Original Research Article

Microbiological and Physicochemical Research of Thermal Spring and Mountain Spring Waters in the District of Sliven, Bulgaria

ABSTRACT

Defined are the physicochemical properties of four healing non-thermal and thermal spring waters in the area of Sliven District, Bulgaria. The spring waters from the given four water sources are characterized by microbiological indicators, and the pathogenic micro-organisms in the samples from the springs water sources mentioned above are determined by the membrane method.

It is shown that according to 18 controlled parameters included in the study, the non-thermal healing spring "Hadji Dimitar" in the area of "Hot water" in the town of Shivachevo with water temperature 22.5°C and the non-thermal spring "Gunchov spring" with water temperature 21.5°C correspond to all controlled parameters according to Ordinance № 9 / 2001, Official State Gazette, issue 30, and decree № 178 / 23.07.2004 about the quality of water, intended for drinking purposes.

It is established that that thermal healing spring Sliven mineral baths with water temperature 48 °C, healing spring Banya village with water temperature 37 °C meet the standard requirements. Non-thermal spring "Gunchov Spring", Sliven district with water temperature 21.5 ° does not meet the required microbiological parameters in regards to coli bacteria levels.

Keywords: spring water, physicochemical properties, microbiological indicators

1. INTRODUCTION

The causes for that lie in the combination between hydrological conditions of the continuing tectonic processes in the Earth's crust (Ignatov, 2010) (1, 2). By their nature the springs can be separated in cold, warm and hot springs. The first group includes the ones with temperature up to 37°C and this is cold mineral water. The second one ranges between 37° C and 60° C and this is warm mineral water. The third one with over 60°C and this is hot mineral water. The hottest mineral spring in Bulgaria is the one at Sapareva Banya with temperature of 101.4° C. The springing waters have different mineralogical characteristics. Their content is defined by the ones of the rocks, where the water has been flowing through, and the solubility of the minerals within them (Ignatov, Mosin, 2012). The research with hot mineral water from Rupite, Bulgaria with HCO_3^--1320-1488 Ca^{2+}-29-36 mg/dm^3 shows the possibility of origin of life in hot mineral water (Ignatov., 2010; Ignatov, Mosin, 2012) (3,4,5). The temperature of the water of the source is 76°C and in the lake is around 50° C and is depending with season. There are proofs for cyanobacteria in hot mineral water in Rupite (Strunecky et al., 2019) (6). This water contains the following ions and they are structured the first living organisms – stromatolites.

In Bulgaria, there are mineral and spring waters, which are not subjected to physicochemical and microbiological control by the Regional Health Inspectorate, yet they are the most widely used springs by the population as sources of drinking water. Similar springs are located in the territory of Haskovo District (Valcheva, Denkova, Z., Nikolova, Denkova, R., 2013) (7, 8, 9), Stara Zagora District (Valcheva, Denkova, Z., Nikolova, Denkova, R., 2014) (10, 11), Varna District (Valcheva, Ignatov, 2019) (12) and Burgas District (Valcheva, 2019) (13, 14).

For many of these sources physicochemical and microbiological studies have not been conducted, yet they are used for drinking and household needs (Tumbarski, Valcheva, Denkova, Z., Koleva, 2014).

Although water is an unfavorable environment for the development of microorganisms, studies by many authors, including our research team, demonstrate that microorganisms with valuable

