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Prevalence of *Pseudomonas Aeruginosa* in Oligomineral or Highly Concentrated Mineral Waters and the Possible Impact on their Curative use

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Abstract: *Pseudomonas aeruginosa* is an emergent opportunistic pathogen whose presence in curative mineral waters may become significant in certain scenarios which require health promotion measures; this study aimed the evaluation the degree of contamination of the mineral waters used for therapeutic purposes with *P.aeruginosa* by monitoring this omnipresent bacterial species in aquatic environments in underground hydromineral sources (springs, wells or boreholes) or above-ground hydromineral sources (natural lakes and bathing ponds) using the culture-dependent method. The results show that the curative waters contaminated with *P.aeruginosa* were characterized by a different mineralization and specific chemical profiles, in which the predominant ions (>20% mEq) were Na^+, Cl^-, S^{2-}, HCO_3^{2-} or Br^-; statistical observations showed an interdependence between the level of populations of *P.aeruginosa* and HPC (37^0C) or HPC (22^0C), unlike the magnitude of abiotic factors, that are dynamic and may change at any point along the exploitation chain of these waters. The physicochemical profile of the hydromineral source contaminated with *P.aeruginosa*, regardless of its origin, appears to include a medium mineralization (1-15 $g \cdot dm^{-3}$), a chemical structure with dominant ions Na^+/Cl^-, an optimal temperature <20°C and a basic pH.

Keywords: *Pseudomonas* sp. monitoring; curative waters; contamination; impact on water's curative potential.

1. Introduction

The pseudomonads are a group of bacteria isolated from soil, seawater, and drinking water, generally present in aquatic environments; they can also colonize the surface of plants and animal's mucosa and are frequently found in households and hospitals, in media with a high-level humidity. *Pseudomonas aeruginosa* is generally described as ubiquitous in natural settings. Still, it is usually scarce in pristine environments, being described as a bacterium largely found in locations associated with human activity (in organic waste, pesticide, and

feces-contaminated environments)[1]. Being extremely versatile and able to adapt to a wide range of conditions, these bacteria are known to survive even in distilled water, in the presence of traces of minerals [2], or in the aquatic ecosystems of floodplains [3]. The adaptability and flexibility of *P.aeruginosa* are conferred by a large number of virulence factors from its „arsenal" which allows it to respond adequately to all environmental stressors [4].

Because *P.aeruginosa* has more than 10% of its genes that include virulence factors [5], it is also an opportunistic bacterial pathogen in freshwater supplies that creates a risk for public health [6] or even causes severe health problems in both hospital and household settings [7]. Drinking water distribution systems can host pathogenic amoebae and many bacteria genera in the biofilm community; the main bacterial genus found in this environment is *Pseudomonas*, which represents one of the main members of biofilms in chlorinated drinking water distribution systems, associated with different types of pipe materials and hydraulic conditions [8,9]. Microbial contamination with potentially pathogenic microorganisms (*Escherichia coli, P.aeruginosa, Klebsiella pneumoniae*) is favored by deposits and corrosion scales in the water distribution systems [10]. Also, studies concluded that bathroom water from hospitals with permanent bathtubs is a potential reservoir of antibiotic-resistant *P.aeruginosa* [11]. *P.aeruginosa* is a pathogenic bacterium resilient towards chloramine [12] which, together with *Escherichia coli*, can adhere to any plumbing water delivery installation, the interaction being more or less thermodynamically active depending on water composition [13].

Several species of *Pseudomonas* are known to cause opportunistic human infections. For example, *P.fluorescens* is able to proliferate at 40°C and sometimes causes infections through contaminated blood, whereas *P.puckettii* has been isolated from sterile water for injection [14]. *P.aeruginosa* is a superbly adapted organism; plant pathologists and surgical pathologists recognize it well, and several of its basic microbiologic characteristics are of great clinical importance [15]. Among the bacterial strains, *P.aeruginosa* showed maximum decolorization activity; the dyes were the most challenging pollutants for the aquatic environment that are toxic and interfere with photosynthesis, reducing the light penetration into deep water [16].

