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PHYSICOCHEMICAL AND MICROBIOLOGICAL RESULTS FROM HOT MINERAL WATER IN THE VILLAGE OF VARVARA, DISTRICT OF PAZARDZHIK, BULGARIA

IGNAT IGNATOV [a*], NEDYALKA VALCHEVA [b], TEODORA P. POPOVA [c], NIKOLAI NESHEV [d], FABIO HUETHER [e] AND ALEXANDER I. IGNATOV [a]

[a] Scientific Research Center of Medical Biophysics, Sofia, Bulgaria.
[b] Faculty of Agriculture, Department Biochemistry, Microbiology, Physics, Trakia University, Stara Zagora 6000, Bulgaria.
[c] Faculty of Veterinary Medicine, University of Forestry, 10 Kl. Ohridski Blvd., Sofia 1756, Bulgaria.
[d] Faculty of Physics, Sofia University "St. Kliment Ohridski", Sofia 1000, Bulgaria.
[e] EVODROP AG, Zürich, Switzerland.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

There are 225 mineral springs in Bulgaria, and out of them, 148 are in southern Bulgaria and 77 in northern Bulgaria. The region with mineral water "Varvara" is located in Rhodope mountains, Pazardzhik region, Bulgaria. Physicochemical and microbiological research of two springs from the village of Varvara is presented in the article. The springs are "Drilling No. 3 - Varvara" and "Drilling No. 5 – Varvara". The physicochemical research of mineral waters showed that it is characterized as hydrothermal, mineralized, hydrocarbon-sulphate, sodium, silicon-rich and fluoride waters. The hyperthermal mineral spring "Drilling No. 3 – Varvara" is with t=79.9°C. The hyperthermal mineral spring "Drilling No. 5 – Varvara" is with t=83.7°C. The presence of single spores of species of genus *Bacillus* (*Bacillus subtilis*, *Bacillus licheniformis*), phylum *Bacillota* were found in the tested mineral waters. *B. subtilis* is a Gram-positive bacterium that forms heat-resistant spores. *B. licheniformis* is a Gram-positive, mesophilic bacterium and its optimal growth temperature is around 50°C. *B. licheniformis* can survive at higher temperatures. *Geobacillus stearothermophilus* also was registered in the tested mineral waters. It is a rod-shaped, Gram-positive bacterium, a member of the phylum *Bacillota*.
The results show that the waters from both examined sources in Varvara are useful for human health according to their physicochemical composition. The content of silicon and calcium is a prerequisite for their application

**Corresponding author: Email: mbioph@abv.bg;*

in strengthening the musculoskeletal system. The rich fluoride content allows their controlled use for reduction in dental caries. The waters from "Drilling No. 3 - Varvara" and "Drilling No. 5 - Varvara" did not show any sanitary- chemical and microbiological signs of contamination. This reveals opportunities for balneological therapy and tourism in this region. Bottling these waters would allow for their wider use.

Keywords: *Varvara region; Bulgaria; hot mineral water; physicochemistry; microbiology.*

1. INTRODUCTION

Varvara is a village located at the foot of the Western Rhodopes, Southern Bulgaria, on the northern slope of the Alabak ridge. The Western Rhodopes are the highest part of the Rhodope Mountains and since the XIX century they are known for the largest number of long-lived people and centenarians, contributing to this is the quality and composition of the water.The thermo- mineral area "Varvara" covers around 0.06 km^2, with an approximately triangular shape, as the geometric height of the figure coincides with the narrow valley of Chepinska river. Two naturally ascending springs and four wells with boreholes are located at the bottom of the valley. These are captured natural springs (CNS) No. 1 "Varvara" and CNS No. 2 "Vetren dol". The springs are linked to the faultcrack system of the gneiss fold structures in the region. They have served as first indicators for carrying out further exploration for additional drilling hydrogeological surveys in order to detect thermomineral water from gneisses. In hydrological terms the region with mineral water "Varvara" is located in the northernmost part of a large hydrothermal system. Springs are included in the valley of river Chepinska. The mineral springs of spa resort Velingrad are also taken into consideration. According to Bulgarian State Standard, the mineral water is considered as cold at temperatures up to 37° C. From 37 to 60°C it is warm, and over 60°C it is hot or hydrothermal. Drilling in the area with hot mineral water "Varvara" is also situated at the bottom of the basin and in the vicinity of the springs. So in the lower part of the valley, occupied by a thin river terrace, is sheltered a proven zone of thermo-mineral waters with high temperature and artesian origin.

