STUDY FOR A COMPARATIVE ANALYSIS OF SEASONAL MICROBIAL LOAD IN SOME NATURAL SALTED WATERS FROM ROMANIA

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ABSTRACT. The task of our studies was represented by the seasonal microbial load analysis from some Romanian natural salted water sources (springs and lakes) in order to check the requested conditions for their use as balneal treatment in a safe way. In order to check if the seasonal environmental factors can change the microbial load of some natural salted springs, a set of microbiological analyses were carried out at a laboratory level. Our study was focused on the next microbial groups: mesophilic bacteria growing at 22° C, mesophilic bacteria growing at 37° C, total number of coliform bacteria, *Pseudomonas aeruginosa, Staphylococcus aureus* and *Salmonella sp*. The methods used for this purpose were those recommended by the specialized standards. The obtained results were compared with the literature data and indicated the importance of keeping a safe area in the neighborhood of those natural water sources which are selected as balneal treatment resorts.

Keywords (4-6): natural salted waters, microbial seasonal analysis

1. INTRODUCTION

The task of our studies was represented by the seasonal microbial load analysis from some Romanian natural salted water sources (springs and lakes) in order to check the requested conditions for their use as balneal treatment. Our previous researches have indicated that these water sources have a high salt concentration, which means that they can be used for balneal treatment or for different industrial and agricultural applications. There is at our company another research team that studies the saline environments and the microbes selected from saline soils and the halophilic and halotolerants' ecology (Caterina Tomulescu, 2021). According to the literature data, there are a lot of natural mineralized water resources all around the world. Due to this situation, there are many studies focused on the use of this kind of

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renewable sources (salted or thermal waters), looking for the water use, for salt obtaining starting from these salted waters or also for the valorization of some valuable microbial strains able to produce different useful bioproducts. However, these biotechnological pathways must be established according to many factors, such as the geographical origin of the water spring (or lake) and possibly according to the seasonal microbial development.

As an example, we can present a study realized in Poland (Agnieszka Kalwasińska et al., 2020) where the authors are presenting their obtained results concerning the microbial composition of different deep-subsurface hot brines in northwest Poland. Their conclusion was that „although a few studies revealed the existence of microbial life at the depths", they had found some bacterial communities involving Proteobacteria and Firmicutes Bacterial genera potentially involved in sulfur oxidation and nitrate reduction (Halothiobacillus and Methylobacterium).

Another article presenting the microbial load from the European natural water sources (Maren Emmerich et al., 2012) was focused on the anaerobic microbial iron metabolism in hypersaline sediments of Lake Kasin, from southern Russia, where they found some species dominating the iron(III)-reducing enrichment cultures (relatives of Halobaculum gomorrense, Desulfosporosinus lacus, and members of the Bacilli). According to their conclusion, combined with the presented geochemical and mineralogical data, their findings suggest the presence of an active microbial iron cycle at salt concentrations close to the solubility limit of NaCl.

Also from Europe, we can indicate a study (Camille Mazière et al., 2021) related to the bacterial and eukaryotic diversity discovered in the exploited and abandoned salterns at the Ré Island (France). In this case, their researches indicate that „the abandoned saltern was dominated by sulfate-reducing bacteria and Nematoda, while the exploited saltern was characterized by the presence of halophilic bacteria belonging to Marinobacter, Salinivibrio and Rhodohalobacter genera, and the larger abundance of Hypotrichia (ciliates)".

Another study related to the comparative microbial diversity (Peter T. Podar et al., 2020) was realized in Yellowstone National Park (North America) and Iceland, but in this case the analyzed areal was represented by the hot springs and their issue was to emphasize the different microbial strains found across the temperature gradient in three alkaline hot springs. I this case, they found a large diversity of mesophilic and mildly thermophilic heterotrophic as well as photosynthetic bacteria, including Alpha and Betaproteobacteria (Roseomonas, Rhodobacter, Tepidimonas), Bacteroidetes (Chitinophaga, Saprospira) and Cyanobacteria (Cyanobium, Leptolyngbya).

