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MICROBIOLOGICAL AND CHEMICAL CHARACTERISTICS OF WATER AND SEDIMENT FROM VRELO CAVE, REPUBLIC OF MACEDONIA

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Abstract: Vrelo Cave is the deepest cave in Macedonia, located in the canyon Matka which is home to many endemic species not found anywhere else in Europe. Until now, Vrelo Cave has not been investigated in terms of its composition and biodiversity. The purpose of this study was to offer some preliminary data for physical and chemical parameters of water and sediments from Vrelo Cave, as well as its microbiological diversity. Samples were taken from 5 locations. They were analysed for a wide array of physico-chemical parameters, macro- and microelements and concentration of selected organic pollutants. All samples were investigated for several groups of bacteria, yeasts and moulds by a conventional selective media approach. Molecular identification of the isolated bacterial species was done by sequencing of the bacterial 16S ribosomal RNA gene. Regarding the total dry components, total hardness, dissolved oxygen, biochemical and chemical consumption of oxygen, water from Vrelo Cave belongs to Class I. All of the investigated groups of microorganisms except anaerobic sporogenic bacteria were present in water and sediment samples. Notably, a large number of coliformic bacteria (total and faecal) were isolated from all of the investigated samples which classify this water in Class IV, as ecologically unsuitable drinking water. Most of the identified non-coliformic bacteria belonged to the genus *Bacillus*. We have also identified representatives from *Staphylococcus*, *Proteus*, *Brevundimona*s and *Enterobacte*r. Overall findings suggest a possible connection between

the water from the cave and surface waters. Further investigation should be performed to determine the origin of these waters.

Key words: cave, microbiological diversity, 16S ribosomal RNA gene, chemical composition, trace elements.

Introduction

There has been a growing awareness and concern about biodiversity worldwide over the last decade. Rapidly increasing amounts of information about patterns of biodiversity for many groups of organisms has become available [1, 2]. The same can be said for the fauna of caves and other subterranean habitats.

The term cave is defined as the space beneath the earth's surface that exists without daylight [3]. Caves, with relatively limited organic matter, stable and with low temperatures, high humidity and mineral substances may be considered extreme living environments and as such represent ecological niches for highly specialized microorganisms [4]. The main inhabitants of caves are microorganisms [5] and their presence in terrestrial and aquatic caves has been confirmed by various methods [6–11].

Vrelo Cave (www.canyonmatka.com) is the deepest cave in Macedonia and is ranked 14th on the list of the deepest caves explored by humans. The cave is located in the Matka canyon, in the lower course of the river Treska, 15 km southwest of Skopje. With its geological, geomorphological and hydrological characteristics, flora and fauna, Matka canyon represents an exceptional object of nature. It boasts 1000 plant species, 20% of which are endemic, including various butterfly species not found anywhere else in Europe. However, Vrelo Cave has not been investigated in terms of its composition and biodiversity until now. The purpose of this study is to offer some preliminary data for the microbiological biodiversity of the Vrelo Cave. Beside microbial diversity, this study also gives information on physical and chemical parameters of water and sediments from different locations in Vrelo Cave. To the best of our knowledge, this is the first study to investigate the Vrelo Cave's water and sediment composition and microbiological diversity.

Material and methods

Samples

Samples of water (500 ml) and sediment (~ 10 g) were taken in the period from 16 to 20 of July 2010, from 5 locations in Vrelo Cave. The positions of these locations are marked in Figure 1. The positions of the sample collection spots were: 30 m from the cave entrance and 14.5 depth (position 1); 120 m

from the cave entrance and 15 depth (position 2); 200 m from the cave entrance and 47.5 depth (position 3); 400 m from the cave entrance and 40 depth (position 4) and 100 depth (position 5). Samples were taken in sterile containers and kept on ice until processing in the laboratory.

