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11. Biochemical parameters of salt lakes sapropelic sludge from Buzau County protected area, with different degrees of microbiological attrition

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Abstract: Industrial and household pollution is linked to the alteration of our ecosystems. The presence of large amounts of polluting agents affects oxidation-reduction and acid-base balances systems, causing cascade effects over the microbiota and aquatic environment. In this study we evaluated the biochemical parameters of salt lakes from the protected area of Buzau County, using enzymatic assays in correlation with microbial abundance.
Keywords: sapropelic, pollution, salt lakes, enzymatic assay, halophiles.

INTRODUCTION

There are 2300 lakes in Romania, most of them with fresh water. However, about 90 are salted, with salinity greater than 3 g L^{-1}. The surface of these lakes have between 0.03 and 1.310 ha, 40 of these salty lakes have black and/or gray organic sludge of at least 1-2 cm. Sediments of these lakes consist of the decomposition of the remains of organisms in the lake and its surroundings, together with the minerals supplied to the lake by the river basin and from the atmosphere. In the case of lakes located in temperate forested areas, the sediment has a high organic content (over 15%), resulting from aquatic plants, plankton and benthic organisms that have been transformed under bacterial influence and mixed with mineral components (Stankevica et al., 2013).
In Romania, the most important salt lakes with therapeutic value are: Techirghiol, Balta Albă, Balta Amara, lake Ursu, thermal salt lakes of Ocna Sibiului, Ocna Şugatag and Slănic-Prahova's "Bride Lake". The salinity of these lakes with therapeutic value ranges from 4 to 223 g of L-1 with Na^+ and Cl^- being the dominating ions. The value of the pH is variable, from 4 to 9.3, and water transparency is between 50-400 cm. The sediments of the lakes generally have a lithological sequence involving deposits of loess, clay, salt or calcareous material which forms the bottom of the lake, including clay sludge, black sludge and other organic matter, all of the mentioned being considered to have therapeutic value. Table 1 presents the mineral data of these sediments (Bulgăreanu, 1993).

Table 1. Mineral and chemical details of the therapeutic sediments from the salty lakes of Romania (Bulgăreanu, 1993)

Parameter		The range of values (g 100-1 dry sediment, excluding pH)
Chemical components	pH	5.5-9.5
	CH_4	0.003-9.040
	CO_2	0.36-95,91
	C organic	0.09-4,34
	Cl	0-36.7
	SO_4	0-11,2

	P	0.02-0.13
	Fe(II)	1.48-14.8
	K	0.7-2.82
	Ca	0.44-16.99
	Mg	0,06-5,65
Mineral components	Quartz	2-72
	Feldspat	0-28
	Calcite	0-46
	Dolomite	0-34
	Mice	0-46
	Chlorite	0-20
	Kaolinite	0-33
	Montmorillonite	0-20
	Gypsum	0-26

As is the case with salt lakes at global level, the number and uses of salt lakes in Romania is limited. The therapeutic qualities of the muds of these lakes include the improvement of several rheumatic, dermatological and gynecological diseases. In most cases, the lakes are exploited economically, commercially, recreationally and, most importantly, for the therapeutic value of sapropelic sludge and highly mineralized water (Quarttini et. al., 2016).

The microorganisms re-mineralize the organic matter to carbon dioxide, water, and various inorganic salts both in aquatic and terrestrial ecosystems and from a microbiological point of view, the importance of the lakes has been shown by isolating several strains of halophilic and archaea bacteria (examples in Romania: Ocna Sibiului and Balta Albă salt lakes) the strains were characterized by their ability to produce extracellular enzymes & biological active compounds (ex. polysaccharides) with putative potential for use in several domains like chemical industry, agriculture and biotechnology (Lazar et. al., 2017). There is a varied field of applications for archaea and halophilic microorganisms due to the fact that strains from both domains possess significant biotechnological potential. For example, halophilic microorganisms contribute to the production of certain carotenoid pigments that facilitate the absorption of light resulting in increased evaporation in saltern crystallizer ponds (Josefa et al., 2002) and *Halobacterium salinarum* produces an integral membrane protein, identified as bacteriorhodopsin, which is being used as data storage material in computer memory. Furthermore, strains from Archaea, belonging to genera *Halobacterium* and *Haloferax* contribute to the degradation of certain hydrocarbons and insecticides with important potential in soil detoxification (Oren, 2010).