The aim of the present research in ecological region Varvara was to show the physicochemical composition of water from both described sources. The studies were also focused on research for the presence of bacteria of genus *Bacillu*s, for which there was evidence for their antimicrobial activity, and producing antibiotics with application in practice [1].

The endospore-forming rhizobacterium *Bacillus subtilis* is able to produce more than two dozen antibiotics with a variety of structures. The produced antimicrobial active compounds include predominantly peptides that are either ribosomally synthesized and post-translationally modified. There are lantibiotics and lantibiotic-like peptides. There are non-ribosomally generated, as well as a couple of non-peptidic compounds such as polyketides, an aminosugar, and a phospholipid [2]. There are developed novel antibiotics to treat infections caused by multi-drug-resistant bacteria [3]. Some research showed cell-free supernatant of B. subtilis (B. subtilis CFS) killed planktonic and biofilm S. aureus, and increased S. aureus susceptibility to penicillin and gentamicin as well [4]. B. licheniformis can replace enramycin in chickens on the growth performance and intestinal health of subclinical necrotic enteritis (SNE) [5].

2. PHYSICOCHEMICAL ANALYSES

The physicochemical properties the following springs in the area with hot mineral water "Varvara", village of Varvara, District of Pazardzhik are researched:

1. Hyperthermal mineral spring "Drilling No. 3 –Varvara" with t=79.9°C.
2. Hyperthermal mineral spring "Drilling No. 5 –Varvara" with t=83.7°C.

"Drilling No. 3 – Varvara" and "Drilling No. 5 – Varvara" are included in the "Register of mineral water facilities from exclusively state-owned deposits". The registrations are with Act No.177 / 17.04.1997.

The total mineralization of the mineral water from the studied springs is 750 ("Drilling No. 3 – Varvara") and 718 $mg.L^{-1}$ ("Drilling No. 5 – Varvara"). It is characterized as hydrothermal, mineralized, hydrocarbon-sulphate, sodium, silicon-rich and fluoride water without sanitary-chemical and microbiological signs of contamination. The contents of studied micro components and the values of radiological indicators are within the norms for mineral waters. The water has a physico-chemical composition and properties that correspond to the requirements of Decree No. 14 regarding the resort resources, resort localities and resorts (Official State Gazette, issue 79 from 1987, and amendment published in issue 70 from2004).

For the natural source of mineral water Varvara there is a document No. 111 of January 2, 2019, Ministry of

Health, Bulgaria for the properties according to Decree No. 14 (Official State Gazette, 79, 1987, and amendment in issue 70, 2004) [6]. The spring water has been defined there as hydrothermal, mineralized, hydrocarbon-sulphate, sodium, silicon-rich and fluoride water without sanitary-chemical and microbiological signs of contamination.

The classification of mineral waters by physicochemical composition by the Ministry of Health is typical for Bulgaria. In this respect the waters are divided into hydrocarbonate, sulphate, chloride; sodium, calcium, magnesium. According to their bioactive parameters, they are silicon, fluorine, iodine-bromine, iron and others.

The aim of the author's team of this research was to conduct a study of physicochemical and microbiological parameters of drinking water according Ordinance No. 9/2001, Official State Gazette, issue 30 [7], and decree No. 178 / 23.07. 2004.

3. MICROBIOLOGICAL ANALYSES

Studies have also been performed for *Bacillus subtilis*, *Bacillus licheniformis* and *Geobacillus stearothermophilus*, which have been reported by other authors in studies of mineral waters [8-10]. There are studies showing thermophilic properties of *B. subtilis* spores at 80 and 90°C [1]. The enzyme retained 100% of its activity when incubated at 80°C for 1 h [11]. The authors show the presence of viable spores of *Bacillus licheniformis* at 83.7 °C. There is also evidence for availability of *B. licheniformis* spores at 70°C [12-15]. The bacterium *Geobacillus stearothermophilus* is active and at 90°C [16].