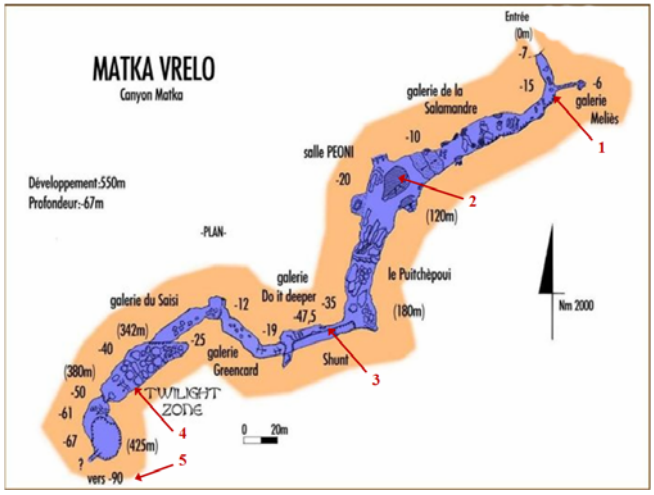

Figure 1 – Map of the cave Vrelo. Cave coordinates from which samples of water and sediment were taken are marked with numbered arrows. The map was constructed by the diving team composed of Roger Cossemyns, Pierre Sciulara, Marc Vandermeulen, Frank Vasseur and Martial Wuyts, which explored the Vrelo cave in 2000 as part of the Matka 2000 Expedition

Physical and chemical parameters of samples

All general physico-chemical parameters in water samples (temperature, colour, odour, transparency, pH, redox potential, electroconductivity, total suspended and dissolved matters, dry matters, alkalinity, water hardness), oxygen status (dissolved O_2, chemical oxygen demand, COD, biochemical oxygen demand for 5 days, BOD-5), nutrient status (total N, NO_3^-, NO_2^-, total P, PO_4^{3-}) and ionic status (HCO_3^-, CO_3^{2-}, OH^-, Cl^-, SO_4^{2-}, S_2^{2-}) were performed according to EPA and ISO methods for water analysis [12].

Trace elements analysis

The water samples were filtered through 0.45 μm acidified at pH 2–4 with concentrated HNO_3 and analysed by an inductively coupled plasma atomic emission spectrometer, ICP–AES, (Varian 715ES, USA) equipped with an ultrasonic nebulizer. Some of the elements (As, Cd) were analysed by an electrothermal atomic absorption spectrometer (Varian, SpectrAA 640Z, USA) according to previously established conditions [13–16].

After drying, the sediment samples were manually wet-sieved through a 125 μm sieve. For digestion of the sediment samples, open wet digestion with a mixture of acids was applied. A precisely measured mass of dust samples (0.5 g) was placed in Teflon vessels and 5 ml concentrated nitric acid, HNO_3 was added, until brown vapours came from the vessels. For complete digestion of inorganic components 5–10 ml HF was added. When the digest became a clear solution, 2 ml of $HClO_4$ were added. Perchloric acid was used for complete digestion of organic matter. After cooling the vessels for 15 min, 2 ml of HCl and 5 ml of H_2O were added for total dissolving of metal ions. Finally, the vessels were cooled and the digests quantitatively transferred to 25 ml calibrated flasks and analyzed by ICP–AES [17–20].

Organic pollutants

Different organic pollutants from the priority list of the Water Framework Directive of the EU [21] were analysed in water and sediment samples. Water samples were filtrated through a glass filter below 0.4 μm. Determination of the organic pollutants was performed by gas chromatography. Previously the analytes were extracted by solid-phase C-18 and ENVI-carb columns allowing a concentration of 200 times. The following groups of organic pollutants were analysed: organochlorine pesticides and their metabolites (aldrin, cis-chlordane, trans-chlordane, oxy-chlordane, 2,4'-DDD, 4,4'-DDD, 2,4'-DDE, 4,4'-DDE, 2,4-DDT, 4,4'-DDT, dieldrin, α-endosulfan, β-endosulfan, endrin, α-HCH; β-HCH; γ-HCH (lindane), δ-HCH; ε-HCH, heptachlor; *cis*-heptachlor epoxide; *trans*-heptachlor epoxide; isodrin; methoxychlor and mirex); nitrogen-phosphorous pesticides (alachlor, atrazine, captan, chlorfenviniphos, chlorpyrifos, diuron, isoproturon, simazine and trifluralin); polycyclicaromatic hydrocarbons – PAH (acenaphthene, acenaphthalene, anthracene, benzo(a)anthracene, benzo(b)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(g,h,i)perilene, benzo(a)pyrene, chrysene, dibenzo(a,h)antracene, fluornthene, fluorene, indo (1,2,3-cd) pyrene, naphthalene, phenanthrene, pyrene); polychlorinated biphenyls – PCB (PCB–28, PCB–52, PCB–101, PCB–105, PCB–118, PCB–138, PCB–153 and PCB–180); chlorinated aromatic hydrocarbons (1, 2, 3-trichlorbenzene, 1,2,4-trichlorbenzene, 1,3,5-trichlorbenzene, pentachlorbenzene (PCB), hexachlorobenzene (HCB)); phtalates (benzilbutilphthalate, dibutilphthalate, bis(2-ethylhexyl) adipate; bis(2-ethylhexyl) phthalate, diethylphthalate, dimethylphthalate) and phenols (2-bromophenol, 4-chloro-3-methylphenol, 2-chlorophenol, 2,4-dichlorophenol, 2,4-dimethylphenol, 2,4-dinitrophenol, 2-methyl-4,6-dinitrophenol (DNOC), 2-methylphenol, 3-methylphenol, 2-nitrophenol, 4-nitrophenol, pentachlorphenol, 2,3,4,6-tetrachlorphenol, 2,4,6-trichlorophenol, 4-*n*-octylphenol, 4-*n*-nonylphenol).

