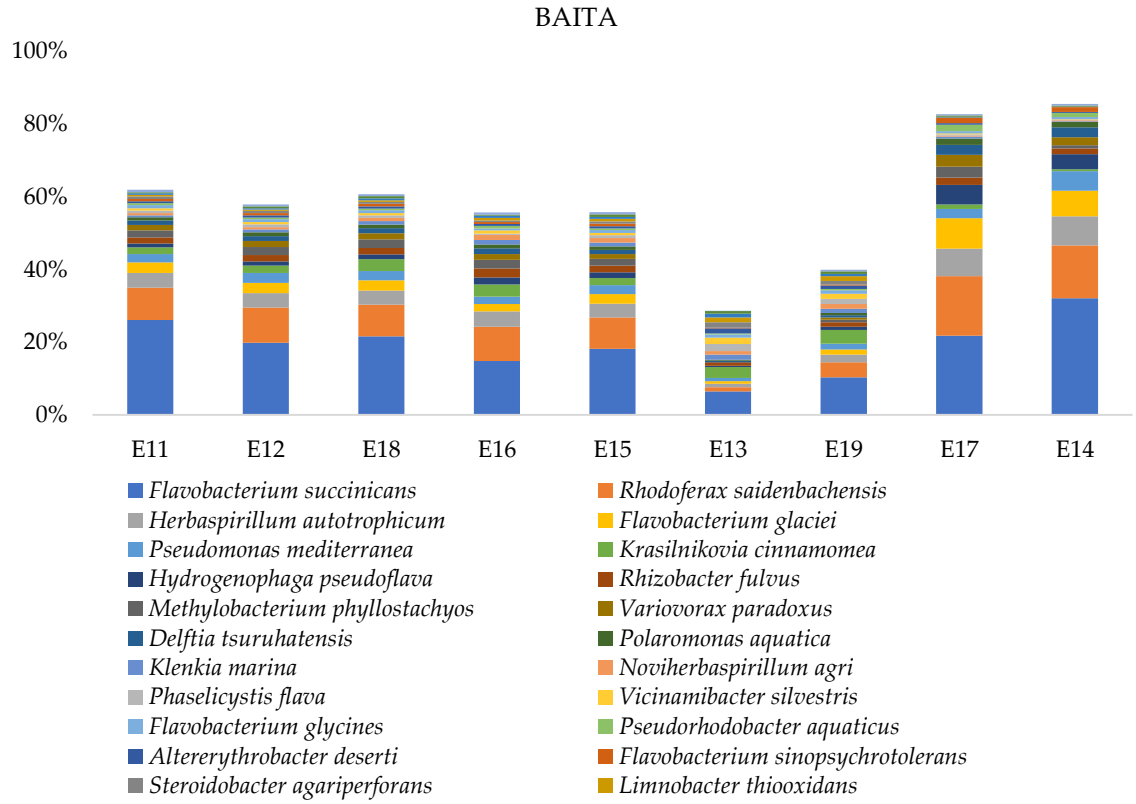

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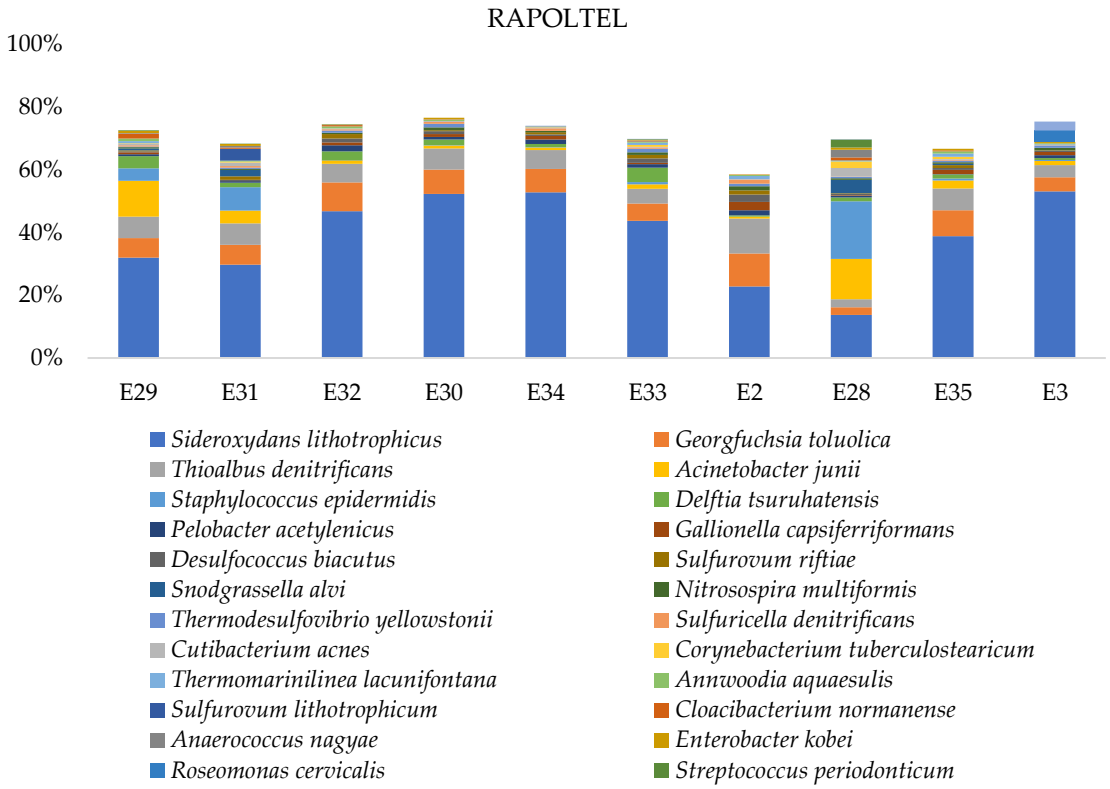

Figure 5. The relative abundance of the 25 best represented taxa in the three studied springs.

We also checked for the presence of the pathogenic bacteria in the different samples (Table 4, Table S1). Baita had no pathogens, while the other two springs had several representatives of known pathogenic bacterial species. The PSR009 and MCE_a membranes were the only that retained pathogenic bacteria in Banpotoc. In Rapoltel, all filters, except for the S-Pak type, had pathogens, represented by one or more species. The PSR009 membrane of Rapoltel had the higher number of identified pathogenic species, although different between the two replicates, 6 species for E28 and only 2 for E35 (Table 4). *Acinetobacter junii* and *Staphylococcus epidermidis* were the pathogenic bacteria found in very high abundances in Banpotoc and Rapoltel springs.

Table 4. The tested membranes for metabarcoding and the most abundant identified pathogenic bacteria.

Spring	Membrane code	Membrane	Biosafety risk species for humans & animals		
			100 – 500 reads/L	501-1000 reads/L	>1001 reads/L
Banpotoc	E20	PES			
	E21	PVDF			
	E22	NC			
	E23	NYLON			
	E24	MCE_a			*Acinetobacter junii*
	E25	MCE_b			
	E26	S-Pak			
	E4	PSR009	*Comamonas testosteroni* *Enterobacter kobei*		

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	E27	PSR010			
Baita	E11	PES			
	E12	PVDF			
	E18	NC			
