

Study of the microbiological status of bottled natural mineral, spring and table waters for the period 2015 – 2024

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Abstract

Natural mineral and spring waters are good examples of pristine aquatic habitats, as their aquifers originate from underground sources protected and are protected from anthropogenic interference. The widespread consumption of these waters necessitates strict adherence to sanitary and hygienic requirements established by national and European regulations, thereby ensuring their quality and safety. Compliance with these requirements is controlled by annual monitoring. The present study is in this regard. Its objective is to perform a comprehensive analysis of the microbiological monitoring results for bottled mineral, spring and table waters over the period 2015 – 2024, aiming to evaluate their microbiological quality and identify the indicator with the highest rate of non-compliance. A total of 696 bottled water samples were analyzed in accordance with the requirements of the Ordinance on the Requirements for Bottled Waters Intended for Drinking Purposes. The examined microbiological indicators are defined by national legislation. The used methods are ISO standards. Throughout the observation period the indicator *Pseudomonas aeruginosa* exhibited the highest degree of deviation from regulatory standards. Out of 45 analyses that failed to meet the requirements of the Ordinance, 33 were non-compliant by the indicator *Pseudomonas aeruginosa* which represents 73.3% of the failures. The high prevalence of *P. aeruginosa* in bottled water can be explained by its capacity for biofilm formation and its inherent resistance to chlorine disinfection.

Keywords: Over-The-Counter (Otc) Cough Syrup Abuse, Non-Codeine Cough Syrups, Diphenhydramine Misuse, Dextromethorphan Abuse, Nigerian Youths, Community Pharmacists, Drug Misuse Prevention.

Introduction

Natural mineral and spring waters originate primarily from intermediate aquifers, characterized by a well-defined and protected catchment area and a prolonged underground residence time [1]. Mineral and spring waters cannot be disinfected by any treatment aimed at removing or destroying microorganisms and in this way their microbiota reflects the autochthonous populations of the aquifer. Mineral water originates from a mineral water deposit and it is extracted from natural springs or engineered boreholes. It possesses a strictly defined and constant discharge (within the limits of natural fluctuations), temperature, mineral composition, trace element content, and other constituents that impart specific properties, clearly distinguishing it from ordinary drinking water. Water is transported to the bottling facility which is constructed within the vicinity of the water source, via a direct pipeline connection under conditions that guarantee the

preservation of its natural characteristics. In the production of natural mineral water, any treatment for disinfection purposes is prohibited, as well as any addition of bacteriostatic elements that could alter its indigenous microflora. Spring water is defined as water originating from a deep-seated aquifer that is not classified as a mineral water deposit.

It is extracted from natural springs or engineered boreholes and is suitable for drinking consumption in its natural state. It must be bottled at the source within a facility connected to the water source via a direct pipeline. During the production of spring water, any treatment for disinfection purposes is strictly prohibited [2].

Table water refers to any water derived from an underground or surface source which is treated or untreated, that does not meet

the specific requirements for natural mineral or spring water but complies with the standards of Ordinance No. 9 of 2001 regarding the quality of water intended for drinking and domestic purposes [3]. In cases when treatment is applied during the production of table water, such processes should not lead to formation of treatment residues in concentrations exceeding established limits that could pose a risk to human health [2].

Ground water is an oligotrophic ecosystem characterized by limited bioavailability. The microbial communities are predominantly aerobic, saprophytic, Gram-negative rods, distinguished by the presence of cytochrome oxidase [4]. Bacteria belonging to the genera *Pseudomonas*, *Acinetobacter*, and *Alcaligenes* are frequently encountered in groundwater. If these bacteria are not adequately removed prior to bottling, microbial proliferation may occur within 1–2 weeks post-bottling, with bacterial counts potentially reaching 103–104 CFU/ml at 37 °C [5]. When water is bottled using compressed air, the microenvironment of these microorganisms is altered. The increased oxygenation of the water, coupled with the potential deposition of nutrients onto the interior surfaces of the bottles, effectively concentrates these substances, making them more accessible for microbial utilization.

