







## Article

# Testing Different Membrane Filters for 16S rRNA Gene-Based Metabarcoding in Karstic Springs

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**Abstract:** Introduction: Karstic springs are used worldwide by rural communities as sources of fresh water for humans and livestock. In Romania, one-third of the population has no direct access to a public water supply. The present study is part of a country-wide project to develop simple, quick and cheap methods for seasonal environmental and microbiological monitoring of karstic springs used as drinking water by rural populations. Critical steps for monitoring workflow consist of evaluating water quality and selecting suitable membrane filters to efficiently capture environmental DNA for further microbial diversity estimation using 16S rRNA gene-based metabarcoding. Methods: Several commercial membrane filters of different compositions 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. Results: We found that different types of applied membrane filters provide varying recovery in diversity and abundance of both overall and pathogenic bacteria. Conclusions: The result of the experiment with different filters shows that mixed cellulose ester, cellulose acetate, and nitrate membranes of 0.20 and 0.22  $\mu\text{m}$  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

A recent review [1] considered the main environmental drivers for 21st century global trends regarding freshwater availability. Changes in freshwater availability not only impact regional food supplies, human and ecosystem health and energy generation but also cause social unrest [1]. Half of the world's 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 such as limestones and dolomites. The high demand for food, in conjunction with climate change, has resulted in economic and social hardships already reported in many parts of the world and are foreseen to accelerate the depletion of available groundwater sources used for drinking and agriculture [4].

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

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

For rural populations that use spring water as their main source of fresh water, our purpose was 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 to evaluate the most suitable membrane filter type and filtration strategy to efficiently recover environmental DNA (eDNA) for further fast and accurate whole community diversity assessment using 16S rRNA gene-based metabarcoding.

## 2. Materials and Methods

### 2.1. Sampling and Sample Preparation

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

In September 2019, samples of spring water were collected from each of the three springs in sterile polyethylene bottles. Bottles were rinsed three times with the sample water and kept refrigerated at 4 °C until chemical analysis. Five liters of water were filtered 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 different types of membrane filters with different pore sizes were tested for their efficiency in retaining biomass that sourced for eDNA from karstic spring water (Table 1). After filtration, each membrane was cut into small pieces and kept in a sterile Petri dish at −20 °C until eDNA extraction.



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

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

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

\* We termed 'sterile' those membrane filters provided sterile by the manufacturer and 'sterilized' those membrane filters provided non-sterile and further steam-sterilized at 121 °C for 20 min. \*\* We define the prices as low, medium and high, relative to each other.

## 2.2. Physicochemical Measurements and Laboratory Analysis

The pH and electrical conductivity (EC) were measured in situ using a portable multiparameter monitoring device with built-in temperature correction (Multi 340i, WTW, Germany).

In the laboratory, 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 via catalytic combustion and infrared detection of CO<sub>2</sub> using a Multi N/C 2100S Analyzer (Analytik Jena, Germany). Total alkalinity was determined via titration with HCl. The bicarbonate (HCO<sub>3</sub><sup>−</sup>) content was calculated by multiplying the alkalinity expressed in mmol/L by 61. Ammonia was determined with UV-Vis spectrometry (Lambda 25, Perkin Elmer, Waltham, MA, USA) using the salicylate method. Anions were determined with 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<sub>3</sub> using inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 5300 DV, Perkin-Elmer, Waltham, MA, USA).

for Na, Mg, Ca and K and inductively coupled plasma mass spectrometry (ICP-MS, ELAN DRC II, Perkin-Elmer, Waltham, MA, USA) for Ba, Sr, Mn, Ni, and Cr.

### 2.3. 16S rRNA Protocol

MiSeq 16S V3-V4 Metagenome Sequencing was performed using a commercial company (Macrogen Europe). The V3-V4 hypervariable regions of the bacterial and archaeal SSU rRNA gene were amplified via PCR using primers 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 operational taxonomic units (OTUs) in QIIME against the last version of the RDP-16Sr DNA database and using a species-level OTU cut-off at 97% sequence identity [20].

### 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 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 both abundance and evenness, of those present in a community.

