

Assessment of the Physiological Groups of Bacteria from Salt Lakes and Euxinic Sediments Involved in Sulfur Cycle, Therapeutical Mud Formation, and Regeneration

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Abstract: The biogeochemical cycle of sulfur is important in the diagenesis of organic matter sedimented in saline and euxinic lakes leading to sapropel formation. The study aimed to determine the numerical densities of physiological groups of sulfur bacteria in water and sediment samples from five Romanian saline and euxinic lakes with therapeutical properties in order to assess their peloidogen regeneration potential. Bacterial communities that metabolize sulfur compounds were monitored in lakes with different salinity values: Techirghiol, Amara, Sovata-Black Lake, Ursu Lake, and Braila Salt Lake by multiple tube method. Statistical results revealed the interdependence between sulfoxidizing bacteria/SO₄²⁻ in sediments, while the reductive microorganisms fraction/metabolic products (H₂S/C_{org}/N_{org}) separately evolved in the economy of peloidogenic ecosystems. In the lakes, Techirghiol, Amara, and Braila Salt Lake, the dominant role in the specific microbiota belonged to sulfate/sulfur-reducing bacteria, and the formed peloid was sulfurous-sapropelic in Techirghiol Lake or sulfurous-mineral in Amara Lake and Braila Salt Lake. In the hypersaline, meromictic, and heliothermal lakes of Sovata, the sulfur metabolism was predominantly oxidative, made by sulfoxidizing bacteria in higher numerical densities, with the accumulation of SO₄²⁻ ions in the strongly hydrated aqueous phase of mineral sediments.

Keywords: sulfur biogeochemical cycle; sulfur bacteria; therapeutical mud; saline and euxinic lakes; aquatic sediments.

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1. Introduction

Sulfur (S) is one of the main biogenic elements in nature, including freshwaters and lakes, that participates in the circuit of matter and energy [1]. Its biogeochemical cycle transforms organic matter and other nutrients, heavy metals from water, and sediments [2]. Sulfur is required for the growth of all organisms and is present in a wide variety of metabolites having distinctive biological functions [3]. Microorganisms catalyze various reactions, mainly

to generate the energy necessary for growth, so they are involved in all biogeochemical cycles of biogenic elements [4].

Sulfur is one of the critical elements in the living matter; aquatic environments are very different from each other in terms of S content; while in oceans, the concentration of sulfates is constantly high, freshwaters are characterized by daily and seasonal variations and by a wide range of sulfur concentration [5]. Sulfate-reducing microorganisms are a diverse group of anaerobic microorganisms that includes over 220 species from 60 different genera [6]. Results suggest that sulfate-reducing microorganism communities inhabiting the subsurface water assemble by the selective survival of members of the surface community [7]. In various aquatic and sedimentary systems, a significant part of sulfide produced during microbial sulfate reduction can be re-oxidized either microbially or abiotically, rather than buried as pyrite [8].

Anthropo-saline lakes in Romania are used mostly for therapeutic purposes because of their high salinity and the sapropelic muds that they contain. In order to preserve their therapeutic properties, it is necessary to maintain the standards that may be different from global recommendations for freshwater and saline (but non-therapeutic) lakes [9]. Hypersaline lakes are considered extreme biotopes for microbial life; previous studies have shown that the taxonomic diversity of microbial populations in hypersaline environments is low. In general, microbial diversity decreases with increased salinity [10].

Sediments are highly heterogeneous systems with different phases (solid, liquid, and gaseous), biotic components (mostly microorganisms), and abiotic components (minerals, humus, organo-mineral aggregates) that are involved in physical, chemical, and biological processes, with all biochemical transformations being dependent on the presence of enzymes [11]. Complete mineralization in anoxic sediments is not mediated by one organism but within associations of different physiological microorganisms groups. Bacterial populations are high in near-surface sediments, reflecting a high mineralization rate and decreasing exponentially with depth [12]. Sediments represent a special ecological zone characterized by low redox potential and a microstratification of physicochemical factors with nutritive substrates that diffuse from one layer to another, allowing specific niches and favoring the growth of different physiological groups heterotrophic bacteria, belonging to microaerophilic and strictly anaerobic species [13]. Sulfur bacteria are prokaryotes that produce their energy by reducing or oxidating sulfur compounds; the availability of S compounds with an adequate redox status is the consequence of the needs for each group of sulfur bacteria [14].