Natural mineral waters represent a valuable therapeutic resource in some circumstances, representing an alternative to pharmacological treatment or an adjuvant; according to their composition, therapeutic mineral waters are recommended for consumption as an internal cure (crenotherapy) or in external cure (hydrotherapy) [17]. According to numerous studies, mineral waters positively affect the functioning of the digestive system, the correction of metabolic syndrome, and the regulation of hormonal or immune processes [18]. In external cure, mineral waters have been indicated in treating different degenerative rheumatic diseases or orthopedic pathologies [19,20].

Natural mineral water is defined as safe, microbiologically wholesome water originating in an underground source, protected from all risks of pollution. This protection is normally provided by the intrinsic physical characteristics of the aquifer material. Natural mineral water is clearly distinguished from ordinary drinking water from an ecological and epidemiological standpoint. Given the fact that *P.aeruginosa* is able to proliferate even in distilled water and has the potential to manifest pathogenicity, its presence in mineral waters must be quickly recognized and remedied. Thus, the two important reasons for monitoring *P.aeruginosa* in mineral waters are first to identify this opportunistic pathogen and use it as an indicator of a vulnerable or poorly controlled bottling process [21]. *E. coli*, *Pseudomonas spp.*,

and *Klebsiella spp*. were the most common bacterial types isolated from bottled water brands consumed in Kenya, most of which exhibited multidrug resistance [22].

P.aeruginosa is often found in natural water bodies such as lakes and rivers, but in most cases, in concentrations of $0,3 \cdot 10^1$ -$0,4 \cdot 10^1$ CFU·cm^{-3}. Because it can multiply in water and organic matter that comes in contact with water, it represents a known cause of hospital-acquired infections with potentially severe complications [23].

In Romania, safe drinking water is generally obtained by complying with specific water quality standards such as European Union Drinking Water Directive (Council Directive 98/83/EC, 1998). The Directive 2000/60/EC for water resources and its Romanian version Law 459/2002 republished in 2011 and 311/2004 (Romanian Law 458, 2011, Romanian Law 311, 2004) imposes microbiological quality parameters for potable water [24].

P.aeruginosa is a major microbiological marker of mineral water quality according to Romanian Government Decision 1020/2005 and bottled drinking water quality according to Romanian Law 311/2004. However, during bottling mineral, spring, and table water, the most common contaminant that exceeds the tolerable upper limit is *P. aeruginosa* [25].

Bottled mineral water is not completely free of microorganisms; typically, the native microbiota of the source is characterized by minimal pathogenicity, with little importance for healthy individuals. After bottling, the bacterial count increases rapidly based on the available organic matter consumed; this increase tends to be higher in non-carbonated water and when plastic recipients are used. The most common bacteria of the microbiota are the pseudomonads, which are opportunistic pathogens. However, confirmed outbreaks following the consumption of bottled mineral water are more likely to be caused by the contamination of the source rather than quality alteration following the bottling process [26].

Natural mineral waters from subterranean sources are oligotrophic ecosystems in which organic matter is limited, and bioavailability is low. Bacterial populations that can grow in these ecosystems are heterotrophic, with survival and degree of starvation determined by the low quantity of nutrients available. After bottling, the number of microorganisms increases rapidly, reaching 10^4-10^5 CFU·cm^{-3} in 3-7 days, following the alteration of ecological conditions [27]. The microbiota of bottled water includes indigenous species from the aquifer and species that contaminate the water during bottling and processing; indigenous bacteria can survive in this type of water for several years [28], giving rise to a secondary growth in which cells belonging to some species die off, generating vital nutrients for other cells.