	E16	NYLON			
	E15	MCE_a			
	E13	MCE_b			
	E19	S-Pak			
	E17	PSR009			
	E14	PSR010			
Rapoltel	E29	PES	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	*Acinetobacter junii*
	E31	PVDF	*Acinetobacter junii* *Escherichia fergusonii*		
	E32	NC	*Acinetobacter junii*		
	E30	NYLON	*Acinetobacter junii*		
	E34	MCE_a	*Acinetobacter junii*		
	E33	MCE_b	*Acinetobacter junii*		
	E2	S-Pak	*Atopobium vaginae* *Corynebacterium tuberculostearicum* *Gardnerella vaginalis* *Moraxella osloensis*		*Acinetobacter junii* *Staphylococcus epidermidis*
	E28	PSR009			
	E35	PSR009	*Acinetobacter junii* *Veillonella dispar*		
	E3	PSR010	*Acinetobacter junii*		

4. Discussion

Groundwater geochemistry, and that of their surface outlets represented by springs, is regulated by the flow paths and groundwater residence time [22, 23]. The rocks and deposits crossed underground by the waterway, length of the underground passages, speed of transit, and the quality of the surface infiltrations shape the chemical composition of the karstic spring waters. Banpotoc and Rapoltel waters cross limestone layers, explaining the high concentration of carbonates. The fast flow of groundwater through the limestone levels explains the low mineralization of Baita spring. Ammonia in Banpotoc slightly exceeded the drinking water quality values [21]. The sources of the nitrogen compounds could be the agricultural activities (use of soil fertilizers based on nitrogen), household activities, leaching from septic tanks, animal debris.