Microbiological analysis of samples

Water samples were filtered through a 0.22 μm filter, while sediment samples were first distributed into a small amount of sterile water, stirred and then the suspension was filtered through a 0.22 μm filter. The filters were put into sterile containers with 50 ml of sterile water and put on a shaker at 100 rpm for 1 hour. The filters were then disposed and the water suspension was used for microbiological analysis.

The samples were investigated for the following groups of microorganisms: aerobic heterotrophic psychrophilic bacteria, aerobic oligotrophic psychrophilic bacteria, total coliform bacteria, faecal coliform bacteria, aerobic sporogenic bacteria, anaerobic sporogenic bacteria, yeasts and moulds. We used different selective media for the specific groups of microorganisms [6, 22, 23] listed in Table 1. The pure bacterial colonies obtained were examined morphologically. Stock culture on agar plates was made for each pure bacterial colony and kept at 4°C. For DNA isolation, each pure colony was grown overnight in the appropriate medium, cells were harvested by centrifugation (14000 rpm, 10 min), washed twice with 1xPBS buffer (140 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and kept at -20°C until further processing.

Table 1

The selective media used for enrichment of the specific groups of microorganisms found in water and sediment samples from Vrelo

Growth medium	Type of microorganism
Andrade Lactose Peptonic Water	faecal coliform bacteria
Endo agar	total coliform bacteira
Tryptic Soya Agar (1: 10 dilution)	aerobic oligotrophic psychrophilic bacteria
Tryptic Soya Agar	aerobic sporogenic psychrophilic bacteria
Differential Clostridial Medium	anaerobic sporogenic psychrophilic bacteria
Tryptic Soya Agar	aerobic heterotrophic psychrophilic bacteria
Malt Extract Agar	yeasts and moulds

Genotyping by 16S rRNA sequencing technique

The sequence of the 16S ribosomal RNA gene (rDNA) of bacterial strains of interest was determined using the MicroSeq Full Gene Kit (Applied Biosystems), composed of two parts: MicroSeq® Full Gene 16S rDNA Bacterial Identification PCR Kit and the MicroSeq® Full Gene 16S rDNA Bacterial Identification Sequencing Kit. DNA extraction was done using the PrepMan-Ultra reagent (Applied Biosystems), following the protocol for culture broth

samples. The concentration of DNA was determined spectrophotometrically. DNA working solution of 2.7–3.1 ng/ml was prepared by diluting the stock DNA. Amplification of the three fragments of the 16S ribosomal RNA gene was done using a 7.5 ml DNA working solution in a reaction volume of 15 μl on a 2720 Thermal Cycler (Applied Biosystems). Purification of the amplified products was done using ExoSAP-IT® reagent (USB) according to the manufacturer's instructions prior to sequencing. The cycle sequencing was performed with forward and reverse primers for each amplified product according to instructions provided by the kit, with one exception: the final volume of the sequencing reactions was 10 ml. After cycle sequencing, excess dye terminators and primers were removed from the cycle sequencing reactions by precipitation in separate tubes with 2 ml 5M Na-acetate and 50 ml ethanol. After incubation at room temperature for 30 min, the tubes were centrifuged at 14000 rpm for 30 min, supernatant was discarded, precipitate was dried for 5 min at room temperature and resuspended in 20 ml of Hi-Di™ Formamide (GE Helthcare). Sequence analyses were performed on a 3130 Genetic Analyzer (Applied Biosystems).