Therefore, even after a long period after bottling, investigating the microbiological quality of mineral waters is of extreme importance; chloride, magnesium, sodium, and potassium concentrations have been negatively correlated only with HPC at 22°C [29]. The composition of the microbial environment also differs depending on the manufacturing time; total coliforms (16.2%), *P.aeruginosa* (9.9%), sulfate-reducing *Clostridium sp.* (5.0%), and *E.coli* (2.0%) were detected in bottled waters, microbiological contamination being present less in the case of mineral waters with lower manufacturing time [30].

Microbiological investigation of thermal bath led to the identification of the existing microbiota, which contains mainly members of the natural microbial community of the well waters and bacteria originating from the environment but also several opportunistic pathogenic taxa, e.g., *P.aeruginosa, P.stutzeri, Acinetobacter baumanni, Legionella spp., Staphylococcus aureus* [31].

Martins *et al*. [32] evaluated several microbiological indicators of water quality in swimming pools: *Candida albicans, Staphylococcus aureus*, total coliforms, fecal coliforms,

fecal streptococci, total viable counts, and *P.aeruginosa*; of these, *C.albicans* and *P.aeruginosa* were the microorganisms whose presence was not correlated with other indicators, and *P.aeruginosa* was detected with the lowest frequency.

Water treatment for *P.aeruginosa* eradication includes the use of chlorine, chloramine, ozone, iodine, and UV radiation. Laboratory data does not suggest that *P. aeruginosa* would resist these disinfection methods. However, this species is more resistant to iodine treatment than other species like *P.fluorescens, P.cepacia, Bacillus sp.*, and *Staphylococcus* [33]. Inactivation of *P.aeruginosa* in mineral water can also be done by the polymers containing immobilized phages that are able to reduce 0.53 log of bacteria population present inside mineral water bottles after 14 days [34].

2. Materials and Methods

The present study was conducted between 2018 and 2019. It aimed to identify and quantify the level of contamination with *P.aeruginosa* in two categories of samples: underground therapeutic mineral waters (TMW's) coming from springs, boreholes, or wells and used for internal use (crenotherapy) or external use (hydrotherapy, inhalation, irrigation) and TMWs from surface sources (lakes or hydrotherapy pools) for bathing.

P.aeruginosa was monitored in 93 samples of TMW's, of which 88.4% were subterranean, from natural sources (26 springs) or artificial ones (54 boreholes or wells), and 11.6% originated from surface sources (11 natural lakes and 1 bathing pond). Particular physical and chemical properties characterized the TMW's included in the study being analyzed 17 oligomineral water samples (<1g·dm^{-3} mineral composition), 55 water samples with medium mineralization (1-15 g·dm^{-3} mineral composition), and 9 water samples with high mineralization (>150 g·dm^{-3} mineral composition)).

In the study, temperature, and pH were also monitored, which are important ecological factors that influence the normal biological activity of microorganisms [35]. For the determination of the pH from the matrix, the electrometric method was used in conformity with the SR EN ISO 10523:2012 standard; the obtained results represent mean values±SD (n=3). *P.aeruginosa* was monitored both in cold curative waters (T=(20-31)°C) and thermal curative waters (T=(32-38)°C), the results obtained also representing mean values ±SD (n=3).

The chemical parameters of TMW's (levels of anions, cations, mineralization) were determined using current standard laboratory methods for mineral water quality analysis. The results obtained were expressed in mg·dm^{-3}.

The samples were taken from natural lakes and bathing ponds from depths of 0.5 m; from springs, boreholes, or wells, the sampling procedure was carried out according to SR EN ISO 19458:2007, with measures taken in order to avoid external contamination.

The method used for microorganism quantification was membrane filtration (cellulose ester membrane, 0,45 µm pore size). The number of *P.aeruginosa* bacteria was obtained by counting the specific colonies developed on selective culture media according to SR EN ISO 8199:2019. The isolated strains were oxidase-positive, able to synthesize pyocyanin, and fluorescent under UV radiation; these criteria are considered sufficient for species identification according to SR EN ISO 16266:2008. The results of the microbiological analysis were expressed in colony-forming units (CFU) per unit volume.