Another article presenting the microbial diversity of the salted waters from SUA (Swati Almeida-Dalmet et al., 2015) was realized on samples taken from the north arm of Great Salt Lake, Utah. Their results indicated that in the domain, Bacteria, the Salinibacter group dominated the populations in all samplings. However, in the case of Archaea, many of the temporal communities were distinct from each other, which may indicate that these species are affected by biotic factors, such as nutrients or viruses, that are independent of seasonal temperature dynamics.

A study realized in the Guerrero Negro solar saltern, Baja CA Sur, Mexico (Jesse G Dillon et al., 2013) had as main goal to discover the patterns of microbial diversity along the salinity gradient.

They hypothesized that diverse communities of heterotrophic bacteria and archaea would be found, but that bacterial diversity would decrease relative to archaea at the highest salinities and their results suggest that the communities of saltern bacteria and archaea vary even in ponds with similar salinity and further investigation into the ecology of diverse, uncultured halophile communities is warranted.

An article concerning the bacterial diversity from manmade salterns (Konstantinos T Konstantinidis et al., 2021) is suggesting that even within a single saltern site, only a small minority of these genes appear to be functionally important during environmental perturbations and that the studies need to be continued if peoples want to detect and track such populations and their ecologically important genes that should be broadly applicable.

From Bolivia, we can find a study (Götz Haferburg et al., 2017) concerning the microbial diversity of Salar de Uyuni, situated in the Southwest of the Bolivian Altiplano, which is considered as the largest salt flat on Earth. The samples taken from those brines of this hypersaline environment rich in lithium and boron revealed the occurrence of an exclusively archaeal community comprising 26 halobacterial genera including only recently identified genera like Halapricum, Halorubellus and Salinarchaeum.

A study carried out in Iran (Ali Naghoni et. Al., 2017) was focused on the microbial diversity in the hypersaline Lake Meyghan. The authors are saying that this lake, which is the largest and commercially most important salt lakes in Iran, in fact is composed by some various basins characterized by different salinities. They had studied three basins by isolation and culturing of microorganisms and by analysis of the metagenome and they found 57 strains of Bacteria and 48 strains of Archaea. Bacteria dominated the low salinity brine, with Alteromonadales (Gammaproteobacteria) as a particularly important taxon, whereas the high salinity brines were dominated by haloarchaea.

Another study presenting the situation of the salted natural waters from Asia is that one realized in China (Rui Han et al., 2017) concerning the microbial community structure and diversity within hypersaline Keke Salt Lake environments. According to the authors, Keke Salt Lake is located in the Qaidamu Basin of China, is a unique magnesium sulfate-subtype hypersaline lake that exhibits a halite domain ecosystem, but with its microbial diversity remained unstudied until their work. Their actions indicated finally a high diversity of operational taxonomic units, detected for Bacteria and Archaea (734 and 747, respectively), comprising 21 phyla, 43 classes, and 201 genera of Bacteria and 4 phyla, 4 classes, and 39 genera of Archaea. Saltsaturated samples were dominated by the bacterial genera Bacillus (51.52%-58.35% relative abundance), Lactococcus (9.52%-10.51%), and Oceanobacillus (8.82%9.88%) within the Firmicutes phylum (74.81%-80.99%). The dominant Archaea belonged to the Halobacteriaceae family, and in particular, the genera (with an abundance of >10% of communities)

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Halonotius, Halorubellus, Halapricum, Halorubrum, and Natronomonas. Additionally, they reported the presence of Nanohaloarchaeota and Woesearchaeota in Qinghai-Tibet Plateau lakes.