Microbial organisms in groundwaters depend mostly on oxidation and reduction of inorganic compounds for energy in this low in nutrients environment [24]. Thus, electron donors and acceptors of water and geological substratum become the major driver of communities' composition and diversity [25-29]. Microbial communities can change drastically when contamination is originating from the surface [30-32].

In our experiment, on the efficiency of different filter membrane types for 16S rRNA metabarcoding, the chemistry was important in shaping the diversity of microorganisms and

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pathogenic bacteria. Different filtrating membranes provided various OTUs diversity in the three springs. Nylon, MCE-b, S-Pak and PSR009 were the best filters for diversity. Table 5 contains the selection of the best filtering membranes for an estimation of the OTUs diversity/abundance and presence of pathogens obtained through metabarcoding. We expected to have similar results with the same filter(s) on different spring waters. However, the same filtering membrane gave various results when used on water from springs which are chemically different.

The filtering membranes also performed differently when pathogen identification is considered. However, for the efficiency of different filtering membranes in detecting pathogenic bacteria, most of the species were obtained filtering water with the PSR009 membrane. For Banpotoc samples, PSR009 and MCE_a membranes provided good results. Rapoltel samples, provided more diverse and abundant pathogens with the PSR009 and also with the PVDF and PES membranes. In the three studied springs, all the identified pathogenic bacteria have low or moderate pathogenic risks, unlikely to cause human or animal diseases. None of the pathogens classified in high risk groups that can cause serious diseases were found in our samples.

Table 5. The comparative analysis of the different membranes for 16S metabarcoding of karstic springs, for diversity and quality analysis.

Spring/Membrane	Pathogens identification	Diversity assessment	Proposed membrane for pathogens	Proposed membrane for diversity
Banpotoc	PSR009, MCE_a	Nylon, PSR009, S-Pak	PSR009	S-Pak, PSR009
Baita		MCE-B, Nylon, S-Pak		
Rapoltel	PSR009, PES, PVDF	MCE_b, S-Pak, PSR009		

No previous studies were undertaken on the chemical and microbiological composition of the three springs chosen in the present studies. We do not know if the ammonia contamination in Banpotoc and the microbiological contamination in Rapoltel are recent or not. However, the presence of pathogenic bacteria, sometimes in high number, in two of the springs (Banpotoc and Rapoltel) that are used as sources of drinking water calls for further quality monitoring. We will continue the monitoring program on these three springs and other springs in Romania and will use the selected filtering membranes as specified in Table 5.

5. Conclusions

For the karstic springs' quality assessment, we proposed a preliminary evaluation of 16S rRNA diversity and abundance by testing different membranes for water filtering. The testing of different sterile or sterilizable commercial membranes was a prerequisite in establishing monitoring protocols. We based our decision in choosing the best filtering membrane on their efficiency in retaining microorganisms and pathogenic bacteria and price. As a result of the experiment we proposed two membranes for diversity and one for pathogenic bacteria. These three membranes will be used in the monitoring program of karstic springs used by rural populations as drinking water sources.

Supplementary Materials:, **Figure S1.** Piper diagram of the chemical elements for the studied springs; **Figure S2.** The heat maps of the first 25 most abundant species (above the most abundant) in each of the studied springs, Banpotoc, Baita and Rapoltel (from left to right). Relative abundance of species was considered in the analysis; **Table S1.** Number of reads for the pathogenic bacteria for humans and animals found in the three studied karstic springs.

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References

[1] Rodell, M., Famiglietti, J.S., Wiese, D.N., Reager, J. T., Beaudoing, H. K., Landerer, F. W., Lo, M.-H. Emerging trends in global freshwater availability. *Nature* **557,** 651–659 (2018).

[2] Zektser, I. S., Everett, L. G. (eds.) Groundwater Resources of the World and Their Use (UNESCO, Paris, 2004); http://unesdoc.unesco.org/images/0013/001344/134433e.pdf.