4. METHODS AND MATHERIALS

4.1 Methods for Physicochemical Analysis

Method for determination of color according to Rublyovska Scale – method by Bulgarian State Standard (BDS) 8451: 1977;

Method for determination of smell at 20°C — method BDS 8451: 1977 using technical device glass mercury thermometer, conditions No. 21;

Method for determination of turbidity - EN ISO 7027, technical device turbidimeter type TURB 355 IR ID No 200807088;

Method for determination of pH – BDS 3424 : 1981, technical device pH meter type UB10 ID NoUB10128148;

Method for determination of oxidisability – BDS 3413: 1981;

Method for determination of chlorides – BDS 3414: 1980;

Method for determination of nitrates – Validated Laboratory Method (VLM) – NO_3 – No. 2, technical device photometer "NOVA 60 A" ID No. 08450505;

Method for determination of nitrites – VLM NO_2 – No. 3, technical device photometer "NOVA 60 A" ID No. 08450505;

Method for determination of ammonium ions – VLM – NH_4 – No. 1, technical device photometer "NOVA 60 A" ID No. 08450505;

Method for determination of general hardness – BDS ISO 6058;

Method for determination of sulphates – VLM – SO_4 – No. 4, technical device photometer "NOVA 60 A" ID No. 08450505;

Method for determination of calcium – BDS ISO 6058;

Method for determination of magnesium – BDS 7211: 1982;

Method for determination of phosphates – VLM - PO_4 – No. 5, technical device photometer "NOVA 60 A" ID No 08450505;

Method for determination of manganese – VLM – Mn – No. 7, technical device photometer "NOVA 60 A" ID No. 08450505;

Method for determination of iron – VLM – Fe – No. 6, technical device photometer "NOVA 60 A" ID No. 08450505;

Method for determination of fluorides – VLM – F – No. 8, technical device photometer "NOVA 60 A" ID No. 08450505;

Method for determination of electrical conductivity – BDS EN 27888, technical device – conductivity meter inoLab cond 720 ID No 11081137 [17-21].

4.2 Methods for Microbiological Research

4.2.1 Growth media

a. Nutrient agar (MPA) with contents (in %) – meatwater, peptone – 1%, agar – agar – 2% .

b. Endo's Agar (for defining of *Escherichia coli* and *coliform* bacteria) with contents (ing/L) – peptone – 5,0; triptone – 5.0; lactose – 10.0; Na_2SO_3 – 1.4; K_2HPO_4 – 3.0; fuchsine– 0,14; agar – agar – 12,0 pH 7.5 – 7.7 (Grabow, du Preez, 1979; Grant, 1997; Pitkänen et al, 2007).

a. Nutrient gelatine (MPD) (for defining of *Pseudomonas aeruginosa*) with contents (in %) – Peptic digest of animal tissue; 25 % gelatin; pH = 7.0 – 7.2.

b. Medium for defining of enterococci (esculin – bile agar).

c. Medium for defining of sulphite reducing bacteria (Iron Sulfite Modified Agar).

d. Wilson-Blair medium (for defining of sulphite reducing spore forming anaerobes (*Clostridium perfringens*) with contents ($g.L^{-1}$) – 3% Nutrient agar; 100 mL 20% solution of Na_2SO_3 ; 50 mL 20% glucose solution; 10 mL 8% solution of Fe_2SO_4 [22,23].

4.3 Methods for Determination of Microbiological Indicators

a. Methods for evaluation of microbiological indicators according to Ordinance No. 9/2001, Official State Gazette, issue 30, and decree No. 178/23.07.2004 about the quality of water, intended for drinking purposes.

b. Method for determination of *Escherichia coli* and coliform bacteria – BDSEN ISO 9308 – 1: 2004;

c. Method for determination of enterococci – BDS EN ISO 7899 – 2;

4.4 Methods for Microbiological Identification

Vitek® MS is an automated system for microbial identification of the company BioMerieux. The device is based on innovative technology mass spectrometry [24,25]. Identification are needed microbial cultures. In the conducted analysis were used 48 h pure cultures over nutrient agar. Each culture was mixed with a matrix over a special plate, positioned in a preparatory station with a bacteria protocol, inserted in the device and influenced by a laser. To control the process a standard strain of *E. coli* ATCC 8739 was used, which has well known characteristics. As a result were generated the so called MALDI-ToF spectra that were analyzed by the means of a software, and compared to existing profiles in the database.

5. RESULTS

a. Method for determination of sulphite reducing spore anaerobes – BDS EN 26461 – 2: 2004;

b. Method for determination of total number of aerobic and facultative anaerobic bacteria – BDS EN ISO 6222: 2002;

c. Method for determination of *Pseudomonas aeruginosa* – BDS EN ISO 16266: 2008.

d. Determination of coli – titre by fermentation method – Ginchev's method Determination of coli – bacteria over Endo's medium – membrane method.

e. Determination of sulphite reducing anaerobic bacteria (*Clostridium perfringens*) – membrane method [26].