Analysing sequencing data

The obtained sequences for each bacteria analysed were queried against the GenBank – public sequence database of The National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST). BLAST uses statistical theory to produce a bit score and expect value (E-value) for each alignment pair. The bit score gives an indication of how good the alignment is while the E-value gives an indication of the statistical significance of a given pair-wise alignment and reflects the size of the database and the scoring system used. Only sequence alignments that had an E-value of 0 and a bit score > 1000 bits signifying identity $\geq$ 99% were taken into consideration.

Results and discussion

Water samples from Vrelo Cave were analysed for a wide array of chemical parameters: general physico-chemical parameters, oxygen status, nutrients and ionic status, macro- and microelements as well as the concentration of selected organic pollutants. In the sediment samples, besides the content of macro- and trace elements, the presence of selected organic pollutants was also checked.

Regarding the total dry components, total hardness, dissolved oxygen, biochemical consumption of oxygen and chemical consumption of oxygen (Table 2), water from Vrelo Cave belongs to Class I, according to the Regulation of Water Safety in the Republic of Macedonia [24]. The exception to the above statement is the concentration of total nitrogen and total phosphorous which indicate possible contamination.

Table 2

General physico-chemical parameters in water samples from Vrelo

Parameter	Sampling location					Regulation					
	1	**2**	**3**	**4**	**5**	**Class I[a]**	**Class II[a]**	**Class III[a]**	**Class IV[a]**	**Class V[a]**	**Drinking water[b]**
Temperature, °C	14	14.1	13.1	13.1	13.2						
Odour	No	No	No	No	No	No	No	Sl. cloudy	Cloudy	–	No
Colour, Pt-Co units	1.6	1.9	1.8	1.8	2.1	< 15	15–25	26–40	> 40	–	20
Transparence, NTU	0	0	15	1.3	1	< 0.5	0.5–1.0	1.1–3.0	> 3	–	1.5
pH	6.86	3.5	6.93	6.96	6.94	6.5–8.5	6.5–6.8	6.3–6.0	6.0–5.3	< 5.3	6.5–9.5
Electroconductivity, mS/cm	456	-0.5	367	469	224						1000
Dissolved CO_2, mg/l	< 1	387	3.78	< 1	193						
Total matters at 105°C, mg/l	422	362	355	421	379	350	500	1000	1500	> 1500	< 1000
Total matters at 600°C, mg/l	151	136	132	156	141						
Oils and other light liquids	N.D.	N.D.	N.D.	N.D.	N.D.						
Alkalinity (phenolpht.), mE/l	< 0.01	< 0.01	0.02	0.07	< 0.01						
Total alakalinity, mE/l	4.45	3.7	3.7	4.48	3.9						
Total hardness, °DH	14.69	12.5	12.4	14.7	13.2						
Carbonate hardness, °DH	10.71	9.245	7.7	10.6	9.475						
Dissolved oxygen, mg/l	12.87	9.61	14.97	13.4	11.64	> 8	7.99–6.00	5.99–4.00	3.99–2.00	< 3.00	–
BOD-5, mg/l O_2	5.07	2.21	2.84	2.18	1.54	< 2	2.01–4.00	4.01–7.00	6.01–15.0	> 15	–
COD-$KMnO_4$, mg/l O_2	0.63	0.815	0.72	0.74	0.76	< 2.50	2.51–5.00	5.01–10.0	10.0–20.0	> 20	8.0
COD-$K_2Cr_2O_7$, mg/l O_2	10.9	9.0	1.05	5.07	2.95	–	–	–	–	–	–
Parameter	**Sampling location**					**Regulation**					
	1	**2**	**3**	**4**	**5**	**Class I[a]**	**Class II[a]**	**Class III[a]**	**Class IV[a]**	**Class V[a]**	**Drinking water[b]**
Total N, µg/l N	**727**	**777**	**1192**	**845**	**1009**	< 200	200–325	326–450	> 450	> 450	1000
Ammonia, µg/l N	5.31	16.6	20.1	18.0	9.2	16	16	400	400	> 400	400
Nitrates, µg/l N	722	760	1172	827	996	10000	10000	15000	15000	> 15000	10000
Nitrites, µg/l N	0.1	0.55	0	0.53	4.4	10	10	500	500	> 500	30
Total P, µg/l P^-	**7.0**	**14.55**	**11.35**	**6.9**	**9.6**	< 4	4–7	7.1–10	10–50	> 50	-
PO_4^{3-}, µg/l	14.8	13.65	9.3	9.9	12.6						1000
HCO_3^-, mg/l	271	226	223	265.4	237.9						-
CO_3^{2-}, mg/l	N.D.	N.D.	N.D.	4	N.D.						-
OH^-, mg/l	N.D.	N.D.	N.D.	N.D.	N.D.						-
Cl^-, mg/l	9.08	8.01	8.69	9.20	9.57						250
SO_4^{2-}, mg/l	37.6	35.6	31.3	34.3	34.6						250
Total S^{2-}, mg/l	0.0	0.08	0.08	< 0.01	< 0.01						Without
Cr^{6+}, mg/l	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.01	0.01	0.05	0.05	> 0.05	–