## 3. Results

### 3.1. Chemical Analysis

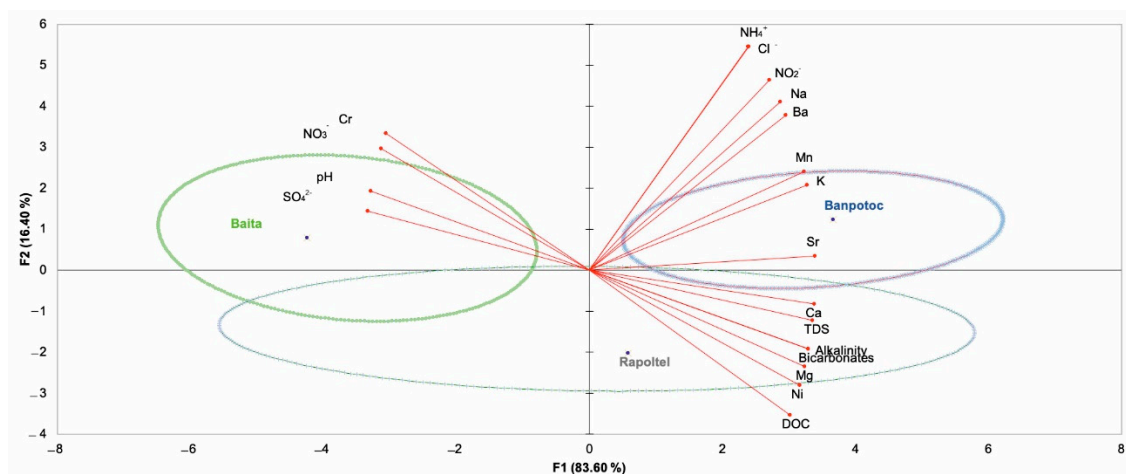
The chemical data quality was assessed by calculating the charge balance between the sum of cations and the sum of anions, expressed in mEq/L. In each case, the charge balance error (CBE) was less than 10% (Table 2).

**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 <sub>4</sub> <sup>+</sup>  | mg/L   | 0.57     | 0.12  | 0.04     |
| Cl <sup>-</sup>               | mg/L   | 4.53     | 2.55  | 2.2      |
| SO <sub>4</sub> <sup>2-</sup> | mg/L   | 2.2      | 19.6  | 6        |
| NO <sub>3</sub> <sup>-</sup>  | mg/L   | <0.2     | 2.4   | <0.2     |
| NO <sub>2</sub> <sup>-</sup>  | mg/L   | 0.32     | <0.05 | <0.05    |



The Piper diagram (Figure S1) shows that Banpotoc, Baita and Rapoltel springs have a Ca-HCO<sub>3</sub> facies. The PCA of the physicochemical variables (Figure 2) separated Baita and Banpotoc along the horizontal axis (F1). The F1 axis explains more than 83.6% of the variation 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 the 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.



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

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

### 3.2. Microbial Diversity Inferred by 16s RNA 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 1106 OTUs following singleton removal. OTUs pertaining to Archaea and Bacteria were identified, with Bacteria-related sequences dominating in all samples.

The identified taxa show high variations of diversity and abundances between the 28 analyzed filter samples (Table 3). The highest diversity was in the Baita samples followed by the Rapoltel and Banpotoc samples. AE23 (nylon) and AE4 (cellulose acetate and nitrate) from Banpotoc, BE13 and BE16 from Baita (mixed cellulose ester and nylon, respectively) and CE33 (mixed cellulose ester) from Rapoltel were the most diverse.

Most Archaea-related OTUs (16 in total) belonged to Euryarchaeota, with *Methanolobus taylorii*, *Methanobacterium aggregans* and *M. palustre* as the most frequently encountered species. Crenarchaeota and Thaumarchaeota were also represented in one to four samples, depending on the membrane type. Sample AE25 from Banpotoc (mixed cellulose ester) 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, Table S1). The other 11.54% of the total OTUs belonged to non-identified phyla. Proteobacteria dominated in all the samples, with Epsilonproteobacteria as the main class in Banpotoc, and Betaproteobacteria as the dominant class in Baita and Rapoltel (Figure 4). In Banpotoc, Actinobacteria was the next diverse phylum, while Bacteroidetes dominated Baita samples. Baita also had the highest diversity in phyla. Rapoltel

samples had no other dominant group except for Proteobacteria, by far the most diverse in this spring. Only CE28 had high OTU abundance belonging to Firmicutes phylum.