The number of the isolated bacteria was presented in colony forming units per ml ($CFU.mL^{-1}$) of the tested waters.

5.1 Physicochemical Analysis of Hyperthermal Mineral Springs in Village of Varvara, District of Pazardzhik

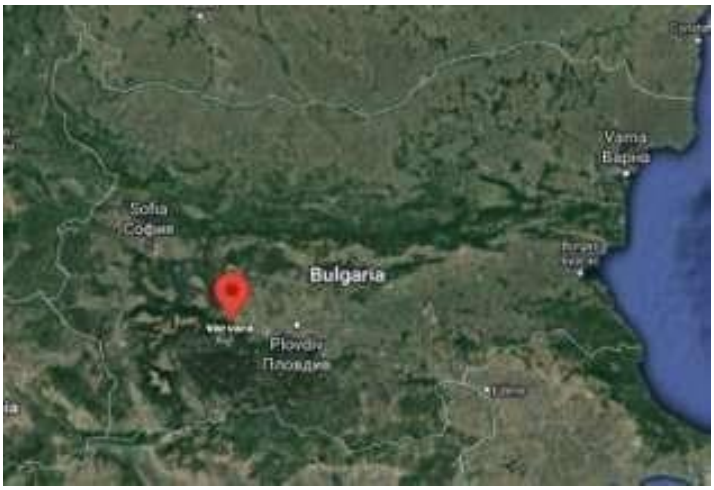

Fig. 1. Illustrates the place of Varvara, Pazardzhik region in Bulgaria

Physicochemical parameters of the waters from, Drilling No 3 – Varvara“ and „Drilling No 5 – Varvara are presented in Table 1.

Table 1. Physicochemical parameters of the waters from„ Drilling No 3 – Varvara“ and „Drilling No 5 – Varvara“

Controlled parameter	Measuring unit	Maximum Limit Value	„Drilling No. 3 – Varvara“	„Drilling No. 5 – Varvara“
1. pH	pH values	≥ 6,5 and ≤ 9,5	8.45±0.1	8.13±0.1
2. Electrical conductivity	$\mu S.L^{-1}$	2000	1035 ±31	1025 ±30
3. Total hardness	$mgekv.L^{-1}$	12	5.1 ±0.5	5.2 ±0.5
4. Color	Chromaticity Values	Acceptable	Acceptable	Acceptable
5. Turbidity	FNU	Acceptable	Acceptable	Acceptable
6. Permanent Oxidation	$mgO_2.L^{-1}$	5.0	1.04±0.01	1.04±0.01
7. Odour	force	Acceptable	Acceptable	Acceptable
8. Calcium (Ca^{2+})	$mg.L^{-1}$	150	12.3 ±1.2	11.5 ±1.1
9. Magnesium (Mg^{2+})	$mg.L^{-1}$	80	15.86 ±1.6	<0.12
10. Iron (Fe^{2+})(Fe^{3+})	$\mu g.L^{-1}$	200	0.04	0.03
11. Manganese (Mn^{2+})	$\mu g.L^{-1}$	50	10±0.1	<0.02
12. Ammonium ion (NH_4^+)	$mg.L^{-1}$	0.50	<0.05	<0.05
13. Sulphates (SO_4^{2-})	$mg.L^{-1}$	250	302.0±30.2	<24
14. Phosphates (PO_4^{3-})	$mg.L^{-1}$	0.5	0.03	0.03
15. Fluorides (F^-)	$mg.L^{-1}$	1.5	6.11±0.61	6.50±0.65
16. Nitrates (NO_3^-)	$mg.L^{-1}$	50	<0.1	<0.1
17. Nitrites (NO_2^-)	$mg.L^{-1}$	0.5	<1.0	<0.05
Additional parameters				
18. Sodium (Na^+)	$mg.L^{-1}$	200	171.5±16.3	163.6±16.3
19. Potassium (K^+)	$mg.L^{-1}$	-	6.6±0.7	7.8±0.8
20. Hydrocarbonates (HCO_3^-)	$mg.L^{-1}$	-	130.6±13.1	96.4±9.6
21. Metasilicic acid (H2SiO3)	$mg.L^{-1}$	-	103.6±11.6	1.2±0.1
22. Hydrogen sulfide (H_2S)	$mg.L^{-1}$	-	<0.05	1.19

Table 2. Total number of mesophilic aerobic and facultative anaerobic bacteria in the studied mineral waters

Examined water source	Indicator, CFU/ml
1. Hyperthemal mineral spring “Drilling No. 3 – Varvara” with t=79.9°C, village of Varvara, District of Pazardzhik	5
2. Hyperthemal mineral spring “Drilling No. 5 – Varvara” with t=83.7°C, village of Varvara, District of Pazardzhik	2 - 3

5.2 Microbiological Research

The results from the studies for determination of total number of mesophilic aerobic and facultative anaerobic bacteria in the waters from „Drilling No 3 – Varvara“ and „Drilling No 5 – Varvara” are shown in Table 2.