A new definition of peloid was proposed by Gomez et al.: "Peloid is a maturated mud or muddy dispersion with healing and/or cosmetic properties, composed of a complex mixture of fine-grained natural materials of geologic and/or biologic origin, mineral water or seawater and commonly organic compounds from biological metabolic activity,, [15].

Sapropel represents sludge sediment in lakes, with a fine structure that contains incompletely divided organic matter and microscopic aquatic life from residues with traces of sand, clay, calcium carbonate, and other rock impurities. Sapropel's complex chemical and biological structure explains its multifunctional effect on the body. The bioactivity of the sapropel is determined by its humic acids, fulvic acids, heratomelic acids, various vitamins, and microorganisms that release antibiotics [16]. But the peloids can also be prepared by mixing mineral-medicinal water with kaolin and bentonite (9:1, w:w); a modification of the ionic concentration of the interstitial liquid was observed concerning the initial mineral-medicinal water, namely an increase in the concentration of Na^+ and K^+ , and a decrease in Ca^{2+} and Mg^{2+} , due to the exchange between cations of the solid phase and the ions of the mineral-

medicinal water [17]. However, it is difficult to assume a variability range of the physical and chemical properties of the peloids with therapeutic properties because literature data does not express enough publications concerning the determination of these properties [18].

The term sapropel or sapropelic mud has been used loosely to describe any discrete black or dark-colored sedimentary layers (>1cm) that contain greater than 2% organic carbon. Sapropels largely contain amorphous (sapropelic) organic matter derived from planktonic organisms (such as planktonic or benthic algae in lakes). Sapropel contrasts with the term gyttja, which is also sediment high in organic carbon content but which is formed under inferred oxygenated conditions in the water column down to the sediment-water interface [19]. The main microorganisms detected in peloids used for therapeutical purposes were microalgae, diatoms, and thermophilic blue-green algae (*Cyanobacteria*) that are part of the so-called “biogloea”, which develops in some hot springs peloids and with a certain composition of mineral-medicinal water and solid phase [20]. Biogloea seems to have an important role in the maturation of thermal mud with a high content of organic matter [21].

Mud which contains organic and mineral ingredients, is used to treat several degenerative diseases; it is assumed that the beneficial effect of the therapeutic mud is not only due to its local thermal action but also because of its chemical composition [22]. The Turkish peloids that are used in pelotherapy contain various amounts of clay minerals, non-clay minerals, organic matter (mainly humic acid), cations (sodium, magnesium, and calcium), anions (chloride, sulfate, and bicarbonate), and insoluble compounds (mainly meta-silicic acid) [23]. Dead Sea mud has a pronounced antimicrobial action, probably due to the combination of high concentrations of salt and sulfide, plus a low pH [24]. Also, the natural clays have a bacteriostatic and bactericidal effect; such clays can be used to prepare sanitary, safe peloids addressed to treat rheumatic disabilities or infectious skin disorders [25].

One of the basic elements of the medicine practiced in health resorts is the efficiency in the prevention and treatment of diseases and improving the functioning of the whole organism (rehabilitation) through the medical use of natural mineral waters, gases, and peloids [26]. Many of the treatments in spas are concentrated on mud therapy; clays are included in the formation of thermal mud as vehicles of the medicinal mineral water. To be suitable for therapeutic use, some mineralogical, rheological, and thermal properties should be respected in order to be topically applied [27]. Balneotherapy that uses peloids as therapeutic factors influences the innate and inflammatory responses, thus representing an immuno-physiological mechanism responsible for the curative benefits of this medical procedure [28]. Pelotherapy is a very important operation. Thus, the concentrations of metal contaminants (elements such as Sr, Ba, Mn, Fe, Sb, Zn, Cu, Pb, Ti, Ni, Cr) must be smaller than the international recommended values [29]. Peloids in medical spa centers are used as thermotherapeutic agents for treating musculoskeletal diseases, especially osteoarthritis of the knee [30], and as a conservative treatment for orthopedic pathologies [31].