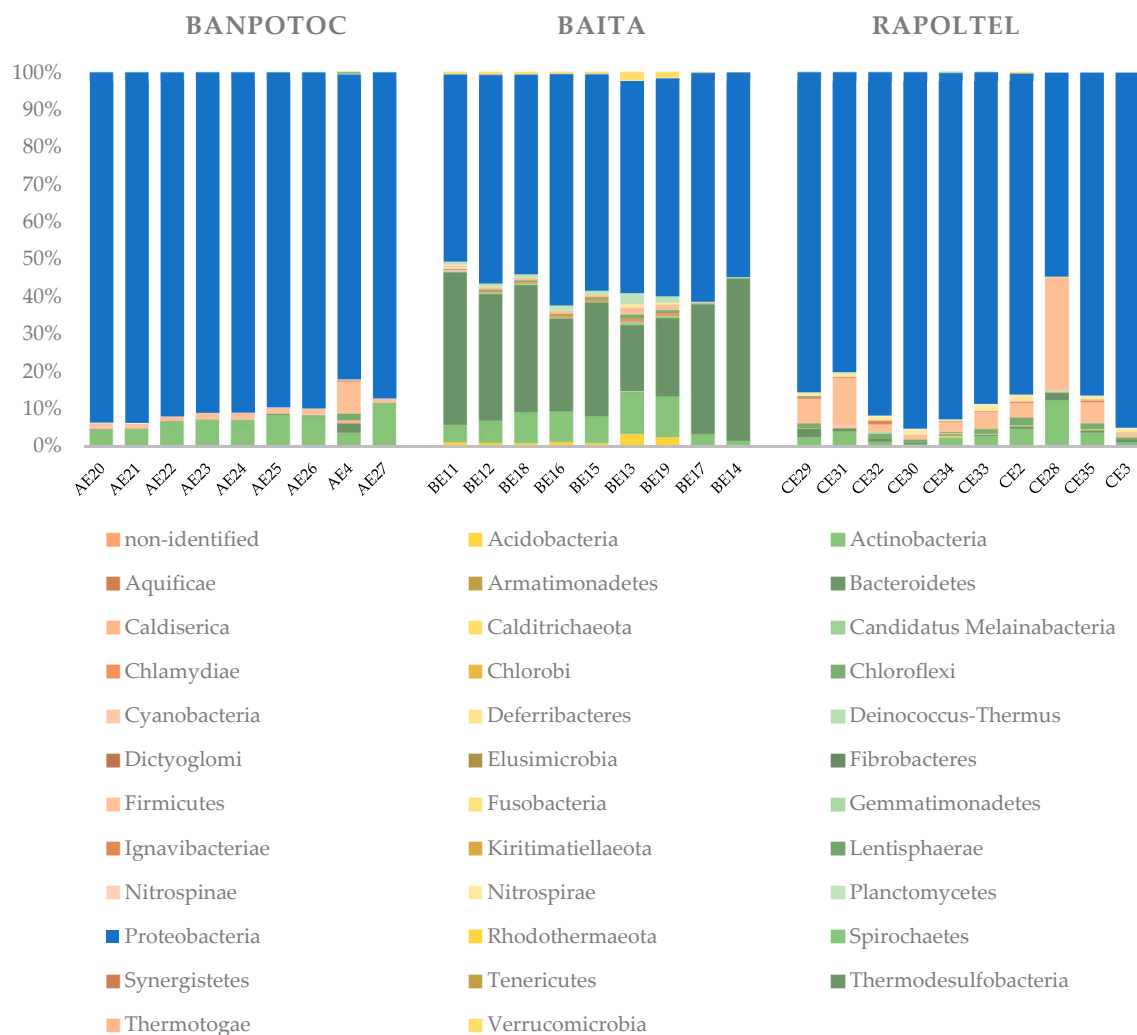
**Table 3.** Collected samples in the three karstic springs with their corresponding diversity indices.