This provides a probable explanation for the observed phenomenon when microbial counts in bottled water increase after bottling. However, it is also possible that the rise in culturable bacteria results from the resuscitation of a great number of viable but non-culturable (VBNC) cells, rather than the active growth or reproduction of a few cells which are already present at the time of bottling [6]. Every country maintains its own national regulatory framework which is subordinate to EU directives and regulations regarding the requirements for bottled natural mineral, spring, and table waters. Both European and national legislations prohibit the treatment of natural mineral and spring waters for the purpose of microbial removal. Consequently, microbiological monitoring of bottled waters is imperative to ensure compliance with safety standards and to minimize the potential occurrence of pathogenic microorganisms.

Aim

The objective of this study is a presentation of a comprehensive analysis of the microbiological monitoring results for bottled mineral, spring and table waters over the period 2015–2024. The aim is evaluation of their microbiological quality and identifying of the parameter with the highest rate of non-compliance.

Materials and Methods

The present study tracks the microbiological monitoring of bottled natural mineral, spring, and table waters (including 19-liter carboys) from 20 Bulgarian brands for the period 2015–2024, in accordance with the requirements of the Ordinance on the Requirements for Bottled Natural Mineral, Spring, and Table Waters Intended for Drinking Purposes [2]. During the 2015–2024 period, a total of 696 bottled water samples were analyzed from 9 brands of mineral water (Devin, Mikhalkovo, Lenovo, Hisar, Staro Zhelezare, Dragoyново, Hisar Millennium, Hisarya, Velingrad), 6 brands of spring water (Aronitsa, Devin, Bachkovo, Baldaran, Rosa, Pure H2O), and 5 brands of table water (Crystal Voda, Zhivena, Divna, Iceberg, Nice Water). The 19-liter carboys included: Crystal Voda, Zhivena, Divna, Iceberg, Nice Water, Aronitsa, and Devin. A total of 696 samples analyzed based on the following microbiological parameters: Total Viable Count (TVC) post-bottling at 20 °C ± 2 °C and 37 °C ± 1 °C; coliforms; *Escherichia coli*; enterococci/fecal streptococci; sulfite-reducing clostridia (SRC); and *Pseudomonas aeruginosa*. So 3,632 individual analyses have been conducted following the established ISO standards [7-11].

Results and Discussion

In accordance with EU Directive 2009/54 [12] and Bulgarian national legislation [2], bottled waters must be free from *P. aeruginosa*, *Escherichia coli*, and enterococci/fecal streptococci in a 250 cm³ sample, and from SRC (sulphite-reducing clostridia) in a 50 cm³ sample. During the 2015–2024 study period, a total of 696 bottled water samples were processed, involving 3,632 individual microbiological analyses.

Out of these, 34 samples (4.88%) were found to be non-compliant with the Ordinance on the Requirements for Bottled Natural Mineral, Spring, and Table Waters Intended for Drinking Purposes, accounting for 45 failed analytical tests (1.23%). Figure 1 illustrates samples analyzed in 2015 which exhibited deviations in two parameters: *P. aeruginosa* and SRC. In contrast, samples tested during 2016, 2017, 2019 and 2021 showed non-compliance solely regarding the *P. aeruginosa* indicator. In 2020 and 2022, samples failed to meet regulatory standards not only for *P. aeruginosa* but also for coliforms and total viable counts (TVC) at 20 °C. In 2023 deviations were observed across *P. aeruginosa*, TVC at 20 °C, TVC at 37 °C, and coliforms. Finally, in 2024, non-compliance was recorded for TVC at 20 °C and *P. aeruginosa*.