However, not only the microbiota but the aquatic environments including ponds, rivers and lakes, through their physical and chemical features, are essential for the biogeochemical cycles and consequently for the productivity and evolution of the respective ecosystems. Biogeochemical cycles interact with each other in many complex ways. Among these interactions there are two important and strongly related chemical systems: oxidation-reduction and acid-base balances systems. The acid-base balances have a wide range of consequences in a variety of aqueous phase systems, including control of the weathering and solubility of minerals, biological influences (including toxicity), and control of numerous aqueous phase and heterogeneous reaction rates (Robert et. al., 2000).

Many substances, such as lignin, cellulose, chitin, pectin, agar, hydrocarbons, polyphenols, and other organic chemicals, are degraded by microbial action. Microbial degradation of lignin has not been intensively studied in organisms other than fungi, but there are reports of bacteria that can break down lignin. These lignin-degrading bacteria represent mainly by the classes Actinomycetes and Proteobacteria, *(*Bugg et. al., 2011).

Mineralization of organic substances begins in the mass of water and continues with maximum intensity at the sediments surface where bacteria develop multispecific populations embedded in biofilms in which metabolic cooperation relationships are established. Mineralization is a general term for the conversion of organic compounds into inorganic compounds under the action of microbes. Co-metabolism referred to that some chemical substances like insecticides, fungicides, and herbicides, etc. which do not exist in natural conditions, could be degraded by bacteria or fungi easily only by adding some organic matter such as exogenous or iso-biomass as the primary energy source (Zhang et. al., 2010).

Commonly, decomposition of organic compounds depends upon their chemical structure and environmental conditions. The degradable ways include: oxidation (hydroxylation reactions, such as aliphatic hydroxylation, aromatic hydroxylation, N-hydroxylation, epoxidation, P-oxidation, N-oxidation, S-oxidation, oxidative dehalogenation, oxidative dealkylation, and oxidative deamination), reduction (reduction of nitrogen group and reductive dehalogenation), hydrolysis (some esters such as thiophosphate, thiocarbamate, etc., which have ester bonds that can be hydrolyzed by bacteria), dehydrogenation, decarboxylation, dehalogenation, condensation and synthesis (Malony et al., 2001). As studies showed, microbial flora can convert organic macromolecules into small non-toxic molecules, thus avoiding the secondary pollution, this is achieved through two main mechanisms: mineralization and co-metabolism (Ye et. al., 2018; Arora et. al. 2012).

Aquatic sediments represent a distinct ecological zone characterized by a low redox potential and a micro stratification of physical-chemical factors that facilitate the occurrence of specific ecological niches, favoring the excessive growth of heterotrophic bacteria, and particularly of microaerophilic and strictly anaerobic species. The presence of large amounts of organic substances, either dissolved or particulate, as well as of complex ions favors the selection of certain microbial groups, whose specific metabolic activities and physiology could underlie both qualitative and quantitative changes, depending on the variation of environmental conditions (redox potential, temperature etc.). Although the use of chemical fertilizers, pesticides, herbicides and other agricultural inputs derived from fossil fuels have increased agricultural production, there is a growing awareness and concern over their adverse effects on indigenous microbiota and consequently, on the environmental quality. These dissolved or sedimentary sources of pollution affect living organisms with the major polluting effect of biodegradable organic materials being the reduction of oxygen concentration in water (Arora et. al., 2012). Certain strains of bacteria and other microorganisms break down these compounds into simpler, organic or inorganic substances, using oxygen in the process. With the increased growth of oxygen demanding microbial flora there is a higher demand for dissolved oxygen, thus resulting in water eutrophication which favors excessive growth of microaerophilic and anaerobic species.

