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THE SEASONAL DYNAMICS OF THE CULTIVABLE MICROBIAL COMMUNITIES IN LETEA SALINE LAKE

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Abstract

The present paper deals with the seasonal dynamics of microbial communities in Letea saline lake which is located nearby of the village with the same name by classical microbiological methods. The chloride content is variable, with values from 8.9 g/L (spring of 2018) up to 38.3 g/L (autumn of 2017). There are different similarities between the values obtained over seasons. Thus, in the spring of 2017, an average of 11.8 g/L was obtained, a value close to that obtained in the spring of 2018 (8.9 g/L). The average of chlorides in autumn 2016 (25.5 g/L) is relatively different to the autumn of 2017 (38.3 g/L). During the autumn of 2016 and spring of 2018, the dynamics of several microbial communities was assessed as follow: heterotrophic, sulphate reducing, ammonifying and denitrifying bacteria respectively. The recorded results revealed that the best represented communities appear to be ammonifying bacteria, being high in the spring months. Denitrifying bacteria have registered high values in autumn 2017 and sulphate reducing bacteria in April 2017. The dynamics of halophilic bacteria were also recorded and more than 100 strains were randomly selected for purification, characterization and extracellular enzymatic activity analyses for the future biotechnological applications.

Key words*: alkalinity, biogeochemical cycles, halophilic bacteria, physiological groups, saline soda lake.*

INTRODUCTION

The investigated Letea saline lake is located nearby of the village with the same name inner the Danube Delta. The native of the area know it as a "*ghiol*" and use it during the summer time for recreational activities. There are still missing knowledge about origin of the lake but some data supports the hypothesis that it was formed in the year 1970 after a big and strong flowed period. After this period a deep lake with a small surface area (around 190 square meters) remained as a permanent lake that is surrounded in autumn, winter and spring by a temporarily fresh body of water. The term "*ghiol*" in Turkish language is attributed for the deep water swirl (Şăineanu, 1932) and became familiarly between natives considering the available information about the lake formation.
The depth of the lake has been estimated empirically at around four - five meters and the source of the salinity is considered to be the sediments of old salmastrian sea, taking into account the steps of Danube Delta formation (Tudorancea and Tudorancea, 2006).

Saline lake and soda lake as extremely environments are widely distributed all over the world, especially in arid and semi-arid environments (the Rift Valley in East Africa, the rain-shadowed regions of California and Nevada, and the Kulunda Steppe in South Siberia). The water forming this type of ecosystem, namely saline soda lake, is rich in carbon dioxide and pour in magnesium and calcium. In preventing carbonate precipitation, is important the absence of dissolved divalent cations. When the evaporation increases, there is a concentration of carbonate salts (Tindall, 1988).
It is well known that the diversity of the microbial community of a saline soda lake depends of their salinity (Trotsenko and Khmelenina, 2002). Salinity values ranged between 35 and 50 g/L and 50 and 250 g/L, corresponding to low and moderately saline soda lakes, high productivity it is observed and functional and diverse haloalkaliphilic microbial communities are present in the cycling of carbon, nitrogen and sulphur.
At moderate salinity, the activity of bacterial communities involved in the carbon and

nitrogen cycles are partially inhibited by salinity (Sorokin et al., 2000; Minegishi, 2013). Salinity over 250 g/L, corresponding to hypersaline environments is a factor limiting the diversity (Ochsenreiter et al., 2002; Mesbah et al., 2007). The high salt concentration stopped the nitrification (Sorokin, 1998) and reduced the complete oxidation of organic matter (Ollivier et al., 1994).

From freshwater to hypersaline environments, a diversity of microbial communities adapted to life under the existing conditions has been founded so far (Oren, 2002).

A large number of microorganisms have adapted to hostile environments (high salinity and relatively high pH values). For example, alkaline soda lakes in Africa, India, China, where at pH values over 11 and salt concentrations over 300 g/L, a great diversity of microorganisms was found (Oren, 2002). In hypersaline ecosystems, with salt concentrations of over 10%, only invertebrates (such as *Artemia salina*), algae (*Dunaliella salina*), bacteria (members of the families *Halobacteriaceae* and *Haloanaerobiaceae*, etc.) and cyanobacteria (*Oscillatoria* spp.) are found (Williams, 1998).

These represent the major sources of compounds that can be oxidized (Ollivier et al., 1994).