This study aimed to determine the variation of the chemical composition of the peloids depending on the populational dynamics of physiological groups of sulfur bacteria from five Romanian saline and euxinic lakes of therapeutical importance to assess their peloidogen regeneration potential.

2. Materials and Methods

Techirghiol Lake (44° 03' N, 28° 36' E) is located in the south-eastern part of Romania on the Black Sea coast, with an area of approximately 11.6 km² and an average water depth of

3.6 m; the lake is mainly known for its sapropelic mud and hypersaline water, which has been exploited for therapeutic purposes since the mid-nineteenth century [32,33].

Amara Lake (44° 36' N, 27° 20' E; 1.3 km² area; the average depth of 2 m approximately) is a brackish lake located in the eastern part of the Romanian Plain. It is the largest chloride-sulfated plain lake in Romania and has a protected status as a natural bird reservation, and its water and mud have long been utilized for bathing and medicinal purposes [33].

Ursu Lake (46° 36' N, 25° 05' E; 4.1 km² area; the average depth of 12m approximately), and Black Lake (46°59' N, 25°08' E; 3.3 km², maximum depth of 9 m) from Sovata are salt lakes of the Transylvanian Basin (Central Romania) formed by intense salt dissolution of the underlying rock, with a permanently stratified halocline (helio-thermal) and sediments used for therapeutic purposes [33,34].

Braila Salt Lake (45°12057.6" N, 27°54038.4" E; 45°12058.8" N, 27°54037.7" E; 45°12058.4" N, 27°54040.3" E) [35], located in the eastern part of Romania, 16 m above sea level, is an old course of Danube; water has high salinity, and the bottom of the lake is covered by a layer of mineralized sludge with important therapeutic qualities [36].

Water and sediment samples were taken in 2019-2020 from three hypersaline lakes (Techirghiol (with TK1, TK2, TK3 sampling points), Braila Salt Lake (with LSB1-03, LSB1-06 sampling points), Ursu (with SLU-02, SLU-09 sampling points) lakes), one meso-saline lake (Black Lake-Sovata (with SLN-02, SLN-10 sampling points)) and one brackish lake (Amara Lake (with AM-05, AM-10 sampling points)), all recognized as pelagenous lakes whose sediments and water are natural factors with therapeutic properties, that are used in spa-based procedures (hot or cold baths, wraps, poultices, compresses, massage, vaginal swabs, etc.)

The studied samples of water and sediment were taken from the ecosystems of origin, from those recognized as pelogenic areas of the investigated therapeutic lakes, i.e., areas where the exploitation of the sediment is already initiated and continuous and represents a source of therapeutic substances for the nearby treatment bases. The water samples were taken from a depth of 0.5m according to the methodology described in SR EN ISO 19458:2007 in sterile polyethylene containers while avoiding external contamination, and the peloid samples were extracted from the surface layers of the sediment, from a depth of 0.5-20cm with Petersen grab instrument being used for the transport of sterile containers of borosilicate glass.

The activity of heterotrophic eco-physiological bacteria in the biogeochemical cycle of sulfur was evaluated by the determination of the final compounds of their metabolic paths in lake water and the aqueous suspension of peloid; thus, the SO₄²⁻ ions were quantified according to STAS 11277/11-80 (water), SR ISO 11048:1999 (sediment), H₂S according to STAS 11277/13-81 (water) and STAS 7184/7-87, SR EN 16192:2012 (sediment). To determine the chemical composition of the lake sediments and to assess their structural profile (determination of the ratio of organic/inorganic substances, humidity), their overall composition was monitored (according to SR EN 15169:2007, STAS 12586-87), Fe²⁺ ions (which form Fe sulfide (FeS), and represent the main component of mud that gives its therapeutical character, by the atomic absorption spectrometric method according to SR 13315:1996) and the organic C/N ratio existing in the mud (according to SR ISO 14235:2000).