Many nitrogen-fixing organisms exist only in anaerobic conditions, respiring to draw down oxygen levels, or binding the oxygen with a protein such as leghemoglobin. The dissolved organic nitrogen, particulate organic nitrogen and soil inorganic nitrogen will ultimately get converted into dissolved inorganic nitrogen and accumulated in water unbalancing the mineral ratio, where it will be assimilated by phytoplankton and nitrogen fixing microorganisms (Santanu et. al., 2015).

As is the case, the unbalanced mineral content due to pollution also showed an exceeded limit in the investigated Ca: Mg ratio of the studied lakes for Balta Alba and Jirlau lakes, indicating a higher than normal amount of Mg ions, (Lazăr et.al. 2017). Previous assays performed for more than one decade ago have demonstrated that this is a constant feature of the investigated ecosystems, as anthropogenic activities, organic and chemical fertilizers or the industrial pollution could be the potential sources for high Mg concentrations (Chapra et.al. 2012).

A suitable approach in our case in order to evaluate the pollution status was that of various enzymatic activities, such as: catalase activity, dehydrogenase activity, urease activity, or phosphatase activity, these were shown to be related to the degree of inorganic contamination in soils and sediments. Measurement of these enzymatic activities may therefore allow environmental scientists to assess the impact of inorganic pollution on soil/sediment bacterial biota, the activity of these extracellular enzymes can give insights into the rates of ecosystem level processes, such as organic matter decomposition or nitrogen and phosphorus mineralization. Briefly, the assays of extracellular enzyme activity in environmental samples typically involve exposing the samples to artificial colorimetric or fluorometric substrates and tracking the rate of substrate hydrolysis.

Our results showed that the physical-chemical and microbiological assessment of the impact of organic pollution on aquatic ecosystem on all four lakes demonstrated a high degree of hypertrophy, that could represent a positive premise for the productivity of these ecosystems, but also an alarming signal for excessive organic pollution (Lazăr et. al 2017) with risk of high oxygen demand in water and a dangerous decrease of the redox potential which can affect the biodiversity of these lakes.

RESEARCH OBJECTIVE

The present study aims to evaluate the potential of selected enzyme activities in correlation with microbial abundance and heterotrophic activity in the sapropelic sludge of salt lakes from Buzau County protected area (Balta Albă, Amara, Jirlau and Coșteiu lakes) during the summer of 2018 (June).
In order to evaluate this correlation, the following array of methods have been performed: Determination of dry matter, determination of alkaline phosphatase activity, determination of acidic phosphatase activity, determination of urease activity, determination of catalase activity, determination of dehydrogenase activity and Resazurin viability test.

MATERIALS AND METHODS

Samples of sludge were collected from the following lakes in Buzău County (Fig.1): Jirlău (Fig. 2), Costeiu (Fig. 3), Balta Albă (Fig. 4) and Amara (Fig. 5). After the sludge sample collection procedure was completed, all the samples have been kept on ice in order to preserve their properties. The samples were then analyzed in the Biochemistry Laboratory of University of Bucharest.