A comparison between the examined mineral waters according to their microbiological indicators is presented in Table 3.

Table 3. Comparison of examined hyperthermal mineral spring waters in village of Varvara in District of Pazardzhik according to their microbiological indicators

Indicators	Mesuring unit	Maximum limit value	Water from „Drilling No. 3 - Varvara“, t=79.9°C	Water from „Drilling No. 5 - Varvara“, t=83.7°C
Coliforms	$CFU.mL^{-1}$	0/100	0/100	0/100
Escherichia coli	$CFU.mL^{-1}$	0/100	0/100	0/100
Enterococci	$CFU.mL^{-1}$	0/100	0/100	0/100
Suphite-reducing anaerobes *(Clostridium perfringens)*	$CFU.mL^{-1}$	0/100	0/100	0/100
Pseudomonas aeruginosa	$CFU.mL^{-1}$	0/250	0/250	0/250

Table 4. Genus identity of the strains isolated from hyperthermal spring waters in District of Pazardzhik using MALDI-TOF MS identification

Strain	Species of genus identity	reliability, %
Result Hyperthemal mineral spring “Drilling No. 5 – Varvara” with t=83,7°C, village of Varvara, District of Pazardzhik	*Geobacillus stearothermophilus 3 Bacillus licheniformis (spores) 7-1 Bacillus subtilis (spores) 6-1-1*	99.9 %
Result Hyperthemal mineral spring “Drilling No. 3 – Varvara” with t=79,9°C, village of Varvara, District of Pazardzhik	*Geobacillus stearothermophilus 3 Bacillus licheniformis (spores) 7-1 Bacillus subtilis (spores) 6-1-1*	99.9 %

5.3 Identification of Microorganisms, Isolated by Hyperthermal Mineral Springs Drilling No. 3 and Drilling No. 5 – Varvara, District of Pazardzhik

By the identification research with Vitek® MS, the following bacterial species were shown in the mineral waters in Varvara – *Geobacillus stearothermophilus, Bacillus licheniphormis* and *Bacillus subtilis* (Table 4).

Along with the known biological species, extreme niches are a habitat of also new, unknown so far species. The current research proves the presence of representatives of genus *Bacillus* and genus *Geobacillus* in the tested hyperthermal mineral spring waters in very small quantities (single spores per L).

6. DISCUSSION

There are analyzes showing that the deeper the mineral water spring is, the purer it is in terms of presence of pathogenic microorganisms and rain waters [27]. The aquifer system in Varvara[3] is cracked and there is an anomaly through which the hot mineral water springs out. There are mainly granites and gneisses [25]. The presence of bacteria that, according to Ordinance No. 9/2001 make mineral water unfit for drinking, was not proven in the tested waters. These bacteria are coliforms, *E. coli*, enterococci, *Clostridium perfringens* and *Pseudomonas aeruginosa*. We proved the presence of *G. stearothermophilus, B. licheniphormis* (spores), *B. subtilis* (spores). These bacteria are not pathogenic to humans and animals, and their presence does not pose an epidemiological risk. Due to their ability to form spores, these species are adapted to survive in these conditions. Their spores are resistant to high temperatures and can remain viable for a long time in hot mineral waters. The thermophile *G. stearothermophilus* that normally develops in hydrothermal waters was also found. There are reports from some authors for isolation of these and other species of these genera from mineral waters – *G. stearothermophilus* [14], *B. licheniformis* [13], *B. subtilis* [28,8,29,30], *B. vallismortis* [8], *B. amyloliquefaciens* [10]. A similar previous research with Vitek ® MS was performed of hot mineral spring Rupite 1, District of Blagoevgrad, Bulgaria [20,29,30].