[a] Maximal permitted concentrations, Regulation for water classification, Official Gazette of R. Macedonia, 18, 1999

[b] Maximal permitted concentrations, Regulation for water safety, Official Gazette of R. Macedonia, 46, 2008

The results from trace element analysis in water and sediment samples showed very low contents of all trace elements (Tables 3 and 4) except Cd. The Cd level in water samples was higher than allowed for Class I (samples 2 and 5), but is in the range of acceptance for drinking water [24].

Table 3

Content of major and trace elements in water samples from Vrelo

Element	Sampling point					Comparison with the Regulations		
	1	**2**	**3**	**4**	**5**	Class I–II[a]	Class III–IV[a]	Drinking water[b]
In mg/L								
Al	0.03	0.033	0.018	0.033	0.017	1.5	1.5	0.2
Ca	83.9	63.6	64.9	79.9	71.1	-	-	-
Mg	13.8	10.4	11.0	13.0	11.7	-	-	-
K	0.92	0.83	0.82	0.95	0.94	-	-	12
Na	5.53	4.24	4.25	5.01	4.46	-	-	200
In µg/L								
Ag	< 1	< 1	< 1	< 1	< 1	2	20	10
As	15.2	9.07	6.32	11.2	5.65	30	50	10
B	79.8	61.4	61.9	74.9	64.4	200	750	1000
Cd	< 0.1	**3.12**	< 0.1	< 0.1	**1.87**	0.1	10	5
Cr	< 1	< 1	< 1	< 1	< 1	50	100	50
Co	< 1	< 1	< 1	< 1	< 1	100	2000	-
Cu	< 1	< 1	< 1	< 1	< 1	10	50	2000
Fe	6.45	7.49	14.8	27.7	10.7	300	1000	200
Hg	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	0.2	1	1
Mn	< 1	< 1	< 1	< 1	< 1	50	1000	50
Ni	< 1	1.37	< 1	< 1	< 1	50	100	20
Pb	< 2	< 2	< 2	< 2	< 2	10	30	10
Se	< 5	< 5	< 5	< 5	< 5	10	10	10
Zn	< 5	< 5	< 5	< 5	< 5	100	200	3000

[a] Maximal permitted concentrations, Regulation for water classification, Official Gazette of R. Macedonia, 18, 1999

[b] Maximal permitted concentrations, Regulation for water safety, Official Gazette of R. Macedonia, 46, 2008