| Spring   | Code | Fabric                        | Membrane Code | OTUs | Diversity Indices |         |
|----------|------|-------------------------------|---------------|------|-------------------|---------|
|          |      |                               |               |      | Chao1             | Shannon |
| Banpotoc | AE20 | polyethersulfone              | PES           | 186  | 188.6250          | 2.8280  |
|          | AE21 | polyvinylidene fluoride       | PVDF          | 226  | 264.2778          | 2.5738  |
|          | AE22 | cellulose nitrate             | NC            | 228  | 306.9643          | 2.6665  |
|          | AE23 | nylon                         | NYLON         | 264  | 294.3571          | 2.7375  |
|          | AE24 | mixed cellulose ester         | MCE_a         | 251  | 318.0000          | 2.7688  |
|          | AE25 | mixed cellulose ester         | MCE_b         | 241  | 257.5000          | 2.8318  |
|          | AE26 | mixed cellulose ester         | S-Pak         | 261  | 320.2941          | 2.9322  |
|          | AE4  | cellulose acetate and nitrate | PSR009        | 263  | 267.5000          | 4.9879  |
| Baita    | AE27 | cellulose acetate and nitrate | PSR010        | 115  | 126.5500          | 1.2992  |
|          | BE11 | polyethersulfone              | PES           | 1280 | 1699.2250         | 6.4304  |
|          | BE12 | polyvinylidene fluoride       | PVDF          | 1335 | 1679.3142         | 6.8048  |
|          | BE18 | cellulose nitrate             | NC            | 1368 | 1652.6980         | 6.6819  |
|          | BE16 | nylon                         | NYLON         | 1538 | 1803.8583         | 7.1422  |
|          | BE15 | mixed cellulose ester         | MCE_a         | 1271 | 1569.4933         | 7.0039  |
|          | BE13 | mixed cellulose ester         | MCE_b         | 1566 | 1734.7447         | 8.5057  |
|          | BE19 | mixed cellulose ester         | S-Pak         | 1455 | 1752.9286         | 7.9813  |
| Raportel | BE17 | cellulose acetate and nitrate | PSR009        | 794  | 1009.5301         | 4.9305  |
|          | BE14 | cellulose acetate and nitrate | PSR010        | 718  | 915.3356          | 4.3866  |
|          | CE29 | polyethersulfone              | PES           | 200  | 202.0000          | 4.8509  |
|          | CE31 | polyvinylidene fluoride       | PVDF          | 221  | 224.7500          | 5.1441  |
|          | CE32 | cellulose nitrate             | NC            | 235  | 242.2000          | 4.4465  |
|          | CE30 | nylon                         | NYLON         | 174  | 174.6000          | 3.9005  |
|          | CE34 | mixed cellulose ester         | MCE_a         | 214  | 217.3333          | 4.1399  |