On the other hand, in saline soda lakes a wide biodiversity is also observed, like bacteria (aerobic or anaerobic) with several nutrition types, archaea, etc (Grant and Jones, 2016).

There are very few studies on the diversity of bacterial populations, the biogeochemical cycles of saline soda lakes or on the entire microbiological community. Generally, studies have been carried out on the isolation and characterization of microorganisms for their putative biotechnological potential (Humayoun et al., 2003).

The aim of our study is to present a few aspects of the cultivable microbial communities at seasonally varying slightly alkaline pH and salt concentrations in Letea saline lake.

Not only halophilic bacteria were discussed, but the whole cultivable microbial community, since that this is one of the first reported study relating to the microbiological ecology aspects of this ecosystem.

MATERIALS AND METHODS

Sample collections

Three sampling points, noted L1, L2 and L3, were established for water samples harvested from Letea saline lake (Amoozegar et al., 2016) and their geographical location determined with GPS devices as follows: 45º16'51.978" (N), 29º33'10.4184" (E) for Letea 1 (L1), 45º16'53.0688" (N), 29º33'11.6244" (E) for Letea 2 (L2) and 45º16'51.8628" (N), 29º33'12.0204" (E) for Letea 3 (L3). The samples were taken in five seasonal sampling trips over three years, namely: autumn 2016; spring, summer and autumn 2017; spring 2018. Of the three sampling points previously set, water samples were collected in sterile bottles and kept at 4°C during transportations to the laboratory for investigations (Azhar et al., 2014).

Physical and chemical analysis of water samples

With the aid of multiparameter Hanna Instruments water parameters were measured *in situ* as follow: pH, salinity, oxidation reduction potential (ORP), conductivity and oxygen saturation (DO %). The chloride content has been determined following previously described protocol (Enache et al., 2000).

Isolation and determination of halophilic bacteria and cultivable microorganisms involved in biogeochemical cycles of carbon, nitrogen and sulphur

For *quantitative determination of the number of halophilic bacteria,* a MH medium was used with the following composition (g/L): yeast extract, 10; proteose peptone, 5; glucose, 1; NaCl, 100; $MgCl_2$ x $6H_2O$, 7; $MgSO_4$ x $7H_2O$, 9.6; $CaCl_2$ x $2H_2O$, 0.36; KCl, 2; $NaHCO_3$, 0.06; NaBr, 0.026; (Ventosa et al., 1972).

For *quantitative determination of the number of heterotrophic bacteria*, nutrient agar medium was used. Both media were sterilized at 120°C for 30 minutes. For solidification form 20 g/L agar was added. For the investigations, the serial dilutions were performed with two repetitions per dilution. One mL from each dilution was spread in sterile Petri dishes. Approximately 20 mL of molten agar medium cooled at 55-60°C was added (Cojoc et al.,

2013). The Petri dishes were incubated at 37°C for 48 h for heterotrophic bacteria and at 28-30°C for one week in the case of halophilic bacteria. After this period of incubation, the colonies were quantified (Halder et al., 2016). Approximately 10% of the colonies of halophilic bacteria were randomly selected for further purification steps and characterization by morphology, color, edges, Gram's staining, etc. (Lazăr et al., 2004).

The Most Probable Number method was used to quantify the microorganisms involved in biogeochemical cycles of nitrogen and sulphur.

For *quantitative determination of the number of sulphate reducing bacteria,* Postgate medium was used with the following composition (g/L): NH_4Cl, 1; K_2HPO_4, 0.5; $MgSO_4$, 2; Na_2SO_4, 0.5; calcium lactate, 3.5. The medium was sterilized at 120°C for 30 minutes. After sterilization, the following was added to 500 ml medium: yeast extract 5% 10 ml, $FeSO_4$ 5% 5 ml, Na_2S 1% 2 ml, $NaHCO_3$ 10% one up to five ml until the pH reached 7.2-7.4. These solutions were separately sterilized under similar conditions before use (Lazăr et al., 2004).

For the studies, the serial dilutions were performed with three repetitions per dilution. Two ml of each dilution was dispensed into sterile tubes and the medium was poured into the high column. After an incubation period of seven days at 28°C the tubes were deemed positive if a black precipitate of FeS appeared (Lazăr et al., 2004).