Table 4

Content of major and trace elements in sediment samples from Vrelo

Element	Sampling point					Dutch standards[a]	
	1	2	3	4	5	Referent values	Intervention values
In mg/kg							
Al	12821	33280	47229	10280	9373		
Ca	52087	70001	24995	93195	88059		
Fe	9365	18880	28438	5165	8829		
Mg	766	8274	17556	22932	3731		
K	1969	16344	10372	16613	14027		
Na	7604	3904	4760	2756	1998		
Ag	< 1	< 1	< 1	< 1	< 1		
As	11.5	27.3	26.4	38.7	33.1	29	55
B	2459	3569	92.6	1922	876	-	-
Ba	147	195	492	36.7	404	200	625
Cd	0.20	0.21	0.14	0.29	0.26	0.8	12
Co	6.68	12.2	3.80	18.3	3.61	20	240
Cr	28.8	78.8	79.9	14.3	18.6	100	380
Cu	16.4	30.6	33.7	7.85	8.87	36	190
Li	8.81	30.6	33.6	7.06	8.37	-	-
Mn	317	171	952	472	174	-	-
Ga	< 5	< 5	< 5	< 5	< 5	-	-
Mo	19.7	64.7	87.9	19.7	14.9	10	200
Ni	19.9	50.2	45.9	14.5	14.5	35	210
Pb	25.2	10.3	3.52	32.1	3.93	85	530
Sr	53.0	48.5	54.5	44.6	44.1	-	-
V	51.6	67.2	89.6	21.8	28.2	-	-
Zn	64.8	63.9	114.9	21.7	33.7	140	720

[a] The New Dutchlist, http://www.contaminatedland.co.uk/std-guid/dutch-l.htm

It was found that nitrogen-phosphorous pecticides, polycyclicaromatic hydrocarbons (PAH), polychlorinated biphenyls, chlorinated aromatic hydrocarbons and phenols were not detected in the water samples. Some of the chlorinated pesticides or their metabolites (2,4-DDE, 2,4'-DDE, 4,4-DDT and γ-HCH-

lindane, δ-HCH) were detected but the concentration was below the detection limit (< 5 ng/l). It was known that these pesticides were in use in Macedonia in the past [25]. Bis (2-ethylhexyl) phthalate was also detected in all water samples in the range of 0.7 to 5.8 ng/l which was established during the analysis of surface waters in Macedonia [25]. Bis phthalate is known as a product present in some plastic materials which could be extracted in the water. The same pollutants were also detected in sediment samples collected from the same sampling points for water, but at very low concentrations. Of the other organic pollutants traces of benzo(a)pyrene (from PAHs) and PCB–138 were also detected. These findings, as well as the results of the chemical analysis, suggest a possible connection between the water from the cave and surface waters. Further investigation should be performed to determine the origin of these waters.

Analysis of isolated pure bacterial cultures by conventional microbiological methods revealed numerous bacilli and cocci. The bacilli found were Gram positive bacilli, rare bacilli, sporogenic and asporogenic bacilli, thin bacilli, coccobacilli, small sporogenic and asporogenic bacilli and long bacilli. The cocci were present as single cells and tetriad cocci.

All of the investigated groups of microorganisms except anaerobic sporogenic bacteria were present in water and sediment samples from all 5 coordinates in the Vrelo cave (Table 5). A few exceptions were that aerobic heterotrophic psychrophilic bacteria were not found in water samples from position 1 and aerobic sporogenic bacteria were not found in water samples from positions 1, 2 and 5.

A large number of coli formic bacteria (both total and faecal) were isolated from all of the investigated samples from Vrelo Cave. Regarding the microbiological results for total and faecal coliform bacteria, water from Vrelo Cave is classified into Class IV, as ecologically unsuitable drinking water according to the Regulation for Water Classification of the Republic of Macedonia [26]. We also isolated oligotrophic bacteria which are engaged in the auto purification of the water. However, the number of total and faecal coliform bacteria is much higher than the number of oligotrophic bacteria, which led us to the conclusion that the rate of auto purification of the water from Vrelo Cave is low (Figure 2). The presence of aerobic heterotrophic psychrophilic bacteria as well as yeasts and moulds in most of the water samples points to the conclusion that water in Vrelo Cave is contaminated by organic material. However, since the number of these specific groups of microorganisms is lower than in spring water [24], we have concluded that the organic pollution of water from Vrelo Cave is present but is at low level, which was also confirmed by biochemical analysis of total nitrogen and total phosphorous concentrations (Table 2). The

presence of aerobic sporogenic bacteria in water samples 2 and 3 and in all sediment samples, suggests that water in Vrelo Cave contains soil material.