We also determined the heterotrophic plate count (HPC) at 37°C and 22°C because it can include *Pseudomonas* species among other bacteria. The total number of aerobic

mesophilic microorganisms was determined by counting them using the pour plate method, according to SR EN ISO 6222:2004.

The obtained results were processed in GraphPad Prism 9.0.2.161 and statistically analyzed by multiple linear regression and ANOVA. The results were also analyzed using the Chi-square test; statistical significance was considered ($p<0.05$), taking into account the difference between the critical value and the calculated value for the corresponding degrees of freedom (df), the null hypothesis (lack of association) being confirmed when calculated $\chi 2<$ critical $\chi 2$; for calculated $\chi 2>$ critical $\chi 2$, the alternative hypothesis was confirmed.

3. Results and Discussion

From the subterranean TMW's contaminated with *P.aeruginosa*, 85.71% of samples were from hydromineral sources (naturally flowing springs and borehole springs) used in external therapy (hydrotherapy) in balneary facilities, while the presence of the bacterium in mineral waters for internal use (crenotherapy) was low (14.29%). In the second category of samples (surface TMW's, for bathing), *P.aeruginosa* was identified only in lake ecosystems, in minimal densities ($0.1 \cdot 10^1$ CFU·100ml^{-1}; $1.8 \cdot 10^1$ CFU·100ml^{-1}).

The pH of the analyzed matrices ranged in the entire acidic-neutral-basic spectrum (between 5,56±0.23 and 8,56±0.09), and the temperature index of the samples varied between (6,34±0.38)ºC and (86,3±0.04)ºC. The identified strains produced pyocyanin, with one exception (Well no.1015, Deta, Timis County), which produced pyomelanin.

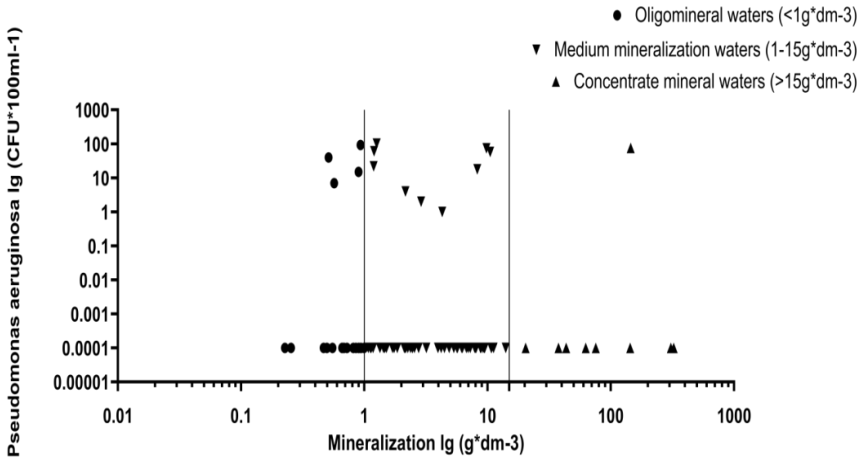

Figure 1. The distribution of *P.aeruginosa* (lg CFU·100ml^{-1}) in TMW's with different degrees of mineralization.

Regarding the effect of TMW's mineralization and chemical composition on the presence and abundance of *P.aeruginosa,* the analyzed TMW's total mineral concentration varied between 0,227 g·dm^{-3} and 324,387 g·dm^{-3}, *P.aeruginosa* being identified in 4 oligomineral TMW's (<1g·dm^{-3}), 9 samples of medium mineralization (1-15 g·dm^{-3}) and 4 highly concentrated ones (>15 g·dm^{-3}). It should be noted that the target bacteria were not identified in concentrated TMW's with 15-35 g·dm^{-3} dissolved minerals and neither in TMW's of high concentration with >150 g·dm^{-3} dissolved minerals.