To *determine the number of ammonifying bacteria,* a medium with the following composition (g/L) was used: standard saline solution Winogradski (K_2HPO_4, 5; $MgSO_4$ x $7H_2O$, 2.5 ; NaCl, 2.5; $Fe_2(SO_4)_3$, 0.05; $MnSO_4$, 0.05; distilled water, 1000 ml) 50, asparagine, 0.2; oligoelement solution (H_3BO_3, 2.8 g; $MnSO_4$ x $4H_2O$, 0.2; $NaMoO_4$ x $2H_2O$, 0.75; $ZnSO_4$ x $7H_2O$, 0.24; $Cu(NO_3)_2$ x $3H_2O$, 0.04); distilled water, 950 ml was distributed in amounts of five mL in test tubes and sterilized for 30 minutes at 120°C (Lazăr et al., 2004).

For the investigation, the serial dilutions were performed with three repetitions per dilution. A total volume of 0.2 mL from each dilution was placed in tubes with culture medium (2.5 mL volume) and incubated at 28°C for 15 days. If a yellow-orange color appears on the addition of a few drops of Nessler reagent, the tube is deemed positive (Lazăr et al., 2004). To *determine the number of denitrifying bacteria,* Pochon medium was used with the following composition (g/L): standard saline solution Winogradski (K_2HPO_4, 5; $MgSO_4$ x $7H_2O$, 2.5; NaCl, 2.5; $Fe_2(SO_4)_3$, 0.05; $MnSO_4$, 0.05; distilled water, 1000 ml) 50; KNO_3, 2; glucose, 10; $CaCO_3$, 5; distilled water, 950 ml. The medium was sterilized at 110°C for 30 minutes. The investigations were performed as described above. If necessary, the incubation period was extended to 15 days. If a red color appears on additions of Griess I and Griess II reagent, the reaction is deemed positive (Lazăr et al., 2004).

RESULTS AND DISCUSSIONS

The investigated Letea saline lake is a permanent lake, surrounded by a temporary body of fresh water, in the Danube Delta, a Biosphere Reserve. Based on the results from our study, the specificity of this lake is seasonally variable salinity and a slightly alkaline pH values.

The results in Figure 1 revealed that the chloride content (results showed as average of the three sampling points) varied from 8.9 g/L, observed in the spring of 2018 up to 38.3 g/L, the maximum value recorded in the autumn of 2017. The data obtained showed that there is a similarity in the values obtained during the spring season. Thus, in the spring of 2017, an average of 11.8 g/L was recorded, a value relatively close to that obtained in the spring of 2018 (8.9 g/L). On the other hand, the average of chloride level in autumn 2016 (25.5 g/L) differed from that for autumn of 2017 (38.3 g/L) by around 13 g/L. One characteristic of this saline lake is therefore salinity variation. Base on our observations during to the trips, the main hypothesis to explain the diminution of salinity from autumn to spring would be the input of fresh water from rain and snow. In the summer, due to water evaporation and small amounts of precipitation, a salinity concentration occurs (Figure 1) (Trotsenko and Khmelenina, 2002).

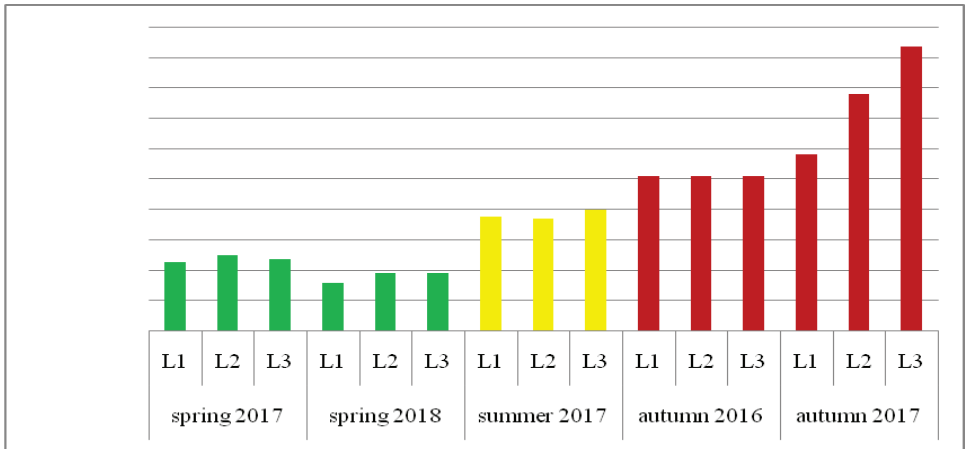

Figure 1. Seasonal variation of chloride content (g/L) between autumn 2016 and spring 2018, in Letea saline lake (L1- L3 - sampling sites)

The following physical and chemical parameters of water were measured *in situ*: pH, temperature, conductivity, oxygen saturation (DO %) and salinity; the recorded data are summarized in the Table 1.