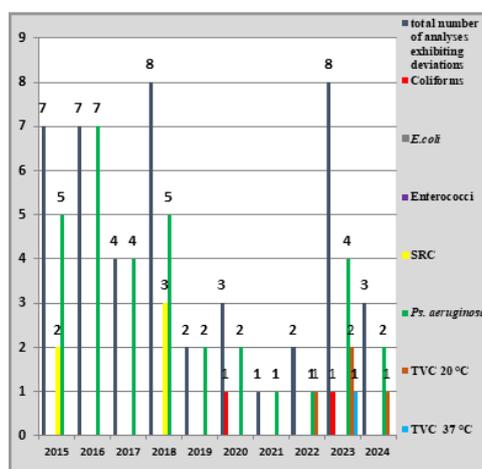


Figure 1: Number of Analyses Exhibiting Deviations by Indicators for the Period 2015 – 2024

The absence of samples exhibiting deviations in *E. coli* and enterococci parameters indicates that the mineral or spring water aquifers are well-protected, with contamination from environmental sources successfully prevented. Consequently, these waters do not pose a risk to public health in this regard. Water found to contain *E. coli* must be considered unsafe for consumption due to the strong correlation between *E. coli* and fecal contamination. Furthermore, *E. coli* and enterococci do not typically colonize the biofilms that form on the moist surfaces of bottling lines. The detection of enterococci/fecal streptococci in water serves as a reliable index of fecal pollution, even in the absence of *E. coli* and/or coliforms. This is attributed to the superior environmental persistence of enterococci in water compared to these other indicator bacteria [6]. The presence of coliform bacteria in bottled water is not necessarily indicative of fecal contamination. Coliforms can also originate from the environment and are capable of establishing biofilms on bottling equipment. Consequently, these microorganisms may signal a decline in the quality of the water source or potential contamination during the bottling process, serving primarily as process hygiene indicators.

While many species of coliform bacteria are associated with nosocomial (healthcare-associated) infections in vulnerable patients, they generally do not pose a direct risk to public health for the general population. Although their presence in bottled water samples is significant from a hygienic perspective, the isolation of coliforms alone is not a robust indicator of the potential presence of enteric pathogens [6]. Sulphite-reducing bacteria (SRB) are spore-forming anaerobes whose spores exhibit extreme resistance to adverse environmental conditions. Their detection in bottled waters may be a consequence of the infiltration of surface waters into the underground source or insufficient hygiene during extraction, transportation or bottling. The presence of these microorganisms casts doubt on the water source protection, stability of the pipeline system and the efficiency use of the sanitary protected zones. Throughout the monitoring period it

is observed a downward trend in the number of non-compliant analyses for the SRC indicator. This decline may be related to excessive control regarding the implementation of Good Manufacturing Practices (GMP) and the advancement of HACCP (Hazard Analysis and Critical Control Points) systems within water bottling facilities.

Elevated values exceeding the maximum permissible limits for the Total Viable Count (TVC) at 20 °C are likely attributable to the presence of autochthonous microflora. These bacterial communities are characterized as psychotrophic, as they are able to grow at low temperatures up to 5 °C. After bottling bacterial counts often increase rapidly which does not necessarily mean pathogenicity or pose a health risk to the consumer. But it indicates:

- A shift in the microbiological equilibrium;
- Potential post-bottling proliferation;
- The presence of biofilms within pipelines, storage tanks, or bottles.

These findings underscore the critical importance of rigorous hygiene protocols and effective equipment sanitation. Evaluation of the bacteriological quality in bottled natural mineral waters marketed in Hungary has been made by a comparative study involving the analysis of 492 samples of both carbonated and non-carbonated mineral waters. These samples were screened for the presence of: spore-forming sulphite-reducing anaerobes (clostridia), coliforms, *Escherichia coli*, *Enterococcus* spp., and *Pseudomonas aeruginosa*. Heterotrophic plate counts (HPC) were also determined following incubation at 37 °C for 24 hours and at 22 °C for 72 hours. The results indicate that 5.3% of the carbonated and 10.2% of the non-carbonated mineral water samples are positive for at least one specific indicator or potentially pathogenic bacterium. In general 38 samples (7.7%) failed to comply with the requirements of Directive 2009/54/EC [13].