The processing of microbiological sediment samples consisted of their homogenization and the realization of a suspension with sterile distilled water in the ratio of 1:10. Five series of decimal dilutions (up to 1/10⁵) with five replicas were prepared in order to obtain a microbial

density convenient for identification, knowing that the number of microorganisms in the sludge is very high even in the absence of pollution. The seeding method used was the multiple tube method (MPN - *most probable number*), and incubation was carried out at $22\pm 2^{\circ}\text{C}$ or $28\pm 2^{\circ}\text{C}$ for 14 days-28 days, depending on the concentration of sulfur products in the samples, in aerobic or anaerobic mode. The number of tubes in which the positive reaction was identified was calculated using statistical tables, according to SR EN ISO 8199:2019, the most probable number of microorganisms per 100 grams of sludge ($\text{MPN}\cdot 100\text{g}^{-1}$). In aquatic sediments, the eco-physiological groups of microorganisms involved in the biogeochemical circuit of the S were monitored: sulfate-reducing bacteria, sulfur-reducing, and sulfur-oxidant bacteria; thus, the following culture sites were used to identify and evaluate their populational densities in the sludge samples: Starkey's Sulphate Reducing Agar Base for sulfate-reducing bacteria, Oppenheimer's environment for sulfur-reducing bacteria and the modified Postgate for sulfur-oxidant bacteria [37]. The obtained results were processed in GraphPad Prism 9.0.2.161 and analyzed statistically by multiple linear regression and ANOVA test.

3. Results and Discussion

In the five analyzed pelagenous lakes, the amount of SO_4^{2-} present in water oscillated between $0.05\pm 0.02\text{ g}\cdot\text{l}^{-1}$ in Sovata-Black Lake (SLN) and $78.01\pm 5.82\text{ g}\cdot\text{l}^{-1}$ in Braila Salt Lake (LSB1), in sediment SO_4^{2-} ions were present in concentrations ranging from $0.38\pm 0.07\text{ g}\%$ in Techirghiol Lake (TK) to $5.47\pm 0.03\text{ g}\%$ in LSB1. It is quite difficult to monitor the *in situ* reactions involved in the metabolization of sulfur and its compounds, between oxidation of SH- and reduction of SO_4^{2-} via microbial loop [38], but it is known that in the warm season, the reduction of sulfates is stimulated by the temperature and sedimentation of organic matter from the plankton [1]; therefore, in the analyzed therapeutic lakes it was observed a variation in the concentrations of SO_4^{2-} during the period of S transfer from the plant debris between water and sediment, the dynamics being specific to each ecosystem, depending on the amount of organic matter, its distribution and by consequence of the densities of bacterial populations; thus, in TK, regardless of the season in which the sampling was carried out (January, June or October), the quantity of SO_4^{2-} /water was higher than the quantity SO_4^{2-} /sediment.

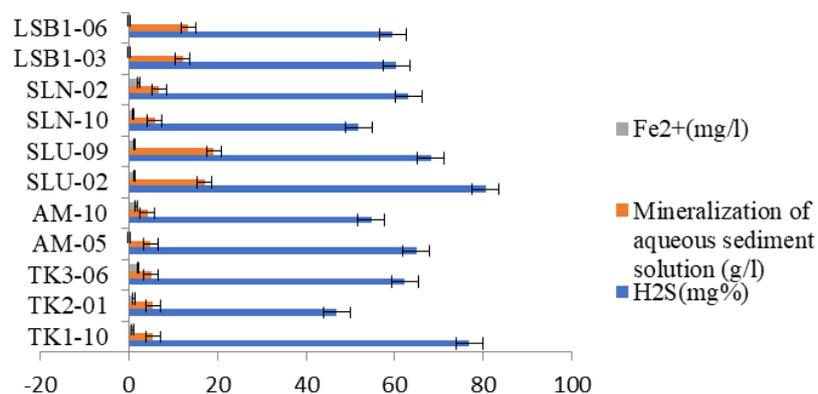


Figure 1. The availability of H₂S and Fe²⁺ in therapeutic sediment from five saline lakes (± standard deviation of three replicates)