Table 1. Physico-chemical parameters of the water samples; DO = total dissolved oxygen; L1, L2 and L3 = Sampling points identification: Letea 1 (L1), Letea 2 (L2) and Letea 3 (L3); bold letter = minimal and maxim values of parameters

Seasons	Spring						Summer			Autumn					
Years	2017			2018			2017			2016			2017		
Sampling points	L1	L2	L3	L1	L2	L3	L1	L2	L3	L1	L2	L3	L1	L2	L3
pH	10.0	9.5	9.4	9.3	9.6	9.5	9.2	**8.7**	9.3	9.6	9.4	9.5	10.2	9.4	**10.4**
Water temperature (ºC)	**5.2**	6.6	5.4	19.4	20.2	20.3	**27.5**	27.4	27.4	19.2	19.2	19.2	8.7	8.7	8.6
Sampling points	L1	L2	L3	L1	L2	L3	L1	L2	L3	L1	L2	L3	L1	L2	L3
Conductivity (mS/cm)	27.7	27.4	27.9	13.9	14.2	**14.0**	42.2	42.1	41.2	59.3	56.2	**59.6**	58.3	58.4	58.4
DO (mg O_2/L)	4.1	4.9	4.4	3.2	**1.2**	**1.2**	2.7	2.6	2.6	6.7	6.9	7.2	**17.0**	11.7	15.6
Salinity (ppm)	16.9	16.8	16.6	**7.8**	8.2	8.1	27.1	27.0	26.6	39.8	38.4	**39.9**	38.6	38.7	38.7

During the five sampling trips, it was observed that the pH value varied between 8.7 in summer of 2017 (L2) and 10.4 in the autumn of 2018 (L3). According to these data, the Letea saline lake could be considered in the category of slightly alkaline waters. The data shown in Table 1 reveals that the water temperature and dissolved oxygen were in an inverse relationship. As generally expected, the dissolved oxygen showed a seasonal decrease as the water temperature increased (Table 1). In autumn of 2016, summer of 2017 and spring of 2018, it was the same decrease in dissolved oxygen with seasonal water temperature. In autumn of 2017, an increase was recorded in dissolved oxygen, which reveals higher solubility than in other seasons, solubility supported by a low value of temperature. These conditions and also a high chloride content (Figure 1) could be correlated with the presence of a number of halophilic bacteria at around 2 x 10^2 c.f.u./mL (Table 2) higher than the number for autumn of 2016.

Table 2. Dynamics of halophilic heterotrophic bacteria between autumn 2016 and spring 2018, in Letea saline lake; C.F.U. = colony forming units; bold letter = minimal and maxim values recorded

Sampling points	C.F.U./mL recorded on MH medium				
	Spring		Summer	Autumn	
	2017	2018	2017	2016	2017
L1	**$3.7x10^3$**	**280**	$4.2x10^2$	770	$1.7x10^2$
L2	$3.6x10^3$	**280**	$4.1x10^2$	410	$1.9x10^2$
L3	$2.1x10^3$	350	$1.4x10^3$	560	$2.3x10^2$

The results from Table 2 showed that the largest numbers of halophilic bacteria were recorded in the spring of 2017; the number varied from 2.1 x 10^3 in L3 to 3.7 x 10^3 at L1, considerably higher than spring of 2018 or other seasons. In the last cases, the total number of halophilic bacteria remained constantly low. The data obtained during this

study revealed a relatively low number of halophilic microorganisms if compared with other studies (Moldoveanu et al., 2015) and, on the other hand, showed a low anthropogenic impact. Similarly, the highest number of heterotrophic bacteria was recorded in the spring of 2017, namely 3.1 x 10^3 c.f.u./mL, in other seasons the number being relatively similar (Table 3). This behaviour could be correlated with the salinity of the investigated samples (Table 1); the low concentrations (below 17 ppm) led to a high number of heterotrophic microorganisms (Table 3).