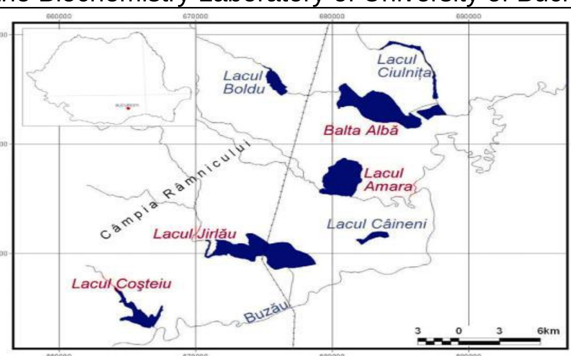

Figure 1. Geographic position of the lakes in Buzău County

Figure 2. Lake Jirlau, satellite image at the time of sampling

Figure3. Lake Coşteiu satellite image at the time of sampling

Figure 4. Lake Balta Albă, satellite image at the time of sampling

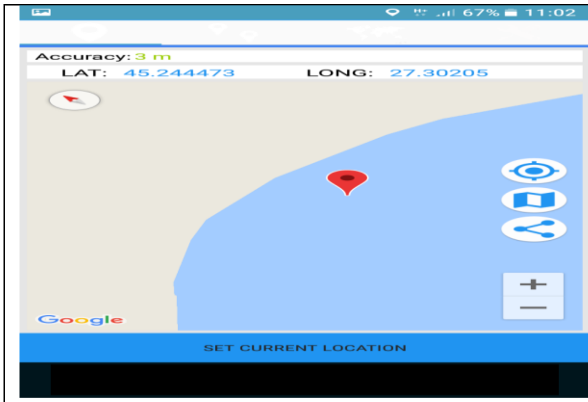

Figure 5. Lake Amara, satellite image obtained at the time of sampling

Determination of dry matter

Determination of dry matter was obtained by Laskri et. al. method (2015). Eight weighted samples were centrifuged (Eppendorf minispin plus) for 5 min at 13000 RPM then placed at 50°C for drying (Venticel oven). The initial weight of samples was between 1.5017g and 1.5044 g.

Determination of alkaline phosphatase activity.

Alkaline phosphatase activity was performed according to the protocol described by Tabatabai and Bremner (1969). This method is rapid, precise and has significant advantages over methods previously proposed for assay of soil phosphatase activity. At the end of the reaction the supernatant was taken and read at the spectrophotometer (Jasco Spectrophotometer V-560) at 405 nm against blank. Table 3 describes the quantity of reagents and parameters used.

Determination of acidic phosphatase activity

The same colorimetric analysis method as in alkaline phosphatase activity was performed, according to the protocol described by Tabatabai and Bremner (1969).

Determination of urease activity

Urease activity was determined with the use of the Nessler reagent, (Greenberg et. al 1992). The test result was read at the spectrophotometer at 500 nm. In order to calculate the enzymatic activity, a graph with the values obtained from the volume (NH4) 2SO4 as well as from the concentration of (NH4) SO4 was made, thus a graph with the the molecular mass of (NH4) SO4 was obtained. The urease estimation was performed using the standard ammonium sulfate curve with one unit of activity defined as the amount of transformed substrate / hour / 250 mg sludge, i.e. 10-3 µmol transformed in 60 minutes / gram.

Determination of catalase activity

The enzymatic activity of the catalase was carried out according to the method proposed by Dragan-Bularda (1974). The following materials were used: 1.4 ml pH 7 phosphate buffer with a concentration of 0.05 M, 0.28 ml 3% hydrogen peroxide, 1.4 ml sulfuric acid 4N, 10.92 ml distilled water and sodium permanganate 0.05 N. After the protocol was performed, the samples were ice-cold for 15 min and centrifuged for 15 min at 5000 RPM at 4 ° C. After centrifugation, 13 ml of supernatant was pipetted into Berzelius beakers, 1.3 ml of 4N sulfuric acid was added and then titrated with 0.05N sodium permanganate

Determination of dehydrogenase activity

Dehydrogenase activity assay method was performed according to the protocol described by Casida et al., (1964). For this method, the following materials were used: 0.025 g CaCO3, 0.125 ml distilled water, 0.05 ml 3% TTC and then 0.5ml methanol was added to stop the reaction. Afterwards the samples were read at the spectrophotometer at 500nm against witness.

Resazurin viability test

Resazurin dye (7-hydroxy-3H-phenoxazin-3-one-10-oxide) has been broadly used as an indicator of cell viability in several types of proliferation and cytotoxicity assays. After performing the protocol proposed by A. Lee (2017) the supernatant was collected and read at the spectrophotometer at two wavelengths 570 and 600 nm. The level of reduction can be quantified by spectrophotometer analysis due to the fact that resazurin exhibits an absorption peak at 600 ηm and resorufin at 570 ηm wavelengths.

RESULTS AND DISCUTIONS

Alkaline phosphatase activity

Alkaline phosphatases play an indispensable role in phosphate metabolism and the production of alkaline phosphatase is regulated by the phosphoester compounds available in the environment. Divalent metal ions are required for the activity of alkaline phosphatase (Fig.6).