A similarity was observed in lakes Amara (AM) and LSB1, which had maximum concentrations of SO_4^{2-} /water in the months 05, 10 (AM), or 03 and 06 (LSB1). In contrast, in

the helio-thermal lakes of Sovata, SO_4^{2-} ions were present in higher concentrations only in sediments of the samples collected in the months 09 – Sovata-Ursu Lake (SLU) and 10 (SLN).

Fe^{2+} ion is an important chemical element in the budget of the peloidogenic ecosystems, and it was identified in small quantities both in water (between $0.13 \pm 0.06 \text{ mg} \cdot \text{l}^{-1}$ and $2.89 \pm 0.82 \text{ mg} \cdot \text{l}^{-1}$) and in sediments (between $0.13 \pm 0.06 \text{ mg} \cdot \text{l}^{-1}$ and $2.36 \pm 0.45 \text{ mg} \cdot \text{l}^{-1}$), sufficient to form iron sulfide (FeS), the main component with pharmacological action of the peloid. Its concentration values varied from one ecosystem to another, depending on the time of sampling (with maximum values in the spring-autumn months for TK, AM, SLU, SLN lakes), water mineralization (LSB1–hypersaline lake with $269.07 \text{ g} \cdot \text{l}^{-1}$ was the ecosystem with the highest amount of Fe^{2+}) or aqueous sediment suspension (SLN with $6.79 \text{ g} \cdot \text{l}^{-1}$ aqueous solution/sludge mineralization included a maximum of $2.36 \text{ mg} \cdot \text{l}^{-1} \text{ Fe}^{2+}$)(Figure 1).

H_2S was not detected in water, but only in the sediments of the analyzed lakes (figure 1); the presence of this chemical compound is important and necessary for the formation of FeS; it must be mentioned that the sampling of water was made from the epilimnion (10-15 cm deep), the presence of H_2S being rather specific to deep layers of water, in areas with maximum depth.

The decomposition of organic matter in ecosystems depends on the existing quantities of SO_4^{2-} [1,39,40] but also on the presence of sulfate-reducing bacteria (BSUR), which reduce sulfates (SO_4^{2-}) to sulfide compounds (HS^-) [38,41], releasing H_2S . Quantities of H_2S determined in the analyzed sediment ranged between $51.89 \pm 2.89 \text{ mg}\%$ (SLN-10) and $80.5 \pm 3.02 \text{ mg}\%$ (SLU-09)(Figure 2). It has to be noticed that in this study, the concentrations of H_2S were determined in its free state, without taking into account its presence in sediments, in the bound state, as iron hydrosulfide.

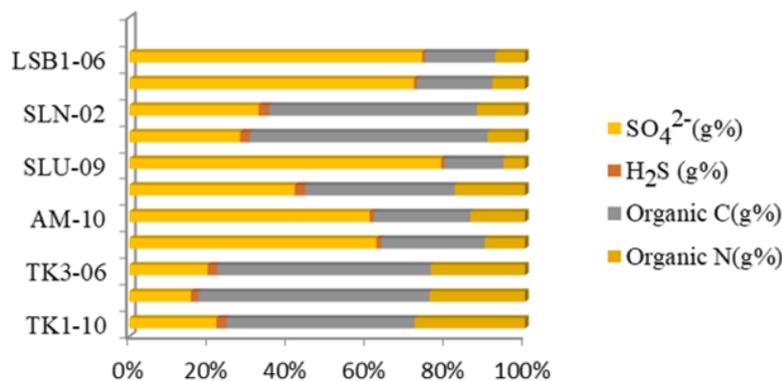


Figure 2. Inorganic (compounds of S) and organic (C and N_2 compounds) chemical profiles of therapeutic sediments from five saline lakes (percentage values)