|          | CE33 | mixed cellulose ester         | MCE_b         | 358  | 360.5000          | 5.0208  |
|          | CE2  | mixed cellulose ester         | S-Pak         | 310  | 312.5000          | 5.7927  |
|          | CE28 | cellulose acetate and nitrate | PSR009        | 260  | 265.1429          | 5.1996  |
|          | CE35 | cellulose acetate and nitrate | PSR009        | 255  | 255.4286          | 5.0427  |
|          | CE3  | cellulose acetate and nitrate | PSR010        | 168  | 169.5000          | 3.8749  |

In a PCA of diversity indices (Figure 5), the samples from Baita separated along the F1 axis (90.85% variance) from Banpotoc and Raportel, while these last two separated from each other along the F2 axis (9.05% variance). Banpotoc membranes are more similar to each other and so are the Raportel membranes, except for AE4 (0.22  $\mu\text{m}$  porosity) and CE3 (0.45  $\mu\text{m}$  porosity), both consisting of cellulose acetate and nitrate. The Baita membranes are more different from each other. Along the F1 axis, BE17 and BE14 (both cellulose acetate and nitrate membranes, with different porosities) have a relatively separate position from the other Baita membranes; their position is explained by the analysis at the species level (see below).

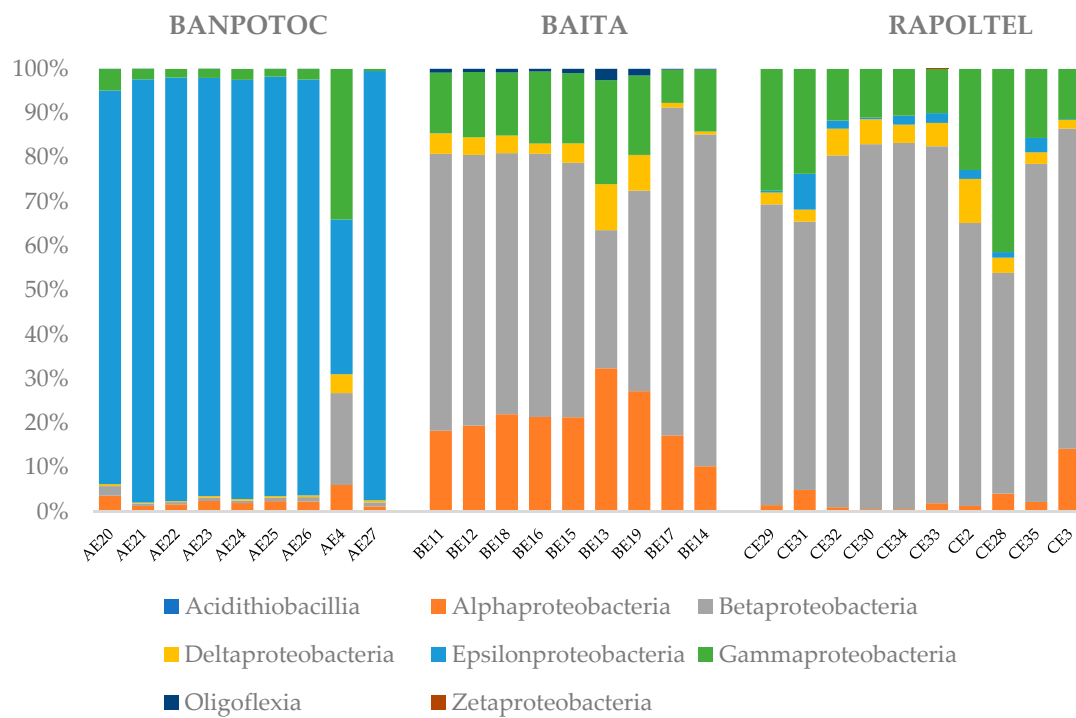
To infer the efficiency of different filtrating membranes, the results were further analyzed separately for each spring at the species level for more evident differences. The relative abundances of the dominant species are represented in Figure S2. The dominant species in Banpotoc samples were *Sulfurovum lithotrophicum* and *Sulfurimonas autotrophica*, both involved in the redox sulfur cycle (Figure 6a). They represented 68.5% of the identified Banpotoc total OTUs. *Flavobacterium succinicans* and *Rhodferax saidenbachensis* represented more than 34% of the identified Baita OTUs (Figure 6b). *Sideroxydans lithotrophicus* was by far the most abundant species in all Raportel samples, representing more than 43% of the total identified OTUs (Figure 6c). The AE4 (0.22  $\mu\text{m}$ ) and AE27 (0.45  $\mu\text{m}$ ) samples, both cellulose acetate and nitrate membranes, provided different results than the other membranes of Banpotoc, all abundant in *Sulfurimonas autotrophica* and *Sulphurovum lithotrophicum*. The AE4 membrane had *Acinetobacter junii* as the dominant taxa, while AE27 was almost entirely represented by *Sulphurovum lithotrophicum*. In Baita, two species were dominant, *Flavobacterium succinicans* and *Rhodferax saidenbachensis*, except for the BE13 and BE19 samples, which were both mixed cellulose