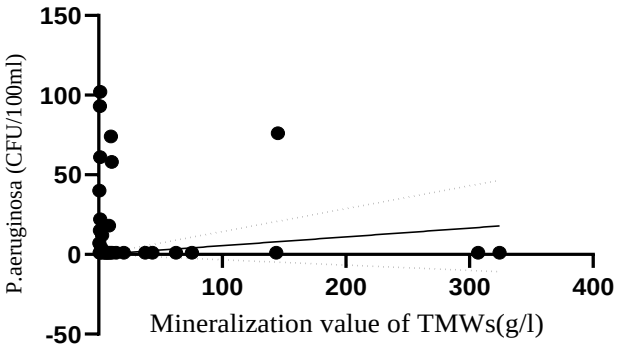

Figure 2. The relationship between the mineralization value of TMW's and the abundance (CFU/100ml) of *P.aeruginosa*.

The maximum density of *P.aeruginosa* ($1,02 \cdot 10^2$ CFU·100ml^{-1}) was found in therapeutic water with a value of mineralization of 1,259 g·dm^{-3} (Well no.2, Baile Boghis, Sălaj County) and the lowest density (0,1·10 CFU·100ml^{-1}) in a sample with 4,287 g·dm^{-3} mineral content (water mixture from the wells F3+F 4771IFLGS Tinca, Bihor County) and also in a matrix with a mineralization value of 69,778 g·dm^{-3} (Lake Techirghiol)(Figure 1). Calculation of the Pearson correlation coefficient (r=0.0295) and the p-value (0.2187)>0.05 (α=0.05), showed independence between TMW's mineralization and *P.aeruginosa* numerical density (Figure 2), the microorganism showing a tendency for lack of adaptation in microhabitats with a high concentration of bioelements (concentrated TMW's (15-35g·dm^{-3}) or highly concentrated TMW's (>150 g·dm^{-3} dissolved minerals)), having a better adaptation in TMW's of medium mineralization (1-15 g·dm^3) (9/17). It is known that *P.aeruginosa* is most frequently isolated from waters with low mineralization, like whirlpools [2], hot tubs (20-9%), tap water (8%), jacuzzis (7-4%) and bottled water (3%) [36].

The metabolic adaptation of *P.aeruginosa* to the varied chemical composition of TMW's was determined by quantification of the dominant (>20% mEq) ions (cations and anions) from contaminated samples (Figure 3). The intervention of this bacterium in the biogeochemical cycles of nitrogen and sulfur is known in the denitrification process, where it can accelerate the metabolism of sulfide [37], with *P.aeruginosa* being recognized as the representative denitrifying bacterial strain [38]; Na^+ and Cl^- ions had the greatest concentration in 12 and 14 of the positive samples, respectively S^{2-} and HCO_3^{2-} were dominant in 7 positive samples, while Br^- had the highest concentration only in 3 samples in which the target bacteria were identified.

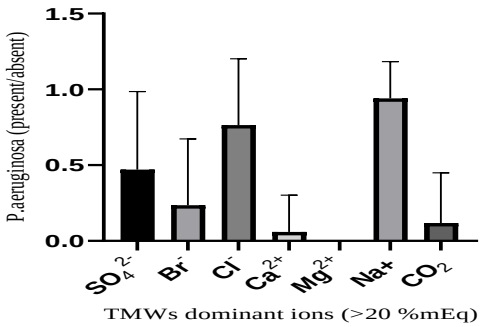

Figure 3. The incidence of *P.aeruginosa* (present/absent) in TMW's with different dominant ions (>20% mEq).

The heterotrophic plate count (HPC) has also been determined to quantify *P. aeruginosa* in associations with other microorganisms at the level of the TMW's microbiota.