BSUR, which are important microorganisms in defining the chemical typology of the newly formed sapropel by their metabolic products, were present in maximum numerical densities of $1.4 \cdot 10^7 \text{ MPN}/100\text{g}$ in AM-10 and minimum densities ($0.6 \cdot 10^3 \text{ MPN} \cdot 100\text{g}^{-1}$) in SLN-10. Because the temperature influences the microbiological growth and catabolic rates (between 15 and 35°C , the growth rate and cell-specific sulfate reduction rate of the sulfate-reducing bacterium increased with temperature [42], the lowest populations of BSUR being identified in January (TK2-01), February (SLU-02, SLN-02), March (LSB1-03) and May (AM-05), and the activity of these bacteria was more intense in the summer-autumn season in lakes LSB1 (June), SLU (September) and TK, AM, SLN (October) (Figure 3).

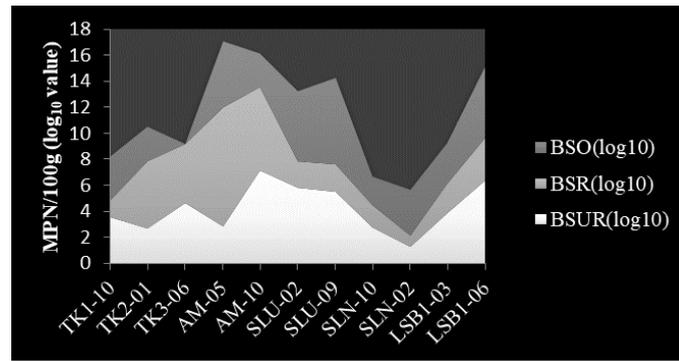


Figure 3. The seasonal presence of sulfur cycle bacteria (spring/autumn) in therapeutic sediments from six therapeutic lakes.

The statistical evaluation of the results did not indicate interdependence between the amount of H₂S ($r(21)=0.3156$; $p=0.1424$) present in sediments and the numerical values of BSUR from the microbiota of the analyzed ecosystems, but a direct proportional evolution of the two components defining the process of peloidogenesis can be assessed (Figure 4).

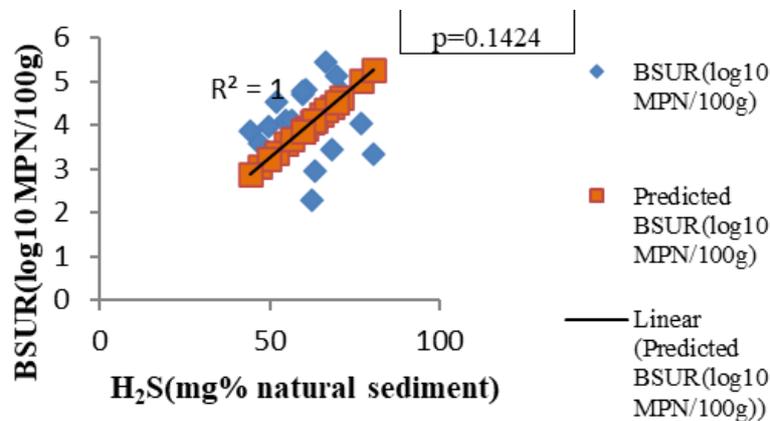


Figure 4. The relationship between H₂S value (mg % native sediment) and the abundance of BSUR (MPN·100g⁻¹).

Sulfur-reducing bacteria (BSR) of the type *Pseudomonas spp.*, *E.coli* or *Clostridium spp.*, which generally mineralize organic S from lake ecosystems by reducing it from amino acids with S to H₂S, have been identified in minimum numerical densities in SLN (0.5·10² MPN·100g⁻¹-0.7·10² MPN·100g⁻¹) regardless of the season in which the samples were taken; instead, in AM this type of microorganisms predominated in the sediment microbiota, being present in densities of 1.4·10⁹MPN·100g⁻¹ (May) and 2.6·10⁶MPN·100g⁻¹ (October).