[3] Siebert, S., Burke, J., Faures, J. M., Frenken, K., Hoogeveen, J., Döll, P., Portmann, F. T. Groundwater use for irrigation – a global inventory. *Hydrol. Earth Syst. Sci.* **14**, 1863–1880 (2010).

[4] Taylor, R. G., Scanlon, B., Döll, P., Rodell, M., Van Beek, R., Wada, Y., Longuevergne, L., LeBlanc, M., Famiglietti, J., Edmunds, M., Konikow, L., Green, T. R., Chen, J., Taniguchi, M., Birkens, M. F. P., Macdonald, A., Fan, Y., Maxwell, R. M., Yechieli, Y., Gurdak, J. J., Allen, D. M., Shamsudduha, M., Hiscock, K., Yeh, P. J. F., Holman, I., Treidel, H. Ground water and climate change. *Nature Clim. Change* **3,** 322–329 (2013).

[5] Eurostat (2020). Available online https://ec.europa.eu/eurostat/web/products-datasets/-/env_wat_pop last accessed on 7.06.2020.

[6] McDonough, L. K., Santos, I. R., Andersen, M. S., O'Carroll, D. M., Rutlidge, H., Meredith, K., Oudone, P., Bridgeman, J., Gooddy, D. C., Sorensen, J. P. R., Lapworth, D. J., MacDonald, A. M., Ward, J., Baker, A. Changes in global groundwater organic carbon driven by climate change and urbanization. *Nat Commun* **11,** 1279 (2020).

[7] Hoaghia, M. A., Cadar, O., Hognogi, G. G., Levei, E., Moisa, C., Roman, C. Quality and human health risk assessment of metals and nitrogen compounds in drinking water from an urban area near a former non-ferrous ore smelter. *Analytical letters*, **52**,1268-1281 (2019).

[8] Pawlowski, J., Kelly-Quinn, M., Altermatt, F., Apothéloz-Perret-Gentil, L., Beja, P., Boggero, A., ... & Feio, M. J. The future of biotic indices in the ecogenomic era: Integrating (e) DNA metabarcoding in biological assessment of aquatic ecosystems. *Science of the Total Environment*, *637*, 1295-1310 (2018).

[9] Deiner, K., Fronhofer, E. A., Mächler, E., Walser, J. C., & Altermatt, F. (Environmental DNA reveals that rivers are conveyer belts of biodiversity information. *Nature communications*, *7*(1), 1-9 2016).

[10] Kuntke, F., de Jonge, N., Hesselsøe, M., & Nielsen, J. L. Stream water quality assessment by metabarcoding of invertebrates. *Ecological Indicators*, *111*, 105982 (2020).

[11] Tolotti, M., Cerasino, L., Donati, C., Pindo, M., Rogora, M., Seppi, R., & Albanese, D. Alpine headwaters emerging from glaciers and rock glaciers host different bacterial communities: Ecological implications for the future. *Science of The Total Environment*, *717*, 137101 (2020).
[12] Paruch, L., Paruch, A. M., Eiken, H. G., & Sørheim, R. Faecal pollution affects abundance and diversity of aquatic microbial community in anthropo-zoogenically influenced lotic ecosystems. *Scientific Reports*, *9*(1), 1-13 (2019).
[13] Szekeres, E., Chiriac, C. M., Baricz, A., Szőke-Nagy, T., Lung, I., Soran, M. L., ... & Coman, C. Investigating antibiotics, antibiotic resistance genes, and microbial contaminants in groundwater in relation to the proximity of urban areas. *Environmental Pollution*, *236*, 734-744 (2018).
[14] Grönwall, J., & Danert, K. Regarding Groundwater and Drinking Water Access through A Human Rights Lens: Self-Supply as A Norm. *Water*, *12*(2), 419 (2020).
[15] Sanganyado, E., & Gwenzi, W. Antibiotic resistance in drinking water systems: Occurrence, removal, and human health risks. *Science of the Total Environment*, *669*, 785-797 (2019).
[16] Moldovan, O. T., Øvrevik Skoglund, R., Banciu, H. L., Dinu Cucoș, A., Levei ,E. A., Perșoiu, A., Lauritzen, S.-E. Monitoring and risk assessment for groundwater sources in rural communities of Romania (GROUNDWATERISK). *Research Ideas and Outcomes*, **5**, e48898 (2019).
[17] Magoc, T., Salzberg, S. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, **27**, 2957-63 (2011).
[18] Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J., Weber, C. F. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.*, **75**, 7537-41 (2009).
[19] Li, W., Fu, L., Niu, B., Wu, S., Wooley, J. Ultrafast Clustering Algorithms for Metagenomic Sequence Analysis. *Brief. Bioinform.*, **13**, 656-668 (2012).
[20] Caporaso, J., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer,N., Pena, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Turnbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, doi:10.1038/nmeth.f.3 (2010).
[21] Law 311. Law 311 for modifying and completing Law 458/2002, regarding the quality of drinking water, Official Monitor, No. 582 from 30 June 2004, Romania (2004).
[22] Tarits, C., Aquilina, L., Ayraud, V., Pauwels, H., Davy, P., Touchard, F., Bour, O. Oxido-reduction sequence related to flux variations of groundwater from a fractured basement aquifer (Ploemeur area, France). *Appl. Geochemistry* 21, 29–47 (2006).
[23] Roques, C., Aquilina, L., Bour, O., Maréchal, J.-C., Dewandel, B., Pauwels, H., Labasque, T., Vergnaud-Ayraud, V., Hochreutener, R. Groundwater sources and geochemical processes in a crystalline fault aquifer. *J. Hydrol.* **519**, 3110–3128 (2014).
[24] Ben Maamar, S., Aquilina, L., Quaiser, A., Pauwels, H., Michon-Coudouel, S., Vergnaud-Ayraud. V., Labasque, T., Roques, C., Abbott, B. W., Dufresne, A. Groundwater isolation governs chemistry and microbial community structure along hydrologic flowpaths. Front. Microbiol., **6**, 1457 (2015).