properties (enzymes, antibiotics, thermophilic and acidophilic stains) are present in mineral and non – thermal spring waters.
This was proved by the results obtained from the experimental work carried out to determine the micro flora of medicinal and spring waters in Haskovo, Stara Zagora, Burgas (Valcheva et al., 2013-2019) and Varna region (Valcheva, Ignatov, 2019).
There are different studies of physicochemical and microbiological properties of hot mineral waters (Schulze-Makuch, D., Kennedy, 2000)(15), (Jeanthon, 2000)(16), Fouke et al., 2003 (17) Belkova et al., 2007) (18) etc.
Isolated bacteria from the healing and spring regions have been identified by physiologico-biochemical and well as molecular genetic tests and the species include *Bacillus subtilis*, *Bacillus cereus, Bacillus thuringiensis, Bacillus methylotrophicus, Aeromonas hydrophila*.
The research of *Bacillus subtilis* in hot mineral water were performed from Asoodeh, Lagzian (2012) (19) and with one of the authors (Ignatov, Mosin, 2013) (2). There was study for effects of *Bacillus subtilis in* heavy waterr with parameters for study in mineral water (Mosin, Svetz, Skladnev, Ignatov, 2013) (20, 21). The study of *Bacillus methylotrophicus* was made in hot water from Tumbarski et al. (2018) (22) and Yuan et al. (2020) (23). For the first time *Aeromonas hydrophila* was observed in hot mineral water from Italian team (Biscardi et al., 2002) (24). One of the authors Valcheva has performed the study with *Bacillus cereus and Bacillus thuringiensis,* (Valcheva, Tumbarski, Denkova, Z., Koleva, 2014) (25).
Strains with high proteolytic, lipolytic and amylolytic activity have been isolated and standardized for further scientific use (Valcheva et al. 2014-2019).
Antimicrobial activity of the strains of *Bacillus sp*., was detected against the following saprophytic and pathogenic microorganisms: *Penicillium sp., Fusariummoliniforme, Rhizopus sp., Aspergillusniger, Aspergillusoryzae, Aspergillusawamori, Mucor sp.*и *Enterococcus faecalis,* in the process of development and growth of the four *Bacillus – Bacillus cereus*, *Bacillus thuringiensis, Bacillus subtilis, Bacillus methylotrophicus* are the most active strains - *Bacillus methylotrophicus*PY5 (Я1), *Bacillus cereus* LH1 (Р1), *Bacillus cereus* WIF15 (ГИ2) и *Bacillus thuringiensis* B62 (ХМБ3) (26-30).
Pathogenic bacteria exhibit resistance and 4 retain their vitality in the process of development and interaction between them and the strains of Bacillus sp. at temperature of 37 C°.
A relatively low bactericidal effect was demonstrated against the (Gr+) bacterium *Enterococcus faecalis*. The isolated strains are likelyto have a higher inhibitory ability against(Gr -) bacteria compared to (Gr +) bacteria.
The yeasts used in the genus *Candida* exhibit a simulating effect onthe growth of *Bacillus sp*. – *Bacillus methylotrophicus* PY5 (Я1), and *Bacillus cereus* LH1 (Р1). This indicates that synergism has occurred between these microorganisms.
The sources from Sliven district, Bulgaria for research are:

1.1. Sliven mineral baths
The mineral water is hot (48 ° C), low-mineralized (1,986 g / dm^3), hydrocarbonate - sulphate - sodium and calcium, slightly fluorine (4.2 mg fluorine per dm^3). It contains 27 mg colloidal metasilicic acid per liter, neutral reaction (pH 6.8) due to its CO_2 content (287 mg / dm^3).Water from this source is effective in the treatment of gastrointestinal diseases, liver and bile diseases, diseases of the musculoskeletal system, diseases of the peripheral nervous system, endocrine diseases, and skin diseases. It has a generalanti-inflammatory effect on the body, improves blood circulation and influences on toxins.

1.2. Non-thermal spring ,,Hadji Dimitar",Hot water" area, town of Shivachevo
The captive natural spring "Hadji Dimitar" is located north of the town of Shivachevo in the southern slopes of the Balkan Range at an altitude of 480 meters. Depending on the season, its flow rate ranges from 4.5 to 5.5 liters per second, with a temperature of 22 - 30 °C. The total mineralization of a captive healing spring, Hadji Dimitar is 392 mg/dm^3. The mineral water is characterized as

hypothermal, low mineralized, hydrocarbonate - calcium - magnesium water. Tested levels of micro-components are within normal limits except for arsenic content. If used for drinking water, it is necessary to reduce arsenic within the normal range. The water from this source is suitable for the treatment of diseases of the musculoskeletal system, the peripheral nervous system, gastrointestinal diseases, bile-liver diseases, and nephro-urological.

1.3. Thermal healing spring Banya village

The village of Banya, with its thermal mineral springs (37 ° C), combined with the beautiful nature and modern infrastructure, provides the perfect conditions for complete recovery~~!~~. It is suitable for the treatment of the following types of diseases – allergies, arthritis, lung disease, back pains, depression, and diabetes.