We found between the literature data also a published article (Srikanta Pal et al., 2020) concerning the microbial taxa dominate the microbiome of hypersaline Sambhar Lake salterns in India. The authors are saying that the inland athalassohaline solar salterns provide unique opportunity to study microbial successions along salinity gradients that resemble transition in natural saltern brines of India's largest inland hypersaline Sambhar Lake. They noticed that microbial assemblages positively correlated to saltern pH, total salinity, and ionic composition and they found specialized extreme halophilic bacterial (Halanaerobiales, Rhodothermaceae) and archaeal (Halobacteriales, Haloferacales) members with recognized salt-in strategy for osmoadaptation. Their conclusion was that microbial compositions in hypersaline lakes are complex and need revisit particularly for their archaeal diversity to understand their hitherto unknown ecological function in extreme environments.

One of the most exhaustive material was presented in 2018 (Bonnie K. Baxter, 2018) and it is related to the historical perspective of the studies realized about the microbial diversity from the Great Salt Lake, from Utah, SUA, also leading to the nickname "America's Dead Sea.". This study is presenting the natural history of Great Salt Lake, the modern Great Salt Lake ecosystem, humans and Great Salt Lake, insights and impacts on microbiology (the early peoples of Utah and their interactions with Great Salt Lake), the recent American Indian inhabitants along the lakeshore and the microbiology of Great Salt Lake (historical studies and also recent microbiology studies).

Our issue of this present work was to verify if there is a connection between the microbial load of the natural salted waters related to the season, which means according to the human or animal frequency of presence, in order to establish if some special security measures are requested in the case of using these resources as a treatment base.

2. DATA AND METHODS

In order to check if the seasonal environmental factors can change the microbial load of some natural salted springs, a set of microbiological analyses were carried out at a laboratory level. There is at our company another research team that studies the saline environments and the microbes selected from saline soils and the halophilic and halotolerants' ecology (Caterina Tomulescu, 2021). Our study was focused on the next microbial groups: mesophilic bacteria growing at $22^{o}C$, mesophilic bacteria growing at $37^{o}C$, total number of coliform bacteria, Pseudomonas aeruginosa, Staphylococcus aureus and Salmonella sp. Our selection was based on the data literature concerning the main microbial factors that can lead to harmful effects on salted waters application as balneal treatment, so that species whose presence must be avoided.

The methods used for this purpose were those recommended by the specialized standards, based mostly on the membrane filtration method and cultivation on

selected culture medium. Membrane filtration method is the physical process required to separate substances using membranes. A filter membrane of the appropriate pore size is placed on a filter holder and the sample to be analyzed is filtered (in the case of the article, the water samples). During the process, the microorganisms in the test sample are retained on the surface of the filter by the action of selecting the filter membrane. After that, the filter membrane is placed on a selective culture medium and incubated at the temperatures mentioned. Bacterial growth means the number of CFU (colony forming units). A colony-forming unit or CFU is a measure of the number of viable bacteria or fungi. Compared to direct microscopy in which all cells are counted, living or dead, the colony-forming unit counts the viable cells. It is agreed that the results be given as CFU/ml for liquids and CFU/g for solids.

The colonies were counted on the plate using the following formula:

$X = N \times D$ / volume, where:

X = no. colonies (CFU) /

mL; N = no. colonies/plate;

D = inverse of the dilution.

3. RESULTS AND DISCUSSIONS

Our obtained results are presented by Tables and Figures, separately for each microbial group mentioned before. The samples mentioned as "Spring" were taken on April, whereas those ones named as "Autumn" were collected on October.

Both categories were brought from a hilly area with salt presence and they were numbered, as follows:

Sample 1 = Mineralized lake- 0, 00 m
Sample 2 = Mineralized lake- 0, 50 m
Sample 3 = Spilled water from lake
Sample 4 = Mineralized spring no.1
Sample 5 = Mineralized spring no.2
Sample 6 = Mineralized spring no.14
Sample 7 = Mineralized spring no.15

This numbering is presented also as a Legend at the end of our list with Tables and Figures, while it is similar for all realized tables.