[25] Akob, D. M., Mills, H. J., Kostka, J. E. Metabolically active microbial communities in uranium-contaminated subsurface sediments. *FEMS Microbiol. Ecol.*, **59**, 95–107 (2007).
[26] Boyd, E. S., Cummings, D. E., Geesey, G. G. Mineralogy influences structure and diversity of bacterial communities associated with geological substrata in a pristine aquifer. *Microb. Ecol.*, **54**, 170–182 (2007).
[27] Flynn, T. M., Sanford, R. A., Ryu, H., Bethke, C. M., Levine, A. D., Ashbolt, N. J., Santo Domingo, J. W. Functional microbial diversity explains groundwater chemistry in a pristine aquifer. *BMC Microbiol.*, **13**, 146 (2013).
[28] Flynn, T. M., Sanford, R. A., Santo Domingo, J. W., Ashbolt, N. J., Levine, A. D., Bethke, C. M. The active bacterial community in a pristine confined aquifer. *Water Resour. Res.*, **48**, W09510 (2012).
[29] Nyyssönen, M., Bomberg, M., Kapanen, A., Nousiainen, A., Pitkänen, P., Itävaara, M. Methanogenic and sulphate-reducing microbial communities in deep groundwater of crystalline rock fractures in olkiluoto, finland. *Geomicrobiol. J.*, **29**, 863–878 (2012).
[30] Hemme, C. L., VanNostrand, J. D., Wu, L., He Z., Zhou J., Green S. J., Rishishwar L., Jordan I. K., Kostka J. E., Prakash O., Pettenato A., Chakraborty R., Deutschbauer A. M., Hazen T. C., Arkin P. Lateral gene transfer in a heavy metal-contaminated-groundwater microbial community. *MBio*, **7**, 1–14 (2016).
[31] He, Z., Zhang P., Wu L., Rocha A. M., Tu Q., Shi Z., Wu B., Qin Y., Wang J., Yan Q., Curtis D., Ning D., VanNostrand J. D., Wu L., Yang Y., Elias D. A., Watson D. B., Adams M. W. W., Fields M. W., Alm E. J., Hazen T. C., Adams P. D., Arkin A. P., Zhou J. Microbial functional gene diversity predicts groundwater contamination and ecosystem functioning. *MBio*, **9**, e02435–17 (2018).
[32] Sonthiphand, P., Ruangroengkulrith, S., Mhuantong, W. Charoensawan, V., Chotpantarat, S., Boonkaewwan, S. Metagenomic insights into microbial diversity in a groundwater basin impacted by a variety of anthropogenic activities. *Environ. Sci. Pollut. Res.* **26,** 26765–26781 (2019).