esters and where no species was particularly dominant. All Rapotl samples, except for CE28 (0.22  $\mu$ m cellulose acetate and nitrate), were dominated by *Sideroxydans lithotrophicus*. CE28 was dominated by *Staphylococcus epidermis*.

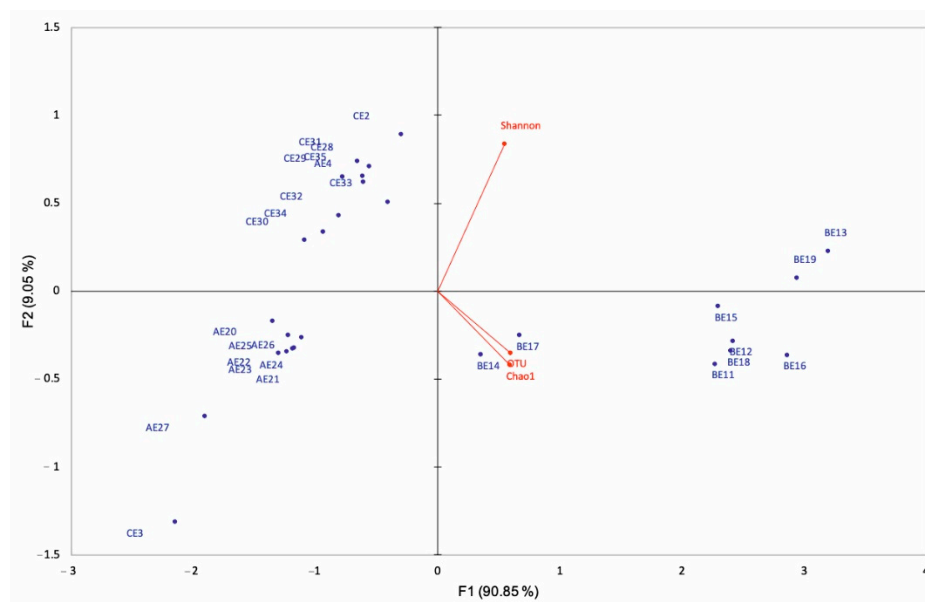


**Figure 3.** The relative abundance of the Bacteria phyla in the analyzed membranes, showing the dominant Proteobacteria in blue (see also Table S1).

We also checked for the presence of pathogenic bacteria in the different samples (Table 4, Table S2). Baita had no pathogens, while the other two springs had several representatives of known pathogenic bacterial species. The cellulose acetate and nitrate of 0.22  $\mu$ m and (PSR009) and mixed cellulose ester (MCE\_a) membranes were the only ones that retained pathogenic bacteria in Banpotoc. In Rapotl, all filters, except for the mixed cellulose ester S-Pak type, had pathogens, represented by one or more species. The PSR009 membrane of Rapotl had the higher number of identified pathogenic species, although there was a difference between the two replicates as CE28 had six species and CE35 had only two (Table 4). *Acinetobacter junii* and *Staphylococcus epidermidis* were the pathogenic bacteria found in very high abundances in the Banpotoc and Rapotl springs.