The established Health Recovery Center combines the most effective recovery package - healthy food, healing gymnastics, many group walks in the unique surroundings, food exclusively of plant origin (vegan), relaxing massages, water treatments with mineral water, with herbs or different salts, compresses and heat-pads, different saunas, inhalations, exercises, juice therapy and herbal teas as needed.

1.4. Mountain spring ,,Gunchov spring", Karandila locality, Sliven district.

The spring is located among the most densely forested areas along the Ravna oriver. The whole area is overgrown with dense beech forest, which is where centuries-old trees can be found here. The spring is located in the Blue Stones Park above Sliven.

2. MATERIALS AND METHODS

This study used the water samples from the following springs in the districtof Sliven. There are "Sliven Mineral Baths", "Hadji Dimitar", "Banya", "Gunchov spring".

2.1. Nutrient media

Nutrient agar (MPA) with contents (in %) – meat water, peptone – 1%, agar – agar – 2% .
Endo's Medium (for defining of Escherichia coli and coliform bacteria) with contents (g/dm^3) – 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 .
Nutrient gelatin (MPD) (for defining of *Pseudomonas aeruginosa*) with contents (in%) – Peptic digest of animal tissue; 25 % gelatin ;pH = 7, 0 – 7, 2.
Medium for defining of enterococci (esculin – bile agar).
Medium for defining of sulphite reducing bacteria (Iron Sulfite Modified Agar).
Wilson-Bleer medium (for defining of sulphite reducing spore anaerobes (*Clostridium perfringens*) with contents (g/dm^3) – 3%.

2. 2. Methods for analysis

2.2.1. Methods for physicochemical analysis

1. Method for determination of color according to Rublyovska Scale – method by Bulgarian State Standard (BDS) 8451 : 1977;
2. Method for determination of smell at 20°C — method BDS 8451 : 1977 technical device – glass mercury thermometer, conditions No 21;
3. Method for determination of turbidity - EN ISO 7027, technical device turbidimeter type TURB 355 IR ID No 200807088;
4. Method for determination of pH – BDS 3424 : 1981, technical device pH meter type UB10 ID NoUB10128148;
5. Method for determination of oxidisability – BDS 3413 : 1981;
6. Method for determination of chlorides – BDS 3414 : 1980;
7. Method for determination of nitrates – Validated Laboratory Method (VLM) – NO3 – No 2, technical device

photometer "NOVA 60 A" ID No 08450505;
8. Method for determination of nitrites – VLM NO_2 – No 3, technical device photometer "NOVA 60 A" ID No 08450505;
9. Method for determination of ammonium ions – VLM – NO_4 – No 1, technical device photometer "NOVA 60 A" ID No 08450505;
10. Method for determination of general hardness – BDS ISO 6058;
11. Method for determination of sulphates – VLM - SO4 – No 4, technical device photometer "NOVA 60 A" ID No 08450505;
12. Method for determination of calcium – BDS ISO 6058;
13. Method for determination of magnesium – BDS 7211 : 1982;
14. Method for determination of phosphates – VLM - PO4 – No 5, technical device photometer "NOVA 60 A" ID No 08450505;
15. Method for determination of manganese – VLM – Mn – No 7, technical device photometer "NOVA 60 A" ID № 08450505;
16. Method for determination of iron – VLM – Fe – No 6, technical device photometer "NOVA 60 A" ID No 08450505;
17. Method for determination of fluorides – VLM – F – No 8, technical device photometer "NOVA 60 A" ID No 08450505;
18. Method for determination of electrical conductivity – BDS EN 27888, technical device – conductivity meter inoLabcond 720 ID No 11081137.

2.2.2. Methods for determination of microbiological indicators
Methods for evaluation of microbiological indicators according to Ordinance No 9 / 2001, Official StateGazette, issue 30, and decree No 178 / 23.07.2004 about the quality of water, intended for drinking purposes.
2. Method for determination of Escherichia coli and coliform bacteria – BDS EN ISO 9308 – 1: 2004;
Method for determination of enterococci – BDS EN ISO 7899 – 2;
3. Method for determination of sulphite reducing spore anaerobes – BDS EN 26461 – 2: 2004;
4. Method for determination of total number of aerobic and facultative anaerobic bacteria – BDS EN ISO 6222 :2002;
5. Method for determination of Pseudomonas aeruginosa – BDS EN ISO 16266 : 2008.
6. Determination of coli – titer by fermentation method – Ginchev's method (Bulgarian standard)
7. Determination of coli – bacteria over Endo's medium – membrane method.
Determination of sulphite reducing anaerobic bacteria (*Clostridium perfringens*) – membrane method.