Table 5

Number of colonies from different groups of microorganisms derived from water and sediment samples from Vrelo

Sample No.	**Water**					**Sediment**				
	1	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
aerobic heterotrophic psychrophilic bacteria (CFU/ml)	0	14	1	1	1	n/d*	1410	n/d	n/d	n/d
aerobic oligotrophic psychrophilic bacteria (CFU/ml)	140	4	2	2	2	n/d	80	n/d	n/d	n/d
total coliform bacteria (CFU/100 ml)	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	10	n/d
faecal coliform bacteria (CFU/100 ml)	500	1500	880	1500	440	880	1500	880	220	220
aerobic sporogenic bacteria (CFU/ml)	0	0	2	3	0	90	70	n/d	37	20
anaerobic sporogenic bacteria (CFU/ml)	0	0	0	0	0	0	0	0	0	0
yeasts and moulds (CFU/ml)	10 moulds	0	yeasts	yeasts	0	400 moulds	100 moulds	yeasts	yeasts	0

* not determined because of very high number

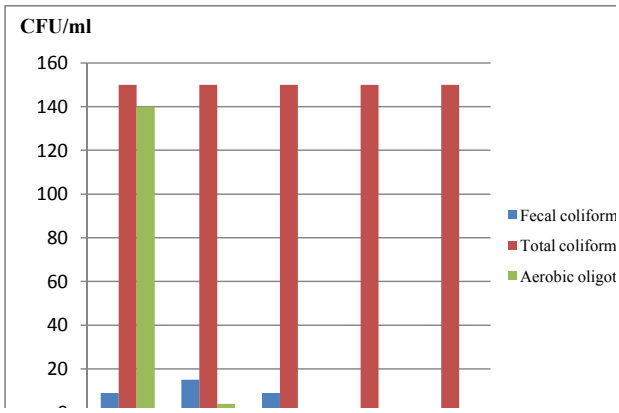

Figure 2 – Proportion of total coliformic bacteria, faecal coliformic bacteria and aerobic oligotrophic bacteria in water samples from the Vrelo cave

In order to achieve rapid and unambiguous identification of the bacterial community of Vrelo Cave, molecular methods of identification were preferred over culture-based methods. Determinative bacteriology based on culture-based methods involves time-consuming isolation, cultivation and characterization of phenotypic traits which often is not discriminatory and can take days to weeks for unambiguous identification [27]. While in a few cases rapid identification can be made using phenotypic methods, the phylogenetic resolution of such methods is usually quite low. Although a variety of nucleic acid based approaches are in use nowadays, we have used comparative sequencing of PCR-amplified 16S rRNA gene [28]. The 16S rRNA gene, universally present in all bacteria, has both highly conserved and more variable domains, which makes it an ideal target for studying phylogenetic relationships.

We have genotyped a total of 23 bacterial strains isolated from samples from 5 different locations in Vrelo Cave. The results from the molecular identification of the isolated bacterial strains are given in Table 6. 19 out of the 23 strains identified (83%), belonged to the genus *Bacillus*. We have also identified representatives from the genus *Staphylococcus*, *Proteus*, *Brevundimona*s and E*nterobacte*r.

Although a very powerful technique for identification and phylogenetic analysis of bacteria, the 16S rRNA gene sequencing does not have the ability to discriminate between some very closely related species. In this study, discrimination could not be made between *Bacillus cereus*, *Bacillus mycoides*, *Bacillus thuringiensis* and *Bacillus weihenstephanensis*. Alignment of the 16S rRNA sequences of these 4 bacterial species revealed an identity of 99% (score > 2600 bits; gaps 0–2 (0%); E-value = 0).

Only in the case of the bacterial strain 4S-02 we got sequence alignments that had an identity lower than 99%. The highest similarity was shown with Bacillus sp. 2_A_57_CT2, Bacillus pumilus SAFR-032, Bacillus megaterium DSM 319 and Bacillus sp. NRRL B-14911 (92–97% identity). These data alone do not allow us to unambiguously identify this bacterial strain and the possibility that we have a new Bacillus species remains open until further analyses are carried out.