Table 1. *Results of the microbiological analysis concerning the mesophilic bacteria growing at 22° C*

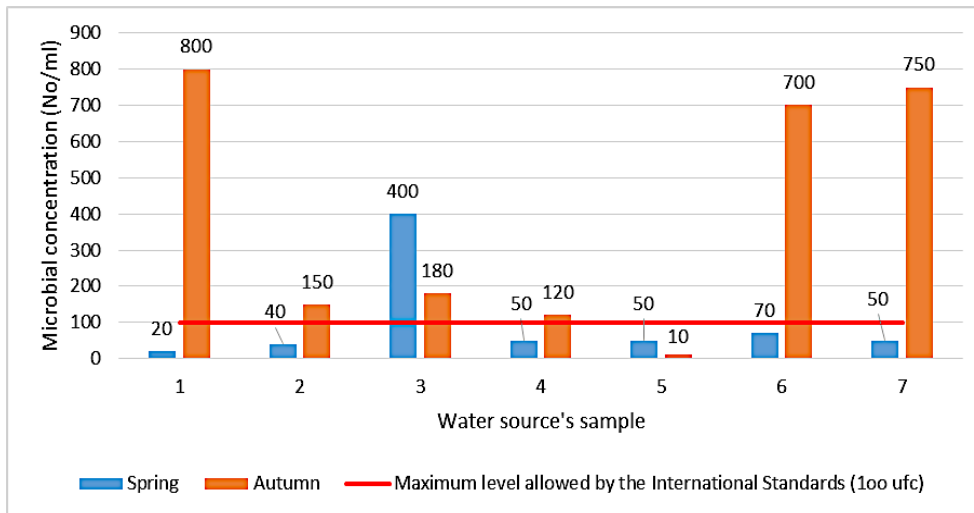| Sample season	Microbial concentration (No/ml)						
	Water source's sample						
	1	2	3	4	5	6	7
Spring	2×10^1	4×10^1	4×10^2	5×10^1	5×10^1	7×10^1	5×10^1
Autumn	8×10^2	$1,5 \times 10^2$	$1,8 \times 10^2$	$1,2 \times 10^2$	1×10^1	7×10^2	$7,5 \times 10^2$

Fig. 1. *Results of the microbiological analysis concerning the mesophilic bacteria growing at 22º C*

Our results concerning the level of mesophilic bacteria rising at 22º C indicated only 2 cases when the autumn samples were richer than those ones taken during the spring season (the samples 3 and 5), so that no matter if the biological test was collected from a natural spring or from a salted lake.
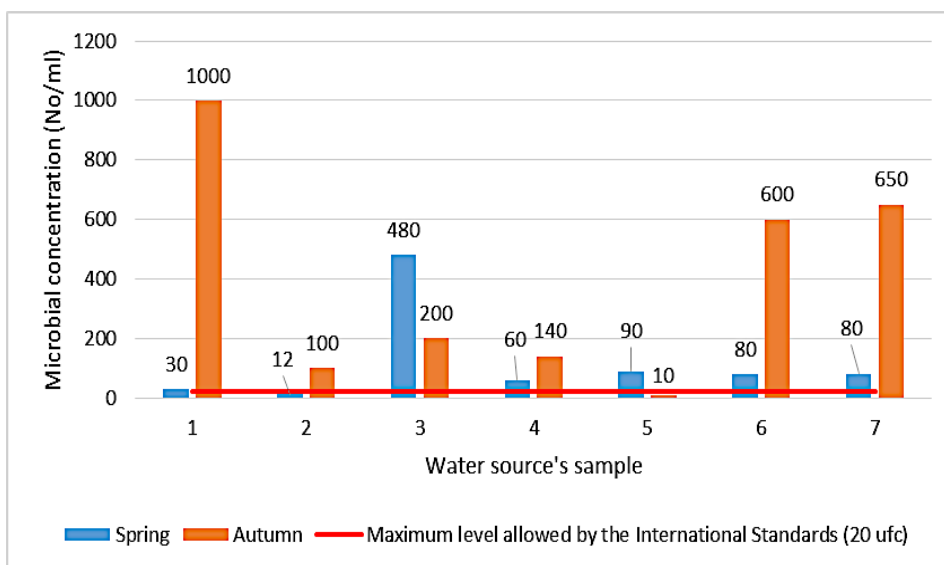