3. **RESULTS AND DISCUSION**
A comparative physicochemical analysis of mineral spring waters at the territory of Sliven District was performed using the main indicators (color according to Rublyovska Scale, smell at 20°C, turbidity, pH, oxidisability, chlorides, nitrates, nitrites, ammonium ions, general hardness, sulphates, calcium, magnesium, phosphates, manganese, iron, fluorides, electrical conductivity). The results from these tests are given in Table 1.

Table 1. Comparison of the examined spring waters in Sliven District by physicochemical properties

Controlled parameter	Measuring unit	Maximum Limit Value	Result Sliven Mineral baths	Result Hadji Dimitar Shivachevo	Result Village of Banya	Result Gunchov spring

1. Color Rublyovska Scale	**Chromaticity Values**	**Acceptable**	**Acceptable**	**Acceptable**	**Acceptable**	**Acceptable**
2. Smell at 20°C	**Rating**	**Acceptable**	**Acceptable to**	**Acceptable to**	**Acceptable to**	**Acceptable**
3.Turbidity	**NTU**	**Acceptable**	**Acceptable**	**Acceptable**	**Acceptable**	**Acceptable**
4.pH	**pH values**	**~~≥~~6,5≤ и ≤ 9,5**	**6.91**	**7.49**	**7.90**	**7.19**
5. Oxidisability	**mgO_2/dm^3**	**≤5.0**	**1.6**	**1.12**	**1.6**	**1.12**
6. General hardness	**mgekv/ dm^3**	**≤12**	**8.55**	**5.2**	**8.55**	**5.32**
7. Chlorides	**mg/ dm^3**	**≤250**	**60.28**	**3.55**	**45.7**	**2.66**
8. Nitrates	**mg/ dm^3**	**≤50**	**<5.0**	**<1.0**	**<0.007**	**0.006**
9. Nitrites	**mg/ dm^3**	**≤0,50**	**<0.05**	**<0.05**	**<0.01**	**0,07**
10. Ammonium ions	**mg/ dm^3**	**≤0,50**	**<0.05**	**<0.05**	**<0.05**	**0.093**
11. Sulphates	**mg/ dm^3**	**≤250**	**475.28**	**16.95**	**165**	**84**
12. Calcium	**mg/ dm^3**	**≤150**	**98.2**	**50.1**	**110**	**88**
13. Magnesium	**mg/ dm^3**	**≤80**	**30.4**	**24.32**	**37.21**	**61**
14. Phosphates	**mg/ dm^3**	**≤0,5**	**0,006**	**<0.02**	**<0.006**	**<0.03**
15. Manganese	**mg/ dm^3**	**≤50**	**0.08**	**<0.02**	**15**	**2**
16. Iron	**μg/ dm^3**	**≤200**	**0.41**	**0.02**	**5**	**0,0037**
17. Fluorides	**mg/ dm^3**	**≤1,5**	**7,73**	**0,4**	**1,48**	**0.01**
18. Electrical conductivity	**μS/ dm3**	**≤2000**	**21.1**	**422**	**450**	**701**

Formatted: Justified

Norm: The indicator with red color is not in norm

Also, the microbiological indicators for the same spring waters were determined by the membrane method. The experimental studies from the determination of total number of mesophilicaerobic and facultative anaerobic bacteria are shown in Table 2.

Table 2 Determination of total number of mesophilic aerobic and facultative anaerobic bacteria

Examined water source	**Indicator, cfu/cm^3**
1. Thermal healing spring Sliven Mineral baths with water temperature of 48°C	**7± 1**
2. Non - thermal healing spring "Hadji Dimitar" locality	**6 ± 1**

"Hot water" town Shivachevo with water temperature 22.5°C	
3. Healing spring Banya village with water temperature 37°C	**5± 1**
4. Non-thermal "Gunchov Spring", Karandila locality with water temperature 21.5 °C	**80 - 90**

According to the standard requirements from the examined water samples from the four springs, the water is clean.
The presence of coliforms and Escherichia coli is determined by the membrane method, and according to Ginchev's method (Bulgarian standard). The experimental results (Table 3 and Table 4) reveal "Sliven mineral baths", "Hadji Dimitar" and "Banya", are in compliance with the requirements for presence of coli bacteria. Non-Thermal healing spring "Gunchev spring" does not comply with the requirements for presence of coli form bacteria and enterococci. The present results for those springs are also confirmed by the analyses via the membrane method (Table 4). All the remaining indicatorsare determined by the membrane method.