In our research it is also impressive that the amounts of calcium ions (Ca^{2+}) in Drilling No. 3 - Varvara” were 12.3, and in Drilling No. 5 - Varvara” were 11.5 $mg.L^{-1}$. Presence of metasilicic acid (H_2SiO^-) has been observed by the testing of the physicochemical

parameters of the examined waters. The results of some studies show that amounts of calcium ions up to 20 $mg.L^{-1}$ are typical for areas where centenarians live. In the regions where most centenarians live in Russia (350 per million), the amount of calcium ions in the water is10 $mg.L^{-1}$ [31-35].

Statistical information shows that the most centenarians in Canada per million inhabitants are in Nova Scotia (210 per million). The calcium content in the water from Nova Scotia is 6.8 $mg.L^{-1}$ [36], lower than this in the mineral waters in Varvara.

A study from China shows a positive correlation between metasilicic acid ($H_2SiO_3^-$), calcium (Ca^{2+}) and iron (Fe^{2+}; Fe^{3+}), and the number of centenarians in Hechi, China [37]. The study found that the amount of calcium ions is 57.18 $mg.L^{-1}$ and the medium content of H_2SiO^- is 11.56 $mg.L^{-1}$, equivalent to its content in the mineral waters we studied.

In the natural mineral water Varvara we found a quantity of fluoride higher than 5.35 $mg.L^{-1}$. In Drilling No. 3 and Drilling No. 5 - Varvara has quantities of 6.11 and 6.50 $mg.L^{-1}$ respectively. Fluoride has a beneficial effect on the teeth. Reduction in dental caries has been observed in all age groups up to 65 years when water with enough fluoride content is consumed. For this purpose, it must act locally on the tooth surface. This can be achieved naturally by regularly drinking water with sufficient fluoride content [38]. With a fluoride content of 5.35 $mg.L^{-1}$, according document No. 111 of January 2, 2019, Ministry of Health, Bulgaria, Varvara waters can be drunk in courses that are not longer than 6-8 weeks and with a dosage from 3 x 100-250 ml per day.

There is evidence that the use of mineral waters with metasilicic acid (H_2SiO^-) reduces Al levels in the human body with positive effects in nitrergic neurons [39]. Unionized silica (SiO_2) increases with enhance of the temperature and kaolinite (Al_2 (OH) $4Si_2O_5$) is increasingly soluble with increase in temperature [40]. There are various compounds of silicon in nature and they are obtained in water (ortho, meta, di, and trisilicates) with silicic acid (41). In the human body, the reduced amounts of silicon adversely affect tendons, joints and cartilage [40,41]. Some studies show that the status of the musculoskeletal system is an indicator of health and life expectancy [42-44]. In Bulgaria metasilicic acid (H2SiO3-) is also proved in mineral waters Devin (42.97) and Hisarya (60.13) $mg.L^{-1}$. We recommend regular use of these waters due to their beneficial effects on the musculoskeletal system.

Based on the results of the present studies, we strongly recommend wider use of the mineral waters from Varvara. We also recommend their bottling, which would allow more people in our country and abroad to take advantage of the health benefits of these waters. It is important to comply with the requirement to drink Varvara waters in courses not longer than 6-8 weeks, and with a dosage from 3 x 100-250 mL per day.

7. CONCLUSIONS

Physicochemical research of mineral waters, village of Varvara, "Drilling No.3 – Varvara" and "Drilling No. 5- Varvara" proved that the water is characterized as hydrothermal, mineralized, hydrocarbon-sulphate, sodium, silicon-rich and fluoride water without sanitary-chemical and microbiological signs of contamination.

The presence of single spores of species of genus *Bacillus (B. subtilis* and *B. licheniphormis)* were found in the mineral waters in Varvara, Bulgaria. The thermophile *Geobacillus stearothermophilus* was also found that normally develops in hydrothermal waters in very small quantities.

The results of the research show that the water from two sources in Varvara is useful for human health according to its physicochemical composition. In this respect, the presence and quantity of metasilicic acid (H_2SiO_3-) and calcium ions (Ca^{2+}) are particularly important. This reveals opportunities for balneological therapy and tourism in this region.

With its rich mineral composition, the examined mineral waters from Varvara, Bulgaria, are especially useful to improve human health. Therefore, we recommend their wider use. The rich content of silicon and calcium is a prerequisite for their application to strengthen the musculoskeletal system, as well as for treatment of disabilities. Due to the waters being rich in fluoride, their controlled use can be successful for reduction of dental cavities. Bottling these waters would allow for their wider use in our country and abroad.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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