**Figure 4.** The relative abundance of the Proteobacteria classes in the analyzed membranes, showing the dominance of Epsilonproteobacteria (blue) in Banpotoc and of Betaproteobacteria (grey) in Baita and Rapotel.



**Figure 5.** The PCA of the samples in the three studied springs separated according to the related OTUs and the diversity indices, Chao1 and Shannon.



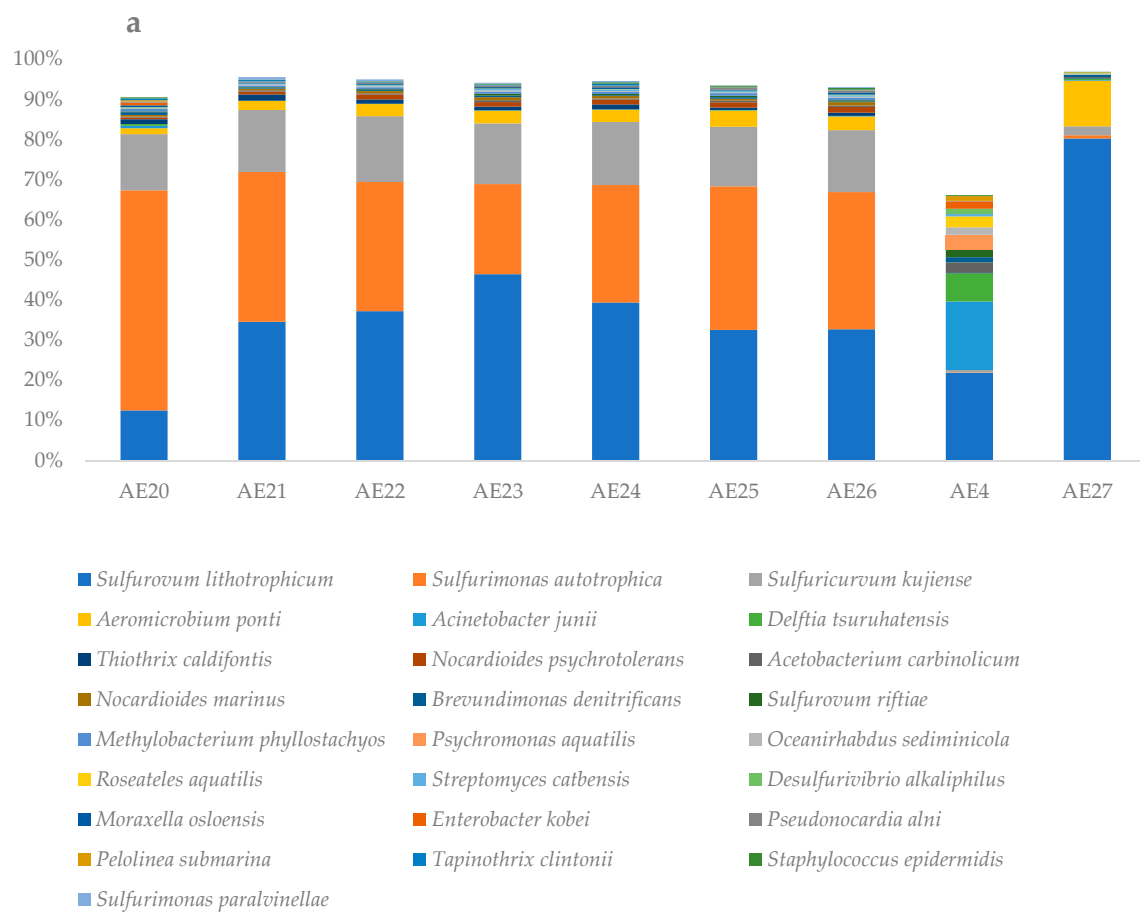
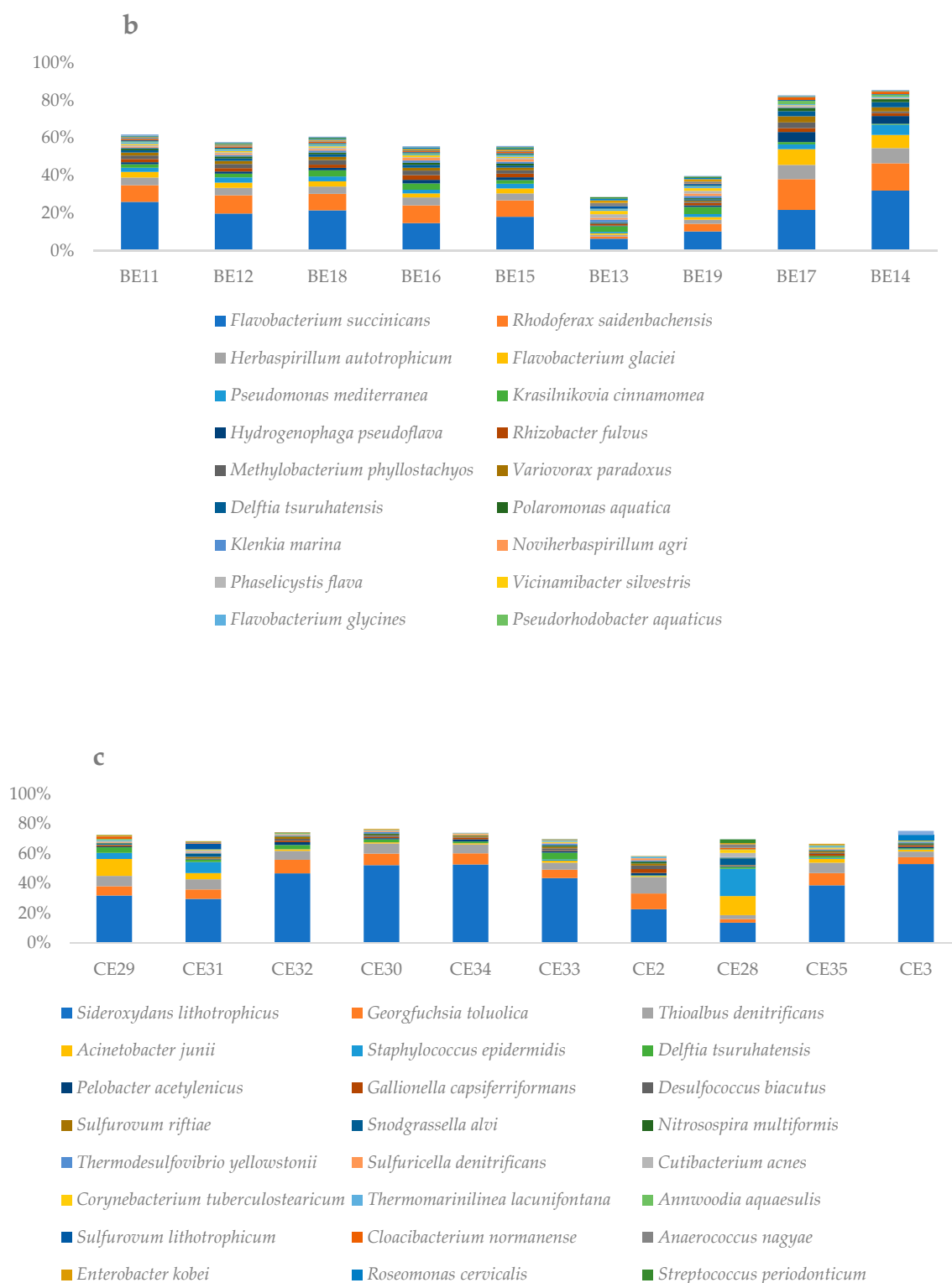