HPC at 37ºC (48h) and 22ºC (72h) of subterranean TMW's contaminated with *P.aeruginosa* varied between $0,1 \cdot 10^1$ CFU·1ml^{-1} and $2,2 \cdot 10^1$ CFU·1ml^{-1}, while for natural lakes in which pseudomonads were found the densities for HPC had a lesser magnitude, in the range of $0,1 \cdot 10^1$ CFU·1ml^{-1} to $1,8 \cdot 10^1$ CFU·1ml^{-1}.

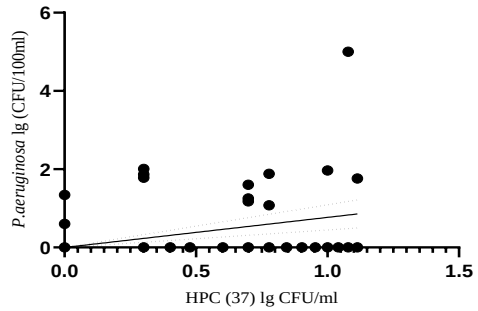

Figure 4. The relationship between HPC (37"C) lg value (CFU/ml) and the abundance of *P. aeruginosa* (lg CFU/100ml) in TMW's.

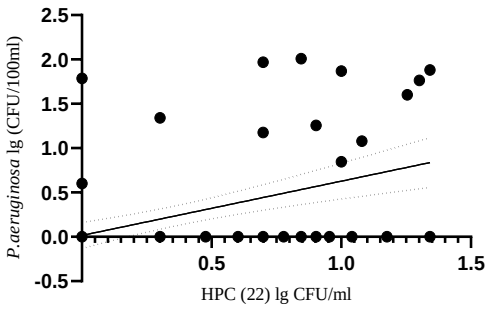

Figure 5. The relationship between HPC (22°C) lg value (CFU/ml) and the abundance of *P. aeruginosa* (lg CFU/100ml) in TMW's.

Statistical results obtained (r=0.2189 and p<0.0001) allowed us to establish a correlation between the variation of CFU/ml for HPC (37°C) in TMW microbiota and the numerical density of *P.aeruginosa* (calculated p<0.05 (α=0.05); dfd=93-2=91)(Figure 4), a similar tendency being observed by Mohammadi *et al*.[39], for HPC(37°C)<20CFU/ml and *P.aeruginosa*. Similarly, for microorganisms found in the same matrix, an association between HPC at 22°C and *P.aeruginosa* was established; the results obtained (p=0.0051; r=0.2146)) with the p value<0.01 showed an interdependence relationship which continuously regulates the numerical densities of these two categories of microorganisms found in TMWs microbiota (Figure 5).

Regarding the effect of TMW's temperature on the presence and abundance of *P. aeruginosa*, it has been established that the species of the *Pseudomonas* genus can tolerate a wide range of temperatures (4ºC-42ºC), *P.aeruginosa* being able to multiply at temperatures of 25°C to 37°C, its ability to grow at 42°C being a criterion used to differentiate it from other species of the genus [40,41].

In the examined subterranean TMW's, the bacteria was identified in 17 samples of different thermal classifications (8 cold waters, 3 hypothermal waters, 3 isothermal waters, and 3 hyperthermal waters) in concentrations of $0,1 \cdot 10^1$ – $1,02 \cdot 10^2$ CFU·100ml^{-1}.

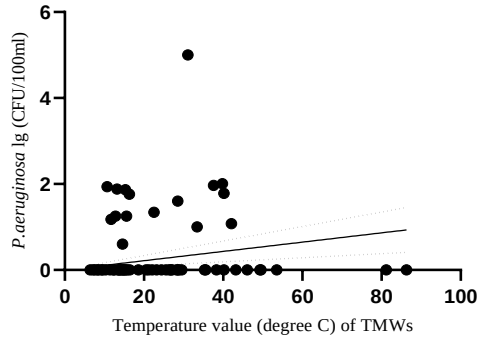

Figure 6. The relationship between temperature value of TMWs and the abundance (CFU/100ml) of *P. aeruginosa.*