The quantities of H₂S released by BSR functionality, usually lower than those resulting from BSUR activity, were not directly correlated with the population level of these bacteria according to the statistical results ($r(21)=0.0257$; $p=0.9074$), but an inverse proportional behavior can still be assessed for the two components of the analyzed peloidogen ecosystems (Figure 5).

The organic C and N₂ quantities that were determined in sediments varied independently from the values of the numerical abundances of BSR according to the statistical results obtained ($r(21)=0.1143$; $r(9)=0.1911$; $p=0.6036$; $p=0.5734$) (Figure 6, Figure 7).

Sulfoxidizing bacteria (BSO) were present in numerical densities that had minimum values in SLN-10 (1.4·10² MPN·100g⁻¹) and maximum values in SLU-09 (4.6·10⁶ MPN·100g⁻¹); through the larger numerical populations in the summer season, they permanently supplied

elementary sulfur to the peloidogenesis process from the hypersaline lakes TK (06) and LSB1 (06) and the brackish lake AM (05), except for the heliothermal, hypersaline lakes of Sovata SLU and SLN, for which higher numerical densities were recorded in spring/autumn season (SLU-09 and SLN-02).

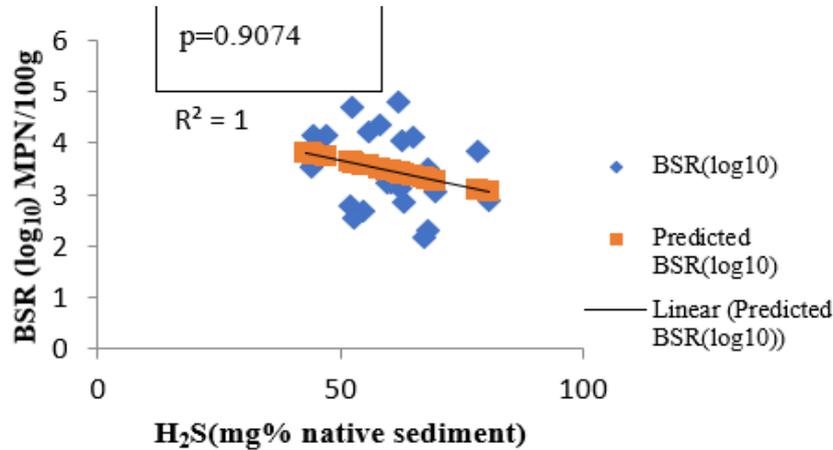


Figure 5. The relationship between H₂S value (mg% native sediment) and the abundance of BSR (MPN·100g⁻¹).

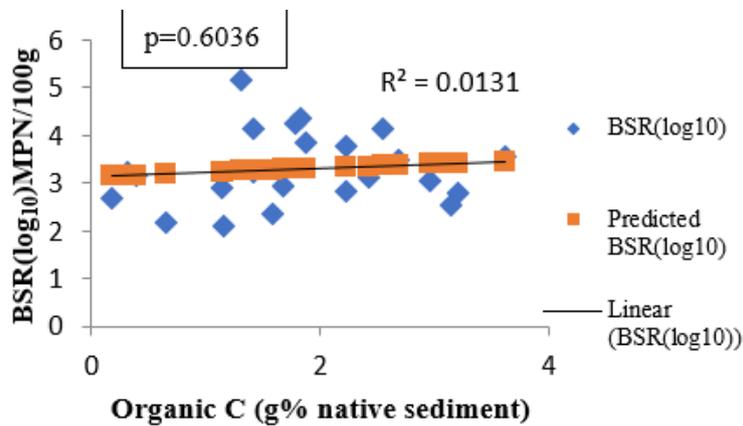


Figure 6. The relationship between Organic C value (g% native sediment) and the abundance of BSR (MPN·100g⁻¹).