Table 3. Coli – titre of thermal healing spring waters in Sliven District

Name of water source	**Coli - titre**	**Culture volumes 50 cm^3**	**Culture volumes 10 cm^3**	**Culture volumes 10 cm^3**	**Culture volumes 10 cm^3**	**Culture volumes 10 cm^3**	**Culture volumes 10 cm^3**
1. Thermal healing spring Sliven Mineral baths with water temperature of 48° C	**> 100**	–	–	–	–	–	–
2. Non - thermal healing spring "Hadji Dimitar" with water temperature 22,5°C	**> 100**	–	–	–	–	–	–
3. Thermal healing spring Banya village with water temperature 37°C	**> 100**	–	–	–	–	–	–
4. Non-thermal "Gunchov Spring", Karandila locality with water temperature 21.5 °C	**80**	**+ Acid**	**+ Acid**	**+ Acid and gas**	**+ Acid and gas**	**+ Acid and gas**	–

Table 4 Microbiological indicators of spring waters in Sliven District

Indicators	**Measuring unit**	**Thermal healing spring Sliven Mineral baths with water**	**Non - thermal healing spring "Hadji Dimitar" locality "Hot**	**Thermal healing spring Banya village with water**	**Non-thermal "Gunchov Spring", Karandila locality**

		temperature of 48°C	water" town Shivachevo with water temperature 22,5°C	temperature 37°C	with water temperature 21.5 °C
Coliforms	cfu/cm^3	0/100	0/100	0/100	4/100
Escherichia coli	cfu/cm^3	0/100	0/100	0/100	4/100
Enterococci	cfu/cm^3	0/100	0/100	0/100	0/100
Sulphite reducing anaerobic bacteria*(Clostridium perfringens)*	cfu/cm^3	0/100	0/100	0/100	0/100
Pseudomonas aeruginosa	cfu/cm^3	0/250	0/250	0/250	0/250

Norm: The indicator with red color is not in norm

4. CONCLUSION

Based on the conducted physicochemical and microbiological evaluations it is established that from the four examined springs at the territory of Sliven district, Bulgaria non-thermal healing spring Banya mineral and non - thermal healing spring "Hadji Dimitar" locality "Hot water" town Shivachevo source correspond to all controlled parameters according to Ordinance № 9/2001, Official State Gazette, issue 30, and decree № 178/23.07.2004 about the quality of water, intended for drinking purposes.

With regards to microbiological parameters thermal healing water Sliven Mineral baths source is in compliance with the requirements for drinking water.

DISCLAIMER

The products used for this research are for scientific research and they are not products of companies. There is absolutely no conflict of interests. The research was not funded by the producing company rather it was funded by personal efforts of the authors.

ETHICAL APPROVAL

1. I, the alone corresponding author, am authorized to submit this manuscript.
2. Submission of the manuscript represent
that the manuscript has not been published previously and is not considered for publication elsewhere.
3. The manuscript, or any part thereof, is in no way a violation of any existing original or derivative copyright.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. 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 producing company rather it was funded by personal efforts of the authors.