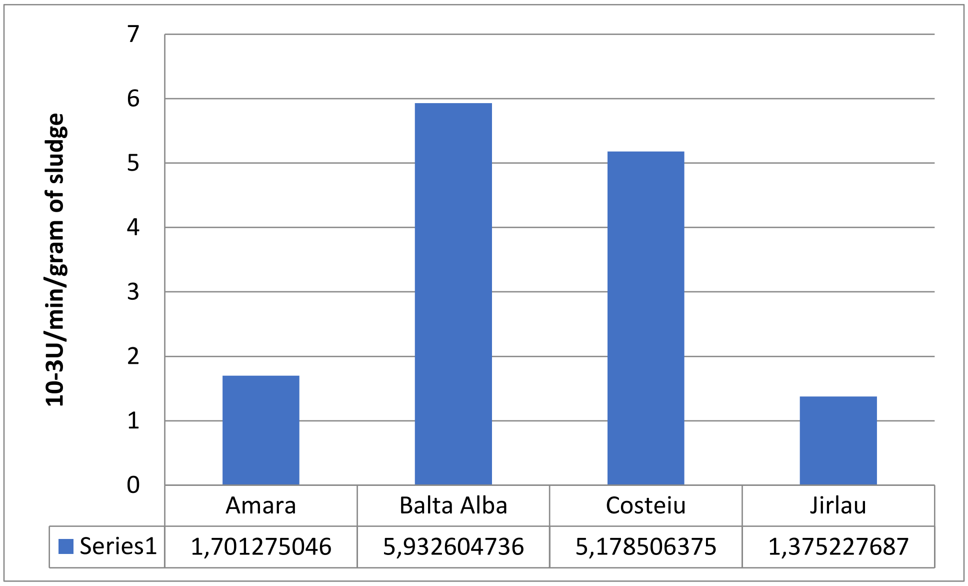

Figure 6. Activity of alkaline phosphatases in salt lakes

Experiments using added fertilization, water availability and other controlled conditions have been performed to identify the drivers of phosphatase activity. The effects of nitrogen and phosphorus or NP fertilization in 34 studies conducted by Marklein et. al., in 2011, revealed that N fertilization increased phosphatase activity and P fertilization decreased phosphatase activity across different microbiotas

Acid Phosphatase activity

Salt lakes mud microorganisms perform the mineralization of organic phosphorus compounds and the solubilization of insoluble mineral compounds, making them available for its metabolism (Fig.7). This mechanism depends on the environmental conditions and biological processes taking place in the soil