Fig. 2. *Results of the microbiological analysis concerning the mesophilic bacteria growing at 37º C*

Table 2. *Results of the microbiological analysis concerning the mesophilic bacteria growing at 37⁰ C*

Sample season	Microbial concentration (No/ml)						
	Water source's sample						
	1	2	3	4	5	6	7
Spring	$3x10^1$	$1,2x10^1$	$4,8x10^2$	$6x10^1$	$9x10^1$	$8x10^1$	$8x10^1$
Autumn	$10x10^2$	$1x10^2$	$2x10^2$	$1,4x10^2$	$1x10^1$	$6x10^2$	$6,5x10^2$

Apart from the sample number 5, all other tests indicate a higher load with mesophilic bacteria rising at 37° C during the autumn season, as we can think, because of the higher temperatures recorded over the summer days.

Table 3. *Results of the microbiological analysis concerning the total number of coliform bacteria*

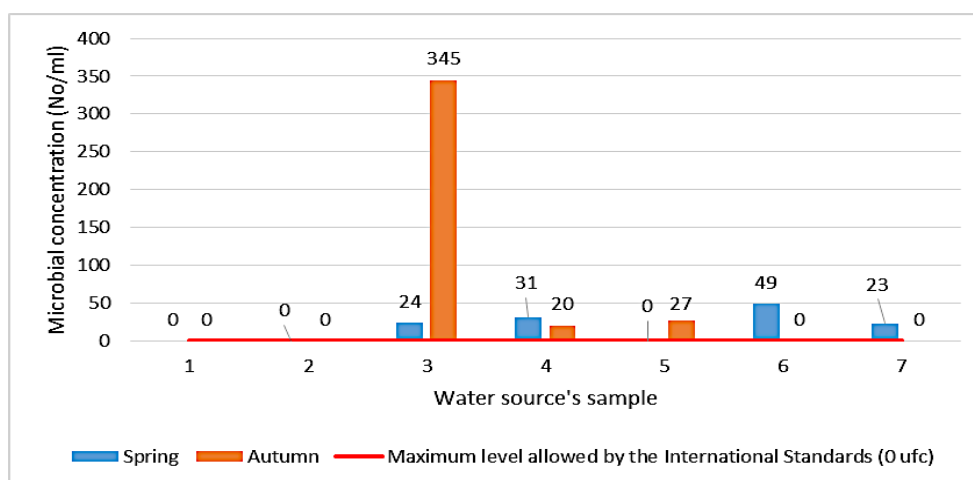| Sample season	Microbial concentration (No/ 100 ml)						
	Water source's sample						
	1	2	3	4	5	6	7
Spring	Abs.	Abs.	$24x10^1$	$3,1x10^1$	Abs.	$4,9x10^1$	$2,3x10^1$
Autumn	Abs.	Abs.	$3,45x10^2$	$2x10^1$	$2,7x10^1$	Abs.	Abs.

Fig. 3. *Results of the microbiological analysis concerning the total number of coliform bacteria.*

The most important aspect that can be noted from the Table 3 and the Figure 3 is represented by the very high level of coliform bacteria found after the summer season at

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the sample number 3. This indicator must be taken seriously, while the coliform bacteria (especially Escherichia coli) are known as very dangerous for the public health.

Table 4. *Results of the microbiological analysis concerning Pseudomonas aeruginosa*