Table 3. Dynamics of heterotrophic bacteria between autumn of 2016 and spring of 2018, in Letea saline lake

Sampling points	C.F.U. / mL recorded on nutrient agar medium				
	Spring		Summer	Autumn	
	2017	2018	2017	2016	2017
L1	**3.1 x 10^3**	18 x 10^2	**340**	1.7 x 10^2	720
L2	7 x 10^2	6 x 10^2	850	2.1 x 10^2	1.5 x 10^2
L3	8.5 x 10^2	12 x 10^2	330	1.5 x 10^2	1.3 x 10^2

The results recorded in Table 4 supported the observation that the highest number of sulphate reducing bacteria was in the sample from the spring of 2017. In other seasons, the number was relatively similar, except for one sample (L1) from the autumn of 2016 (Table 4). As previously mentioned in the case of heterotrophic microorganisms, the physical parameters and chemical composition of the water are reflected also in the total number of microorganisms, including sulphate reducing bacteria.

The ammonifying bacteria were identified in all investigated samples (Table 4), the highest number recorded in the spring of 2017, respectively 11 x 10^3. In other seasons, excepting autumn 2017 and spring 2018, the number is relatively similar. One particular aspect to be noted is the absence of denitrifying bacteria in the spring 2017 (Table 4) in spite of the high number of the ammonifying bacteria in this season. These results are supported by literatures data from which appeared that the salinity partially inhibiting microorganism involved in the nitrogen cycle (Sorokin et al., 2000). In other samples the number of such bacteria was relatively similar and low.

Table 4. Dynamics of sulphate reducing bacteria, ammonifying bacteria and denitrifying bacteria, between autumn 2016 and spring 2018, in Letea saline lake; MPN = most probable number

Physiological groups	Sampling points	MPN				
		Spring		Summer	Autumn	
		2017	2018	2017	2016	2017
Sulphate reducing bacteria	L1	**4.5 x 10^2**	**2.5**	4.5	950	7.5
	L2	**4.5 x 10^2**	**2.5**	**2.5**	**2.5**	7.5
	L3	**4.5 x 10^2**	**2.5**	9.5	**2.5**	2.5
Ammonifying bacteria	L1	**11 x 10^3**	140 x 10^2	25	1.5 x 10^2	**2.5**
	L2	**11 x 10^3**	140 x 10^2	25	9.5 x 10^2	**2.5**
	L3	2.5 x 10^3	45 x 10^2	25	9.5 x 10^2	**2.5**
Denitrifying bacteria	L1	**0**	45	4.5	410	**11 x 10^2**
	L2	**0**	110	4.5	20	150
	L3	**0**	140	9.5	20	**11 x 10^2**

The total number of halophilic bacteria varies in from each season and year. A total number of 113 strains were isolated and purified. From this total number, after successive purification steps, only 73 (82.5%) remained cultivable for further investigation steps. These strains are circular or irregular in shape and have a convex, flat or creased profile. The edges were full, wavy or branched. They were found to have different colors: cream, brick, orange, light orange, white, yellow, light beige, brick, pink or yellow-brick. Also, the strains were described on the basis of Gram's staining (Roohi et al., 2012). In the autumn season, Gram negative bacteria were predominated. Thus, in October 2016, only Gram negative bacteria were isolated. In November 2017, in L1 and L2, the number of Gram negative bacteria was higher than Gram positive bacteria. In L3, only Gram negative bacteria

were isolated. In the spring season (2017, 2018), Gram positive bacteria was predominated. In July 2017, in L2 only bacteria Gram negative bacteria were isolated.

CONCLUSIONS

In the investigated area, the Letea saline lake is a permanent salt lake with seasonally fluctuating salinity (chloride content) varying from 9 to 40 g/L. On the other hand, the pH values suggested a slightly alkalinity in the water, with 8.7 being recorded in the summer of 2017 up to a maximum of 10.4 in the autumn of the same year. During to the winter and spring, the lake is surrounded by temporary bodies of fresh water that are completely evaporated in summer time. Thus, the salinity of Letea lake decreases in spring and has maximum detected values in summer time and the beginning of autumn season. The variation in the two main physico-chemical parameters, namely chloride content and pH values are reflected in the dynamics of microbial communities. An important aspect is represented by the absence of halophilic archaea. No cultivable halophilic micro-organisms belonging to *Archaea* domain could be isolated from the investigated water samples (15 samples over of five seasonally trips). Future investigations will be conducted into the polyphasic taxonomy of selected halophilic bacteria in order to estimate their putative biotechnological implication through the presence of extracellular hydrolytic enzymes, pigments or solutes and other metabolites.

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