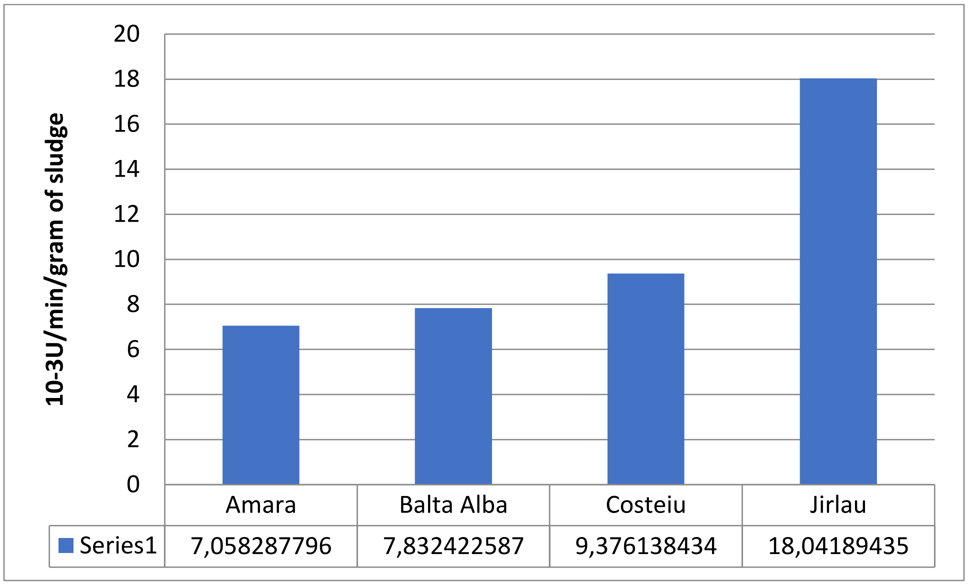

Figure 7. Acid Phosphatase activity in salt lakes

High acid phosphatase activity is often strongly correlated with total nitrogen (TN) and with microbial biomass (Janssens et. al., 2010). Almost all microorganisms are thought to respond to elemental imbalances in their resources by producing of enzymes targeting the element in need, (Mooshamer et.al. 2014). Therefore, phosphorus only becomes growth limiting when availability of other resources, such that of nitrogen, are sufficient. High nitrogen content relative to low phosphorus is required for microorganisms to start producing phosphatase.

Urease activity in the studied samples

In our assay, Lake Amara showed the highest urease activity (**Fig.8**.), results we can correlate with the values obtained in 2015 and 2016 for fecal coliform bacteria (a specific indicator of fecaloid pollution) that indicated an increasing moderate to critical level of bacteriological contamination, Lazăr et. al. (2017).

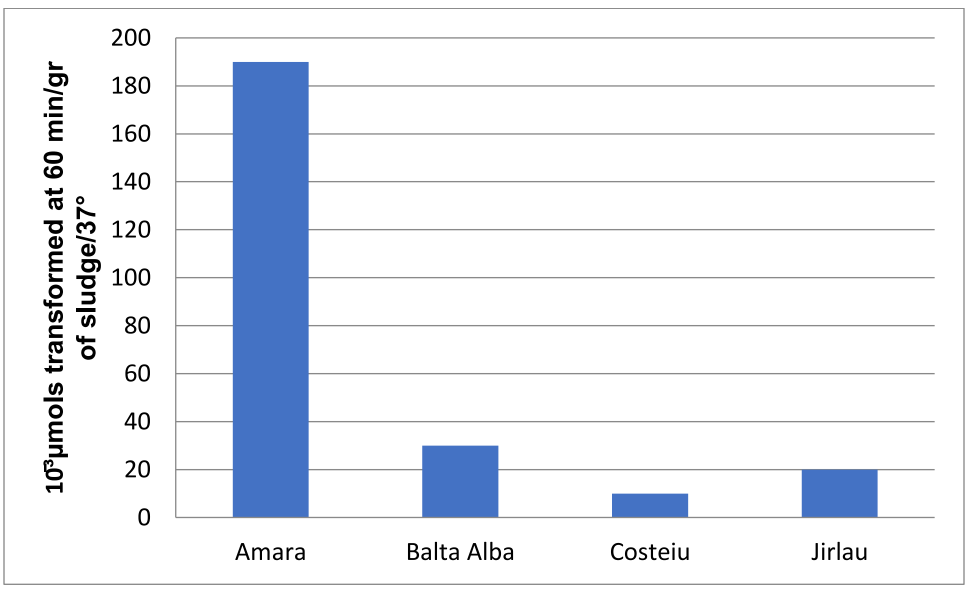

Figure 8. Urease activity in the studied samples

Urea is a significant source of nitrogen for bacteria in both fresh and saltwater (Antia et al. 1991). Within a cell, urea is hydrolyzed by urease resulting in $NH4+$ and $CO2$ that are afterwards used by various biochemical pathways. The measurement of urease activity was necessary for understanding whether urea was or was not assimilated into biomass by bacteria.

Catalase activity in the studied samples

Catalase, superoxide dismutase, peroxidases and oxidases represent the first line of efficient enzymatic antioxidative defense, which prevent the accumulation of ROS and eliminate of those which production cannot be avoided (Fig.9).