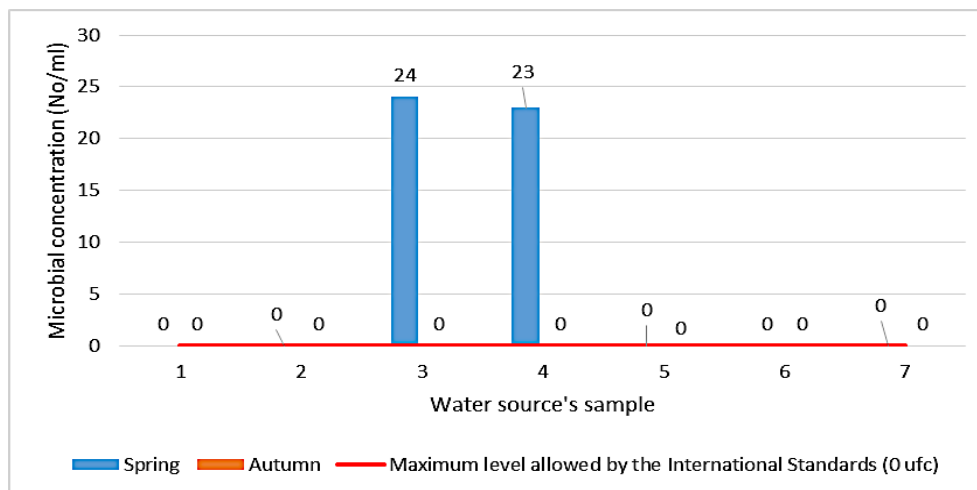| Sample season	Microbial concentration (No/ 100 ml)						
	Water source's sample						
	1	2	3	4	5	6	7
Spring	Abs.	Abs.	24×10^1	$2,3 \times 10^1$	Abs.	Abs.	Abs.
Autumn	Abs.	Abs.	Abs.	Abs.	Abs.	Abs.	Abs.

Fig. 4. *Results of the microbiological analysis concerning Pseudomonas aeruginosa.*

The analysis carried out in order to emphasize the level of Pseudomonas aeruginosa are presenting only 2 samples with this kind of microorganisms (No. 3 and 4). It seems that a salted water is not a medium quite friendly for this species.

Table 5. *Results of the microbiological analysis concerning Staphylococcus aureus (the maximum level allowed by the international standards for Staphylococcus aureus is 0 ufc)*

Sample season	Microbial concentration (No/ 100 ml)						
	Water source's sample						
	1	2	3	4	5	6	7
Spring	Abs.	Abs.	Abs.	Abs.	Abs.	Abs.	Abs.
Autumn	Abs.	Abs.	Abs.	Abs.	Abs.	Abs.	Abs.

The good news is that our analysis concerning the level of Staphylococcus aureus and Salmonella sp. indicate the lack of any trace, while it is known that Salmonella causes an

illness called gastroenteritis, which we may be familiar also as "food poisoning" and, in the same time, we know that sometimes antibiotic resistance can occur in its case.

Table 6. *Results of the microbiological analysis concerning Salmonella sp. (the maximum level allowed by the international standards for Salmonella sp. is 0 ufc)*

Sample season	Microbial concentration (No/ 100 ml)						
	Water source's sample						
	1	2	3	4	5	6	7
Spring	Abs.	Abs.	Abs.	Abs.	Abs.	Abs.	Abs.
Autumn	Abs.	Abs.	Abs.	Abs.	Abs.	Abs.	Abs.

Legend:
Sample 1 = Mineralized lake- 0, 00 m
Sample 2 = Mineralized lake- 0, 50 m
Sample 3 = Spilled water from lake
Sample 4 = Mineralized spring no. 1
Sample 5 = Mineralized spring no. 2
Sample 6 = Mineralized spring no. 14
Sample 7 = Mineralized spring no. 15

5. CONCLUSIONS

1) In order to check if the seasonal environmental factors can change the microbial load of some natural salted springs or lakes, a set of microbiological analyses were carried out at a laboratory level.

2) Our issue was to verify if there is a connection between the microbial load of the natural salted waters related to the season, which means according to the human or animal frequency of presence and to the level of outdoor temperatures, in order to establish if some special security measures are requested in the case of using these resources as a treatment base.

3) Our selection concerning the analyzed microbial groups was based on data literature concerning the main microbial factors that can lead to harmful effects of salted waters applications as balneal treatment.

4) In the majority of cases, the microbial load was higher during autumn, which means that every natural spring or basin arranged for its use as a treatment base needs to receive a surrounding security area.

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