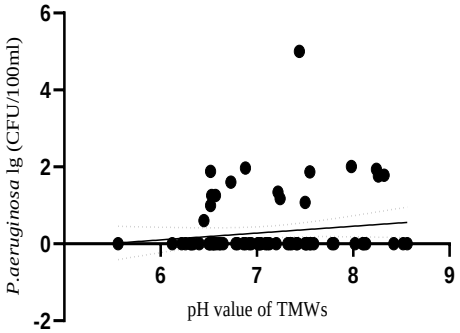

Figure 7. The relationship between TMW's pH value and the abundance (CFU/100ml) of *P. aeruginosa*.

Simple linear regression for temperature (independent variable) and numerical density of *P.aeruginosa* (dependent variable) resulted in the values r=0.0097 and p=0.3865, which is evidence that the variation of temperature factor in TMW's does not have an influence on the numerical density of *P.aeruginosa (*calculated p >0.05 (α=0.05), (Figure 6). The same tendency was revealed after applying the independence Chi-square test, the results obtained (calculated χ^2=1.7649 for df=(3-1)·(4-1)=6; α=0,05 and critical χ^2= 12,59) failing to show an association between the two variables (calculated χ^2< critical χ^2).

P.aeruginosa was also investigated in curative mineral waters with different thermal profiles in the lakes and bathing ponds of balneotherapy resorts (6 cold waters, 5 hypothermal, and 1 isothermal). The results showed a low incidence of the bacteria, *P. aeruginosa* being identified in only 3 of the samples, 2 from cold waters and 1 from isothermal water, all in low concentrations ($0,1\cdot10^1$-$1,8\cdot10^1$ CFU·100ml^{-1}).

Regarding the effect of TMW's pH on the presence and abundance of *P. aeruginosa*, in subterranean TMW's, *P.aeruginosa* had a higher incidence (7/14) in waters with a basic pH (7.22±0.06→8.32±0.2); its incidence was lower (5/14) in slightly acidic samples (6.45±0.21→6.88±0.06), being known that acidic environments inhibit the development of *P. aeruginosa* and *P. fluorescens* [42]. Moreover, a pH<3 for 60 sec. has a bactericidal effect on *P.aeruginosa* [43]. In neutral pH TMW's (6.8<pH<7.2) [44] found that the occurrence of *P.aeruginosa* was minimal (2/14), and even though the optimal pH interval for these species is not well defined, current evidence suggests a value of 7.2 as optimal for the development of the *P.aeruginosa* cells cultivated in an enriched media [45,46].

In surface TMW's, *P.aeruginosa* was identified (1/12) in alkaline media (8.24±0.05), but also in waters (2/12) with a slightly acidic pH (6.52± 0.21→6.57±0.14); surface TMW's contaminated with *P.aeruginosa* were exclusively isolated from salt water natural lakes with curative properties, used in balneotherapy (hydrotherapy).

Based on the results we obtained (simple linear regression for the two variables, pH – independent variable and numerical density of *P.aeruginosa* – dependent variable), the value of p=0.1710 showed a lack of interdependence between the pH value of the microhabitat and the amplitude of numerical density of *P.aeruginosa* (calculated p >0.05 (α=0.05); dfd=93-2=91)(Figure 7).

The causal-comparative study using the Chi-square test (calculated χ^2 < critical χ^2 for Df=6 and p<0.05) also failed to show an association between the proliferation of the target bacteria in TMW's and the pH of the environment. It should be taken into account that these types of waters are living ecosystems, and their chemical properties may change at any point along the exploitation chain (emergence to their practical application). It is possible for abiotic factors that govern the chemical reactivity of the aqueous environment - like the pH and the oxidation-reduction potential - to change over time, causing metabolic disruption for the microorganisms in various degrees, from inhibition of cellular division to cryptobiosis or entering a dormant metabolic state (ultramicrobacteria in the case of Gram-negative aquatic bacteria like *Pseudomonas sp.*)[34,46,47]. The analysis of multiple linear regression used for the evaluation of the convergent effect of the temperature and the pH of therapeutic waters on *P. aeruginosa's* abundance showed independence between the combined variation of the abiotic factors and bacterial proliferation (F(2,79)=1.768, p=0.1774, W=0.5519) (Figure 8).