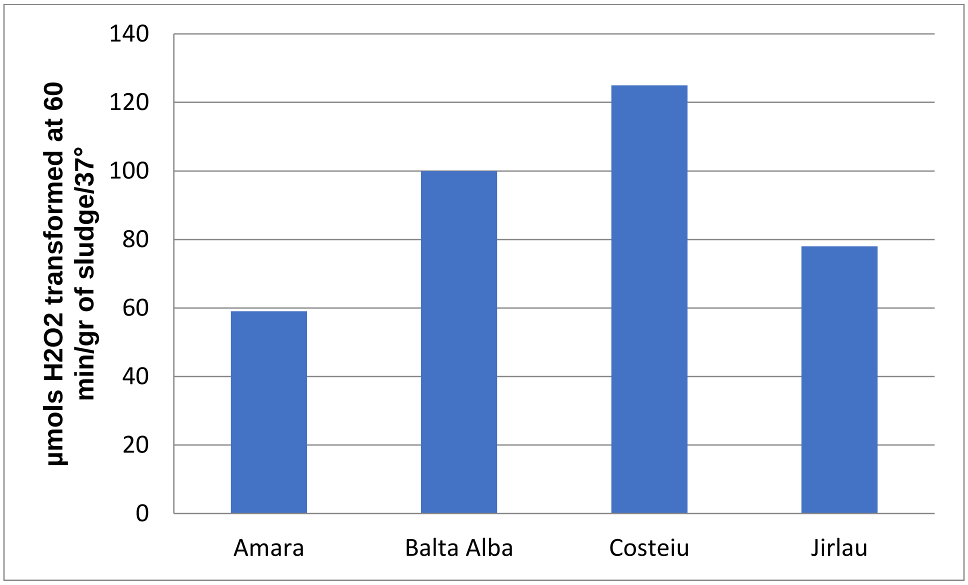

Figure 9. Catalase activity results in the studied samples

The activity of catalase is reduced in ponds containing porous clay, (Tanashita et. al., 1986), and above 5mg/l concentration of heavy metals like Zn, Cd and Co inhibited it in sludges (Patil et. al., 1986).

Dehydrogenase activity

Dehydrogenase activity may be considered as a general indicator of the biological activity of organisms, but was also used as an eco-toxicological test to assess the effects of pollutants on soil microbiota or sediment. Although data from the literature shows the highest microbiological contamination in Lake Amara, mentioned by, Lazăr et. al. (2017). Dehydrogenase activity was at lowest value from all four lakes, indicating the least level of chemical pollution (Fig. 10).

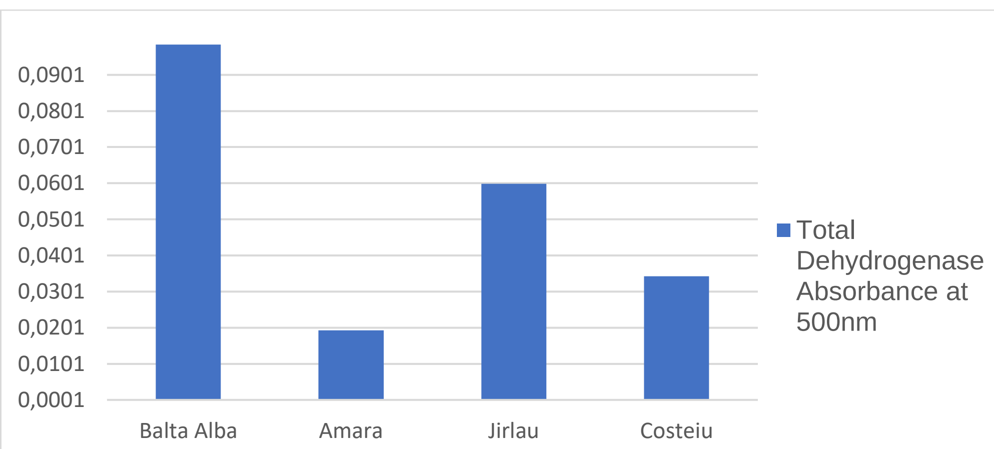

Figure 10. Dehydrogenase activity results in the studied samples

Resazurin viability test in the studied samples

Lake Amara, with a critical level of bacteriological contamination showed the highest level of reduction to resorufin, quantified by spectrophotometer analysis (Fig. 11). The irreversible character of the reduction process from resazurin to resorufin, closely associated with the oxygen uptake rate makes Resazurin a good candidate for a simple, low cost yet effective toxicity assay.