Table 6

Identification of pure bacterial strains isolated from water and sediment samples from Vrelo, by sequencing of the 16S ribosomal RNA gene

Probe No.	Type of speciment	Bacterial strain code	Identification result
1	sediment (1S)	1S-01	Bacillus mycoides / Bacillus cereus
		1S-02	Bacillus mycoides / Bacillus cereus
		1S-03	Staphylococcus epidermidis
		1S-04	Bacillus megaterium
2	water (2V)	2V-K1	Brevundimonas subvibrioides/ Brevundimonas sp. BAL3
		2V-K2	Bacillus thuringiensis/ Bacillus cereus
	sediment (2S)	2S-01	Bacillus megaterium DSM 319/ QM B1551
		2S-02	Bacillus megaterium DSM 319/ QM B1551
		2S-03	Bacillus cereus/Bacillus mycoides
		2S-04	Bacillus mycoides DSM 2048/ Bacillus weihenstephanensis KBAB4
		2S-05	Bacillus megaterium DSM 319
		2S-H1	Bacillus mycoides
3	sediment (3S)	3S-03	Bacillus thuringiensis/ Bacillus cereus
		3S-K2	Bacillus cereus/Bacillus thuringiensis
4	sediment (4S)	4S-01	Bacillus mycoides/ Bacillus cereus
		4S-02	Bacillus
5	water (5V)	5V-01	Proteus penneri ATCC 35198
		5V-H1	Bacillus megaterium
	sediment (5S)	5S-01	Bacillus megaterium
		5S-02	Bacillus cereus/ Bacillus mycoides
		5S-03	Bacillus mycoides DSM 2048/ Bacillus cereus
		5S-04	Bacillus mycoides
		5S-K1	Enterobacter cloacae

Conclusion

Water from Vrelo Cave is classified into Class I regarding the general physical and chemical parameters and presence of major and trace elements.

Regarding the microbiological results for total and faecal coliform bacteria, water from Vrelo Cave is classified into Class IV as ecologically unsuitable drinking water. Overall findings suggest a possible connection between the water from the cave and surface waters but these observations should be confirmed by further investigation. Molecular identification of isolated bacterial cultures from Vrelo Cave by comparison of the 16S rRNA gene sequences allowed differentiation, classifying strains at the level of species and subspecies. We have genotyped all of the pure isolated colonies and most of them belonged to the genus *Bacillus*. However, the occasional exceptions to the usefulness of 16S rRNA gene sequencing in this study related to several *Bacillus* species having the same or very similar sequences.

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Резиме

МИКРОБИОЛОШКИ И ХЕМИСКИ КАРАКТЕРИСТИКИ НА ВОДИТЕ И СЕДИМЕНТОТ ОД ПЕШТЕРАТА ВРЕЛО, РЕПУБЛИКА МАКЕДОНИЈА

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Апстракт: Пештерата Врело е најдлабоката пештера во Македонија, лоцирана во клисурата Матка која е дом за многу ендемски видови кои не се најдени на ниедно друго место во Европа. Досега, пештерата Врело не беше истражувана во поглед на нејзиниот состав и биодиверзитетот.

Целта на оваа студија беше да понуди прелиминарни податоци за физичките и хемиските параметри на водата и седиментот од пештерата Врело, како и микробиолошкиот диверзитет. Беа земени примероци од 5 различни локации. Тие беа анализирани од аспект на нивните физичко-хемиски параметри, содржината на макро и микроелементи и содржината и концентрацијата на одредени органски загадувачи. Сите примероци беа исто така испитувани за присуството на неколку групи на бактерии, квасци и мувли со примена на конвенционални микробиолошки методи, додека молекуларната идентификација на изолираните чисти бактериски култури беше изведена со секвенционирање на бактерискиот 16S рибозомален РНК ген.

Земајќи ги предвид вкупните суви материи, вкупната тврдост, растворениот кислород, биохемиската и хемиската потрошувачка на кислород, водата од пештерата Врело е класифицирана во класа I. Сите испитувани групи микроорганизми со исклучок на анаеробните спорогени бактерии беа присутни во сите примероци од вода и седимент. Голем број на колиформни бактерии (вкупни и фекални) беа изолирани од сите испитувани примероци што од микробиолошки аспект ја класифицира водата од пештерата Врело во класа IV, како еколошки неподобна вода за пиење. Најголем дел од идентификуваните неколиформни бактерии припаѓаат на родот *Bacillus*. Идентификувани се и претставници од родовите *Staphylococcus*, *Proteus*, *Brevundimona*s и E*nterobacte*r.

Целокупните истражувања укажуваат на можна врска помеѓу водите од пештерата Врело и површинските води. Меѓутоа, потребни се понатамошни истражувања за да се потврди оваа опсервација и да се одреди потеклото на овие води.

Клучни зборови: пештера, микробиолошки диверзитет, 16S рибозомален РНК ген, хемиски состав, елементи во траги.

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