Table 1: Number of Samples and Analyses for the Period 2015 – 2024

№	year	number of samples	number of analyses	Samples exhibiting deviations	analyses exhibiting deviations						
					total number	Coli-forms	E. coli	Enterococci	SRC	<i>Pseudomonas aeruginosa</i>	TVC 20 °C
1	2015	82	382	5	7	0	0	0	2	5	0
2	2016	85	445	7	7	0	0	0	0	7	0
3	2017	86	460	4	4	0	0	0	0	4	0
4	2018	111	609	5	8	0	0	0	3	5	0
5	2019	124	560	2	2	0	0	0	0	2	0
6	2020	40	240	3	3	1	0	0	0	2	0
7	2021	36	210	1	1	0	0	0	0	1	0
8	2022	36	212	1	2	0	0	0	0	1	1
9	2023	54	292	4	8	1	0	0	0	4	2
10	2024	42	222	2	3	0	0	0	0	2	1
	total	696	3632	34	45	2	0	0	5	33	4

The presence of *P. aeruginosa* has been recorded consistently throughout the entire monitoring period (Figure. 1). There are numerous studies for microbiological quality of the bottled wa-

ters by other researchers who have reached similar conclusions. Mahmud et al. [14] have investigated bottled mineral water and identified the presence of *P. aeruginosa* in 59 out of 238 analyzed

samples. Scientists from the University of Leeds, England [15] have examined 1 082 samples of bottled natural mineral and spring water across 17 different brands, detecting *P. aeruginosa* in 13 of the samples. Venieri et al. [16]. Have analyzed 1 527 samples of bottled mineral water produced by 10 different bottling companies and have concluded that *P. aeruginosa* is the most frequently isolated contaminant. Eckmanns et al. [17] described a hospital-acquired outbreak of *P. aeruginosa* infection caused by contaminated bottled water in intensive care units (ICUs) at a hospital in Germany. The contaminated bottled water had been used for the preparation of oral medications and as a substitute for oral fluids. It has been established that unopened water bottles contained the specific strain of *P. aeruginosa*, although all bottles haven't been contaminated. The authors have concluded that bottled water should not be used in ICUs unless it has undergone sterilization [17].

From its origin natural mineral water inherently contains bacterial populations in a starvation state which allow to survive for a long period of time. After bottling and in the absence of any disinfection treatments bacterial counts increase rapidly. These microbial communities consist predominantly of aerobic, saprophytic, Gram-negative rods. Bacteria belonging to the genus *Pseudomonas* are a part of the autochthonous microflora of bottled water. According to Daood, 40% of the strains isolated from mineral waters belong to the genus *Pseudomonas* [18]. The prototrophic nature and metabolic versatility, combined with genomic plasticity and the ability to withstand various forms of stress (physical, chemical, and antibacterial compounds), are remarkable characteristics of the members of this genus. These attributes are the primary driving forces behind their adaptability explaining the existence of pseudomonads in all major natural terrestrial and aquatic environments. The bacterial population expands until the available nutrients are depleted and the cells

begin to die. The lysis of deceased cells releases nutrients back into the water, and it causes following reproduction. This process leads to a state of equilibrium within the closed aquatic system, maintaining a constant population density [19] Representatives of the genus *Pseudomonas* are frequently encountered in groundwater due to their exceptional versatility regarding the diversity of organic substrates they can utilize for growth. Bacteria belonging to this genus colonize water reservoirs rapidly, storage areas, food preparation zones, and water processing facilities. Their presence serves as a benchmark for evaluating the overall hygiene of water distribution systems and the quality of bottled waters [20]. Furthermore, the genus *Pseudomonas* includes species that are among the most resistant microorganisms to disinfectants [21].