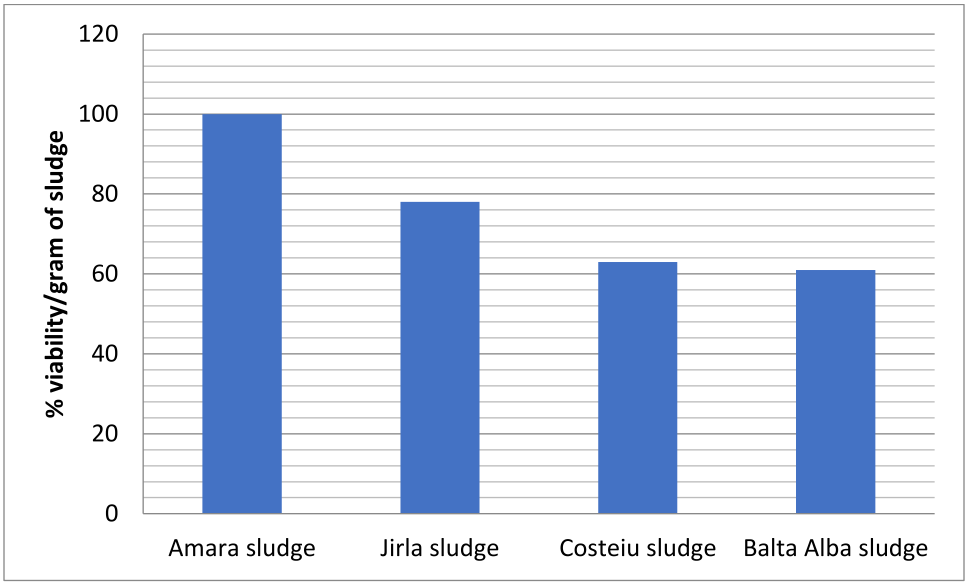

Figure 11. Resazurin viability test results

A similar assay uses a pure culture of Bacillus cereus as test (Liu, 1986), a catalase-positive bacterium classified as an obligate aerobe, though evidence exists that it is a facultative anaerobe (Hoffman et. al., 1995).

CONCLUSIONS

Our results demonstrated that:

Although the data from the literature shows the great water contamination in Amara Lake, the dehydrogenase activity shows a lower level by comparison to the level of Balta Albă, Jirlau and Costeiu lakes sludges;

Viability tests indicate an increased microbiological activity of sludge, which confirms data from other authors who have identified a pathogenic microbial activity in the Amara Lake mud;

The urease activity is correlated with biological contamination of Amara Lake;

Costeiu lake sludge seems to be the least affected by biological contamination, as the value of urease activity in the studied mud is 30% lower than the value obtained in Amara, Jirlau and Balta Alba sludge;

Concerning the activity of acid phosphatase in the studied samples Jirlau lake sludge was estimated approximately 3 times higher than Amara Lake sludge (18,041 vs 7,058 • 10^{-3} U/min/gram sludge wet);

The value of dehydrogenase activity was 5 times higher in Amara Lake sludge comparative with Jirlau and Costeu sludge lakes; the alkaline phosphatase activities in the studied lakes showed that Balta Alba and Costeiu sludges are correlate with the accumulation of organomineral biocomponents.

In studied salt lake sludges, acidic and alkaline phosphatase, urease, catalase and total dehydrogenase activities were found to have a good correlation with microbial abundance measured as colony forming units of heterotrophic bacteria published by other authors one year before (Lazar at al., 2017).

Also, microbial abundance was well correlated with resazurin viability test.

Although the data from the literature shows the great water contamination in Lake Amara, the dehydrogenase activity analyzes performed show a lower level compared to the analyzes obtained from the Balta Albă, Jirlau and Costeiu sludges.

Enzymological tests are as sensitive as microbiological tests. In addition, enzymatic tests are faster than microbiological methods.

Direct discharge of untreated waters into lakes could contribute to microbial pollution and can have negative effects on the quality of lake water. Some measures must be taken to promote environmental norms of behavior among the population and to encourage the private initiative to recycle household waste.

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