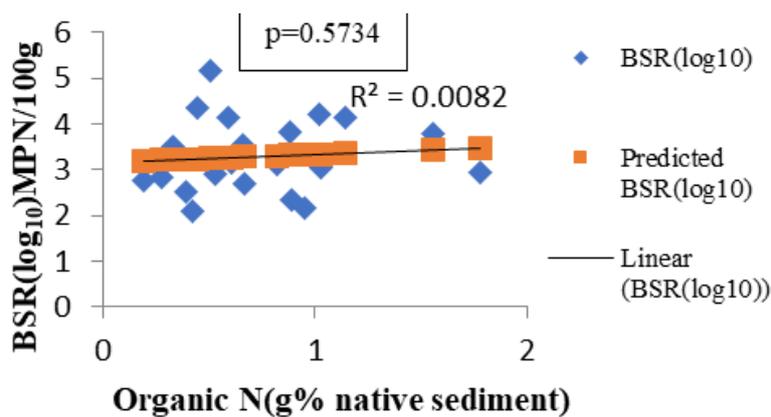


Figure 7. The relationship between Organic N value (g% native sediment) and the abundance of BSR (MPN·100g⁻¹).

The concentrations of SO₄²⁻ ions in the sediments were correlated with the numerical abundance of BSO according to the statistical results ($r(21)=0.518$, $p=0.011$), which indicate a directly proportional evolution for the two factors involved in the peloidogenetic process (Figure 8).

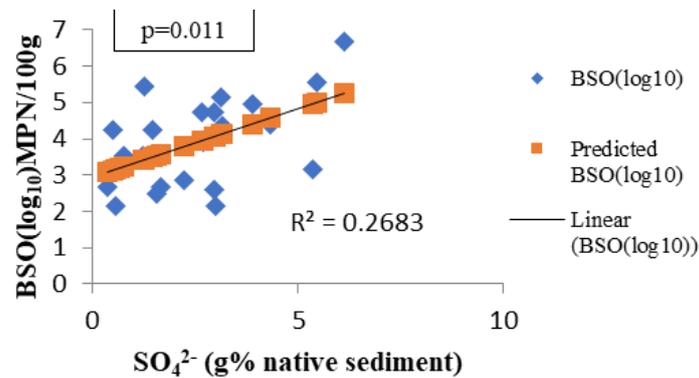


Figure 8. The relationship between SO₄²⁻ value (g% native sediment) and the abundance of oxidizing sulfur bacteria (MPN·100g⁻¹)

4. Conclusions

The presence of sulfur compounds in the composition of the tested therapeutic sediments, coming from five euxinic lakes with salinities that varied over a wide range, was influenced by the biogenic particularities of the ecosystems, but especially by the structure of the active microbiota in the sediment in direct correlation with its mineral and organic content.

In Amara Lake, Techirghiol Lake, and Braila Salt Lake, although the degree of water mineralization ranged between 16.28 g·l⁻¹ and 247.87 g·l⁻¹, all the sediments analyzed had as a common denominator the sulfurous character, containing H₂S > 1 mg%, the accumulation of H₂S due to the reducing-type bacterial processes mediated by sulfate-reducing bacteria and sulfur-reducing bacteria. These physiological groups are present in higher numerical densities in the sediment microbiota (with the highest values in Amara lake).

In the heliothermal, therapeutic lakes of Sovata, Ursu Lake, and Black Lake, sulfur products' metabolism was oxidative and carried out by those sulfoxidizing bacteria that dominated the sediment microbiota, inducing the accumulation of SO₄²⁻ ions in the aqueous solution of mineral-type, strongly hydrated muds.

This study demonstrated that the five Romanian saline and euxinic lakes of great therapeutical importance have a high numerical density of bacterial physiological groups, especially those involved in the biogeochemical sulfur cycle and FeS formation, which can be considered good biomarkers for the peloidogenesis process and mud regeneration potential.

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Conflicts of Interest

The authors declare no conflict of interest.

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