In contrast to other pseudomonads, *P. aeruginosa* is not a typical component of the autochthonous microbial flora in natural mineral waters. It is a representative of the allochthonous microflora, i.e. it is a contaminant. This microorganism is widely distributed in surface and groundwater sources, marine environments, soil, plants, and fecal matter [22]. As a contaminant of natural mineral waters, *P. aeruginosa* is usually in low concentrations. It is characterized by its ability to proliferate in these waters due to its high metabolic adaptability, even in the presence of low nutrient concentrations. Despite the low nutrient concentration in these waters *P. aeruginosa* survives and even proliferates over extended period of time [23, 24]. The high prevalence of *P. aeruginosa* in bottled waters can be explained by its ability to form biofilm and its resistance to chlorine disinfection. These characteristics give it an opportunity to survive for a long time within the piping and bottling systems of production facilities. The presence of this bacterium in the bottled waters we examined is likely associated with such technical and hygienic challenges.

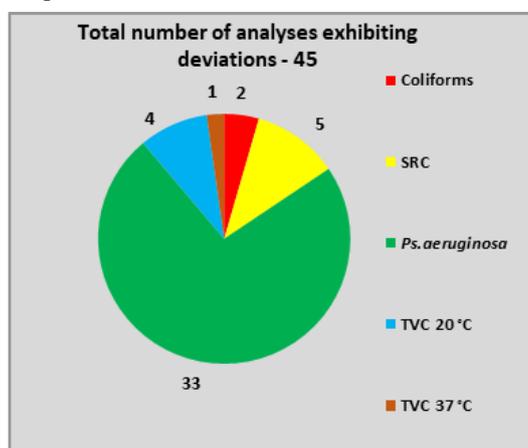


Figure 2: Number of Analyses Exhibiting Deviations by Indicators for the Period 2015 – 2024

In our study, out of all 45 analyses that failed to comply with the regulatory requirements of the Ordinance, 33 are non-compliant specifically for the *Pseudomonas aeruginosa* parameter which means 73.3% of all failures. The detection of *P. aeruginosa* does not pose a risk to the majority of the population, as it rarely causes disease in healthy individuals. However, *P. aeruginosa* is an opportunistic pathogen that migrates from its natural environments to infect immunocompromised patients, potentially leading to healthcare-associated (nosocomial) infections. [25] The presence of *P. aeruginosa* in bottled water is hazardous for

individuals with weakened or compromised immune systems. This includes neonates, young children, and patients suffering from advanced neutropenia, cystic fibrosis, or severe burns. It acts as the primary etiological agent in serious conditions such as urinary tract infections, respiratory system infections, ocular infections, soft tissue infections, dermatitis, bacteremia, and various systemic infections—particularly in patients with cancer or AIDS. Furthermore, *P. aeruginosa* in bottled water presents a significant risk to patients hospitalized in intensive care units (ICUs) and neonatal wards. Reducing the risk of infection

among these vulnerable groups needs infected batches of bottled water to be withdrawn from the market [17, 21].

Conclusion

Natural mineral and spring bottled waters must be safe and wholesome for consumption exactly as they spring from the source treatment or disinfection. It is necessary to guarantee the absence of contamination as the protection of the source is of paramount importance. The quality of bottled waters is ensured by rigorous control across the entire supply chain from securing water protection at the source to distribution channels and the final consumer. Bottling companies must ensure the installation of modern piping systems and the implementation of specialized extraction equipment for natural mineral and spring water. Such equipment should be installed in the source area to provide an additional layer of protection. Ensuring this level of security makes the manufacturers to maintain an effective HACCP-based system. This system must incorporate measures necessary for the protection of the water source and encompass both the extraction and the bottling processes [6]. Frequent quality testing and analysis which are based on HACCP principles and Good Hygienic Practices (GHP), guarantee the safety of the extracted groundwater. In Bulgaria, the Ministry of Health (MoH) defines the health requirements for bottled water. Continuous monitoring provided by the Regional Health Inspectorates (RHI) water quality as well as the conditions for bottling and distribution are strictly supervised. This is how it is guaranteed health safety of the final product.

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Data Availability Statement

Not applicable.

Conflicts of Interest

The authors declare no conflict of interest.

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