References

1. Ignatov, I. Which Water is Optimal for the Origin (Generation) of Life? Euromedica, Hanover, 2010; 34-37.
2. Ignatov I., Mosin O.V. Possible Processes for Origin of Life and Living Matter with Modeling of Physiological Processes of *Bacterium Bacillus Subtilis* in Heavy Water as Model System, Journal of Natural Sciences Research, 2013; 3 (9); 65-76.
3. Ignatov, I., Mosin, O. V. Modeling of Possible Processes for Origin of Life and Living Matter in Hot Mineral and Seawater with Deuterium, Journal of Environment and Earth Science, 2013; 3 (14): 103-118.
4. Ignatov, I., Mosin, O. V. Structural Mathematical Models Describing Water Clusters, Journal of Mathematical Theory and Modeling, 2013; 3(11): 72-87.
5. Ignatov, I., Mosin, O.V. Origin of Life and Living Matter in Hot Mineral Water, Advances in Physics Theories and Applications, 2015; 39: 1-22.
6. Strunecky et al., High Diversity of Thermophilic Cyanobacteria in Rupite Hot Spring Identified by Microscopy, Cultivation, Single-cell PCR and Amplicon Sequencing, *Extremophiles,* 2019; 23: 35-48.
7. Valcheva, N., Denkova, Z., Denkova, R. Physicochemical and Microbiological Characteristics of Spring Waters in Haskovo. Journal of Food and Packaging Science Technique and Technologies, 2013; 2, 21 – 25.
8. Valcheva, N., Denkova, Z. Nikolova, R., Denkova, R. Physiological, Biochemical, and Molecular – Genetic Characterization of Bacterial Strains Isolated From Sping and Healing Waters in Region of Haskovo, *Food, Sciense, Engineering and Technologies*, Plovdiv, 2014; LX: 940 – 946.
9. Valcheva, N., Denkova, Z., Nikolova, R. Denkova, R. Physiological - biochemical and Molecular - genetic Characteristics of Bacterial Strains Isolated from Spring and Healing Waters in the Haskovo region, *N.T. at UCT*, 2013; LX.
10. Valcheva, N., Denkova, Z., Denkova, R., Nikolova, R. Characterization of Bacterial Strains Isolated from a Thermal Spring in Pavel Banya, Stara Zagora Region, *N.T. at UCT*, 2014; LXI.
11. Valcheva, N., The Microflora of Medicinal and Spring Waters in Haskovo and Stara Zagora Region, *Dissertation, University of Food Technology*, 2014; 1 – 142.
12. Valcheva, N., Ignatov, I. Physicochemical and Microbiological Characteristics of Thermal Healing Spring Waters in the District of Varna, Journal of Medicine, Physiology and Biophysics, 2019; 59: 10-16.
13. Valcheva, N. Physicochemical and Microbiological Characteristics of Thermal Healing Spring Waters in the District of Burgas, European Reviews of Chemistry*,* 2019; 6(2): 81-87.
14. Valcheva, N. Physicochemical and Microbiological Characteristics of Thermal Healing Spring Waters in the Districts of Varna and Burgas, Black Sea Region, Bulgaria, European Journal of Medicine, 2019: 7 (2). 120-130
15. Schulze-Makuch, D., Kennedy, J. F. Microbiological and Chemical Characterization of Hydrothermal Fluids at Tortugas Mountain Geothermal Area, Southern New Mexico, USA. Hydrogeol. J. 2000; 8: 295 – 309.
16. Jeanthon, C. Molecular Ecology of Hydrothermal Vent Microbial Communities, *Antonie van Leeuwenhoek*, 2000; 77: 117 – 133.
17. Fouke, B. W., Bonheyo, G. T., Sanzenbacher, B., Frias-Lopez, J. Partitioning of Bacterial Communities Between Travertine Depositional Facies at Mommoth Hot Springs, Yellowstone National Park, USA. Can. J. Earth Sci., 2003; 40: 1531 – 1548.
18. Belkova, N. L., Tazaki, K., Zakharova, J. R., Parfenova, V. V. Activity of Bacteria in Water of Hot Springs from Southern and Central Kamchatkaya Geothermal provinces, Kamchatka Peninsula, Russia. *Microbiol. Research*, 2007; 162: 99 – 107.