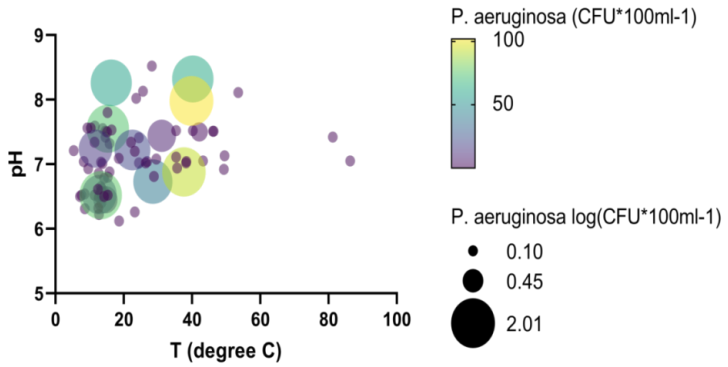

Figure 8. The behavior of *P.aeruginosa* (variation in numerical densities) according to pH and temperature value of TMW's.

Similar results were observed for seawater used for bathing, with physical-chemical parameters (salinity, temperature, pH) showing no relationship to bacterial abundance, which is probably explained by the short sampling timeframe and the stable environment conditions [48].

The higher occurrence of *P.aeruginosa* determined in this study in habitats such as springs and artificial bores with temperatures <20ºC (cold waters) contradicts its predisposition for high temperatures. Thus, the findings may indicate biofilm development in TMW's extraction system, being known that *P.aeruginosa* is a species that can form or attach to the biofilm [35], tending to enter a viable but non-cultivable state when it is in a biofilm state [49].

As a consequence, the abundance of bacteria ($(0.4 \cdot 10^1\text{-}7.6 \cdot 10^1)$ $CFU \cdot 100ml^{-1}$) identified in cold TMW's may correspond in part to the floating, planktonic forms of the microorganism (*P.aeruginosa* is a single-flagellated bacteria which swims using various patterns of motility: oscillatory, helical, sinusoidal, pseudo-helical and torsional [50], but also to its adherent, fixed forms. It is also important to note that *P.aeruginosa* is able to regulate its density through the mechanism of QS (quorum sensing) [35,51] and depending on the concentration of Na/Cl [52].

Also, one possible explanation for low *P.aeruginosa* level in bathing waters, maybe the way the sampling was done, directly from the source (from natural therapeutic lakes that frequently had T<20°C) and not from treatment facilities with hydrotherapy pools, where mineral water is often overheated (33-37)°C).

4. Conclusions

The results obtained in this study show that *P.aeruginosa's* incidence in TMW's with different physical-chemical features (pH, temperature, mineralization, and ionic composition) was very flexible and variable, with different numerical densities. The presence and abundance of *P.aeruginosa's* were independent of the mineralization, temperature, and pH of TMW (spring, well, drilling, or lake) but were correlated with HPC (22°C) and HPC (37°C)'s populational dynamics, specific for each curative water analyzed.

The profile of the hydromineral source contaminated with *P.aeruginosa*, regardless of its underground or above-ground origin, appears to include medium mineralization, between 1-15 $g \cdot dm^{-3}$, a chemical structure where dominant ions are Na+ and Cl-, the optimum temperature <20°C and the basic pH.

However, it should be taken into account that adaptation and survival of *P. aeruginosa* in TMW are complex processes, these types of waters being living ecosystems, and their

physical-chemical properties may change at any given time along the exploitation chain, from the place of harvest to the place of use.

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Conflicts of Interest

The authors declare there is no conflict of interest.

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