Figure 6. Cont.



**Figure 6.** The relative abundance of the 25 best represented taxa in the three studied springs. (a): Banpotoc; (b): Baita; (c): Rapoltel.

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

| Spring   | Sample Code | Membrane Code | Biosafety Risk Species for Humans & Animals  |                                   |   |
|----------|-------------|---------------|--|-----------------------------------|---|
|          |             |               | 100–500 reads/L  | 501–1000 reads/L                  | >1001 reads/L   |
| Banpotoc | AE20        | PES           |  |                                   |   |
|          | AE21        | PVDF          |  |                                   |   |
|          | AE22        | NC            |  |                                   |   |
|          | AE23        | NYLON         |  |                                   |   |
|          | AE24        | MCE_a         |  |                                   | <i>Acinetobacter junii</i>                                      |
|          | AE25        | MCE_b         |  |                                   |   |
|          | AE26        | S-Pak         |  |                                   |   |
|          | AE4         | PSR009        | <i>Comamonas testosteroni</i><br><i>Enterobacter kobei</i>   |                                   |   |
|          | AE27        | PSR010        |  |                                   |   |
|          |             |               |  |                                   |   |
| Baita    | BE11        | PES           |  |                                   |   |
|          | BE12        | PVDF          |  |                                   |   |
|          | BE18        | NC            |  |                                   |   |
|          | BE16        | NYLON         |  |                                   |   |
|          | BE15        | MCE_a         |  |                                   |   |
|          | BE13        | MCE_b         |  |                                   |   |
|          | BE19        | S-Pak         |  |                                   |   |
|          | BE17        | PSR009        |  |                                   |   |
|          | BE14        | PSR010        |  |                                   |   |
|          |             |               |  |                                   |   |
| Rapoltel | CE29        | PES           | <i>Staphylococcus epidermidis</i>  |                                   | <i>Acinetobacter junii</i>                                      |
|          | CE31        | PVDF          | <i>Acinetobacter junii</i><br><i>Escherichiafergusonii</i>   | <i>Staphylococcus epidermidis</i> |   |
|          | CE32        | NC            | <i>Acinetobacter junii</i>   |                                   |   |
|          | CE30        | NYLON         | <i>Acinetobacter junii</i>   |                                   |   |
|          | CE34        | MCE_a         | <i>Acinetobacter junii</i>   |                                   |   |
|          | CE33        | MCE_b         | <i>Acinetobacter junii</i>   |                                   |   |
|          | CE2         | S-Pak         |  |                                   |   |
|          |             |               |  |                                   |   |
|          | CE28        | PSR009        | <i>Atopobium vaginae</i><br><i>Corynebacterium tuberculoostearicum</i><br><i>Gardnerella vaginalis</i><br><i>Moraxella osloensis</i> |                                   | <i>Acinetobacter junii</i><br><i>Staphylococcus epidermidis</i> |
|          |             |               |  |                                   |   |
| Rapoltel | CE35        | PSR009        | <i>Acinetobacter junii</i><br><i>Veillonella dispar</i>  |                                   |   |
|          | CE3         | PSR010        | <i>Acinetobacter junii</i>   |                                   |   |

#### 4. Discussion

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

Microbial organisms in groundwater depend mostly on oxidation and reduction of inorganic compounds for energy in this low-nutrient environment [24]. Thus, electron donors and acceptors of

water and geological substratum become the major drivers of microbial 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 pathogenic bacteria. Different filtering membranes provided various OTU diversity in the three springs. Nylon, mixed cellulose ester and cellulose acetate and nitrate (Nylon, MCE-b, S-Pak and PSR009) membranes were the best filters for diversity. Table 5 contains the selection of the best filtering membranes for an estimation of the OTU 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 was considered. However, for the efficiency of different filtering membranes in detecting pathogenic bacteria, most of the species were obtained by filtering water with the cellulose acetate and nitrate (PSR009) membrane. For Banpotoc samples, cellulose acetate and nitrate and mixed cellulose ester (PSR009 and MCE\_a) membranes provided good results. Rapoltel samples provided more diverse and abundant pathogens with the cellulose acetate and nitrate (PSR009) and also with the polyvinylidene fluoride (PVDF) and polyethersulfone (PES) membranes. In the three studied springs, all the identified pathogenic bacteria had low or moderate pathogenic risks, and were 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.

No previous studies were undertaken on the chemical and microbiological composition of the three springs chosen in the present study. 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.

**Table 5.** The comparative analysis of the different membranes for 16S metabarcoding of karstic springs for diversity and quality analysis. The selected membranes have a porosity of 0.2–0.22 µm.

| Spring/<br>Membrane | Pathogens Identification               | Diversity<br>Assessment   | Proposed Membrane<br>for Pathogens        | Proposed Membrane<br>for Diversity               |
|---------------------|--|---|---|--|
| Banpotoc            | cellulose acetate and nitrate (PSR009) | nylon (Nylon)   | cellulose acetate and<br>nitrate (PSR009) | S-Pak, cellulose acetate<br>and nitrate (PSR009) |
|                     | mixed cellulose ester (MCE_a)          | cellulose acetate and nitrate (PSR009)<br>mixed cellulose ester (S-Pak) |   |  |
| Baita               |  | mixed cellulose ester (MCE-B)   |   |  |
|                     |  | nylon (Nylon)   |   |  |
|                     |  | mixed cellulose ester (S-Pak)   |   |  |
| Rapoltel            | cellulose acetate and nitrate (PSR009) | mixed cellulose ester (MCE_b)   |   |  |
|                     | polyvinylidene fluoride (PVDF)         | mixed cellulose ester (S-Pak)   |   |  |
|                     | polyethersulfone (PES)                 | cellulose acetate and nitrate (PSR009)                                  |   |  |



## 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:** The following are available online at <http://www.mdpi.com/2073-4441/12/12/3400/s1>: **Figure S1.** The 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 Rapotel (from left to right). Relative abundance of species was considered in the analysis; **Table S1.** The relative abundance of the Bacteria phyla in the analyzed membranes, with the dominant Proteobacteria (see also Figure 3); **Table S2.** Number of reads for the pathogenic bacteria for humans and animals found in the three studied karstic springs.

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