19. Asoodeh, A., Lagzian, M. Purification and Characterization of a New Glucoamylopullulanase from Thermotolerant Alkaliphilic *Bacillus subtilis* DR8806 of a Hot Mineral Spring, *Process Biochemistry*, 2012; 47 (5): 806-815
20. Ignatov, I. Origin of Life in Hot Mineral Water from Hydrothermal Springs and Ponds. Effects of Hydrogen and Nascent Hydrogen. Analyses with Spectral Methods, pH and ORP, European Reviews of Chemical Research, 2019; 6(2): 62-73.
21. Mosin, O. V., Shvets, V. I, Skladnev, D. A., Ignatov, I. Microbiological Synthesis of [^{2}H]-inosine with High Degree of Isotopic Enrichment by Gram-positive Chemoheterotrophic Bacterium Bacillus Subtilis, Applied Biochemistry and Microbiology, 2013: 49 (3): 255-266.
22. Mosin, O. V., Shvets, V. I, Skladnev, D. A, Ignatov, I. Microbial Synthesis of ^{2}H-labelled L-phenylalanine with Different Levels in Isotopic Enrichment by a Facultative Methylotrophic Brevibacterium Methylicum with RuMP Assimilation of Carbon, Supplement Series B: Biomedical Chemistry, 2013; 7(3): 247-258.
22. Tumbarski et al. Isolation, Characterization and Amino Acid Composition of a Bacteriocin Produced by Bacillus methylotrophicus Strain BM47, Food Technology&Biotechnology, 2018; 56(4): 546-552.
23. Yuan. X. et al. Bacillus Methylotrophicus Has Potential Applications Against Monilinia fructicola, formerly Central European Journal of Biology, 2019; 14 (1): 410-419.
24. Biscardi et al. The Occurrence of Cytotoxic Aeromonas Hydrophila Strains in Italian Mineral and Thermal Waters, Sci Total Environ, 2002; 292 (3): 255-63.
25. Tumbarski, Y., Valcheva, N., Denkova, Z., Koleva, I. Antimicrobial activity against Some Saprophytic and Pathogenic Microorganisms of Bacillus species Strains Isolated from Natural Sping Waters in Bulgaria, British Microbiology Research Journal, 2014; 4 (12): 1353 – 1369.
26. Aanniz, T. Thermophilic Bacteria in Moroccan Hot Springs, Salt Marshes and Desert Soils, Brazilian Journal of Microbiology, 2015; 46(2): 443–453
27. Igbinosa et al. Emerging Aeromonas Species Infections and Their Significance in Public Health, The Scientific World Journal, 2012; 1-13.
28. Minnan et al. Isolation and Characterization of a High H2-producing Strain Klebsiella oxytoca HP1 from a Hot Spring, Res. Microbiol., 2005; 156 (1):76-81.
29. Takai, K., Horikoshi, K. Molecular Phylogenetic Analysis of Archaeal Intron-containing Genes Coding for rRNA Obtained from a Deep-subsurface Geothermal Water Pool, Appl. Environ. Microbiol.. 1999; 65, 5586 – 5589.
30. Velichkova, K., Sirakov, I., Rusenova, N., Beev, G., Denev, S., Valcheva, N., Denev, T. In vitro Antimicrobial Activity on LemnaMinuta, Chlorella Vulgaris and Spirulina Sp. Extracts, Fresenius Environmental Bulletin, 2018; 27 (8): 5736-5741.

Standards

1. Ordinance № 9 / 2001, Official State Gazette, issue 30.
2. Decree № 178 / 23.07.2004 about the quality of water, intended for drinking purposes.
3. BDS 8451 : 1977 – defining of color according to Rublyovska Scale, determination of smell at 20 °C.
4. EN ISO 7027 – determination of turbidity.
5. BDS 3424 : 1981 – determination of pH.
6. BDS 3413 : 1981 – determination of oxidisability.
7. BDS 3414 : 1980 – determination of chlorides.
8.BDS ISO 6058 – determination of calcium, determination of general hardness.
9. BDS EN 27888 – determination of electrical conductivity.
10. VLM – NH4 – № 1 – determination of ammonium ions.
11. VLM – NO3 – № 2 – determination of nitrates.
12. VLM – NO2 – № 3 – determination of nitrites.
13. VLM – SO4 – № 4 – determination of sulphates.
14. VLM – PO4 – № 5 – determination of phosphates.
15. VLM – Fe – № 6 – determination of iron.

16. VLM – Mn – № 7 – determination of manganese.
17. VLM – F – № 8 – determination of fluorides.
18. BDS 7211 : 1982 – determination of magnesium.
19. BDS EN ISO 7899 – 2 – determination of nitrates.
20. BDS EN ISO 9308 – 1: 2004 – determination of Escherichia coli and coliform bacteria.
21. BDS EN 26461 – 2 : 2004 – determination of sulphite reducing anaerobic bacteria (Clostridium perfringens) .
22. BDS EN ISO 16266 – determination of Pseudomonas aeruginosa.
23. BDS EN ISO 7899 – 2 – determination of eneterococci.
24. BDS EN ISO 6222 : 2002 – determination of total number of aerobic and facultative anaerobic bacteria.