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Original paper

Physiological profiles of the microbial communites from muddy volcanoes Pâclele Mari and Pâclele Mici, ***Buzău county****, Romania*

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Abstract

The purpose of this paper was to establish whether there are differences between the physiological profiles at the community level (CLPP) in two important lakes in Buzău County and in the mud of the Muddy Volcanoes from Pâclele Mari and Pâclele Mici.

Comparisons between substrate use patterns showed differences in the composition of microbial communities, reflecting the heterogeneous distribution of microorganisms in Lake Balta Albă and Lake Amara. The results obtained for the mud samples from the top of the mud volcanoes, showed that there are no differences between the physiological profiles at the level of the microbial communities of the two mud samples; this can be observed both by the average color development (AWCD) and by the types of substrates used by existing microbial communities.

Keywords Microbial communities, EcoPlates metabolic profile, Substrate utilization pattern, AWCD, Shannon's index.

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Introduction

Carbon is a major factor in microbial growth in soil and functional aspects related to substrate utilization can provide important information. Community-level physiological profiling (CLPP) based on sole carbon substrate utilization profiles (CSUP) was first demonstrated by Garland and Mills (1991) using BIOLOG microplates to distinguish between different communities of heterotrophic soil bacteria (LOPES et al, 2016).

Characterization of the diversity of physiological profiles at community level is a method that offers a rapid characterization of the ecological state of the samples collected from the environment (AGATA et al, 2014), such as sediments (LOPES et al, 2016), wastewater (ZHANG et al, 2014), activated sludge (PAIXÃO et al, 2007) and soils (RUTGERS et al, 2016; AL-DHABAAN and BAKHALI, 2017; AMARESAN et al, 2018).

The aim of this study is to determine the physiological profile at the level of the existing communities in the water samples: Amara Lake, Balta Albă Lake, respectively mud of the Muddy Volcanoes from Pâclele Mari and Pâclele Mici: Sample 1, Sample 2. Four samples were taken from different lakes in Buzău County, to investigate the functional diversity obtained by CLPP analysis.

Materials and Methods

1. Site and sampling

The samples were collected in July 2019, from different areas of the protected area in Buzău County. Thus, two water samples were collected from Lake Balta Albă and Amara, respectively 2 mud samples from the surface of the Muddy Volcanoes from Pâclele Mari and Pâclele Mici. They were stored in sterile plastic bottles, at cold (4°C) and in the dark.

2. Microbial community metabolic profiles

Samples collected from different sites were inoculated on EcoPlates Biolog™, in triplicate, to observe the pattern of use of the substrate by the microbial communities in the water samples. The Biolog EcoPlates System consists of 96-well microplates; every well is a coat lyophilize substrate (31 different carbon sources in three replications divided in six main groups (CHOI & DOBBS, 1999): carbohydrates, amines, carboxylic acids, phenolic compounds, polymers, amino acids). The metabolism of the carbon source by the microbial communities is indicated by the reduction of the color indicator (tetrazolium). These substrates allow the evaluation and characterization of the functional diversity of the communities of microorganisms by analyzing the specific color changes associated with the metabolization of carbon sources. The population of microorganisms gives a characteristic pattern of response, wich is called a metabolic fingerprint.

In this study, were analysis four water samples (Lake Amara, Lake Balta Albă, Sample 1, Sample 2). The water samples were ten-fold diluted (10^{-1} Lake Amara and 10^{-2} Lake Balta Albă, Sample 1, Sample 2) in sterile distilled water, and then was used to inoculate (120 μL per well) the Biolog™ EcoPlates. After inoculation, the samples were incubated at 25°C for 12 days, in the dark, and their analysis was performed dynamically. Absorbance in the microplates was measured using a microplate reader at 590 nm every 24 h.

3. Statistical analysis

EcoPlates color development models are extremely varied due to the variability of the initial species composition, the initial community density (inoculum size) and the changes in the color development model during the incubation period, as well as the species composition during the incubation (KONOPKA et al, 1998; PRESTON-MAFHAM et al, 2002; STEFANOWICZ, 2006).

Average well color development (AWCD) is an expression of the activity of microorganisms in the sample, being influenced by factors such as cell density and diversity in substrate use. AWCD is a parameter that describes the average use of C sources by the microbial communities. It represents the sum of all the absorbance values of the substrates divided by 31 and is calculated according to the following formula:

$$\mathbf{AWCD = \sum OD_i / 31}$$

where: OD_i is the average of the optical density values of each normalized well, (GARLAND and MILLS, 1991; WEBER et al, 2007).

Functional diversity (S) was calculated by determining the Richness index (R) (ZAK et al, 1994), which represents the number of different carbon sources that have been used by microbial communities (i.e., normalized absorption values ≥ 0.25) and is determined by the sum of the number of positive responses observed after incubation (GARLAND, 1996). It is considered to be a positive response, when the optical density value, after normalization is greater than or equal to 0.25.

The value of the Shannon's diversity index was determined to assess microbial diversity at the community level. This parameter can be determined according to the formula:

$$\mathbf{H = -\Sigma (p_i \times \ln p_i)}$$

where *pi* is the ratio between the optical density value for a particular substrate to the sum of the optical density values for all substrates. Microbial communities that are capable of metabolizing multiple carbon sources and / or metabolizing them with similar efficiency will have higher values of Shannon's diversity index (KEYLOCK, 2005; SPELLERBERG, 2008).

The Simpson's diversity index (D) was calculated using the formula:

$$\mathbf{D = 1 - \sum (p_i)^2}$$

where *pi* is the ratio between the optical density value for a given substrate and the sum of the optical density values for all substrates, for a given EcoPlates (KREBS, 1972; MAGURRAN, 1988).

Substrate evenness (E) was calculated as follows:

$$\mathbf{E = H / \ln S}$$

where *S* represents the total number of metabolized carbon sources (the number of wells that vary in color) (KEYLOCK, 2005).

Results and Discussions

Biolog™ EcoPlates is considered as a tool for studies of functional diversity and for comparing communities of microorganisms in the environment (SMALLA et al, 1998). Biolog™ EcoPlates technology provides metabolic imprinting of microbial communities (GARLAND and MILLS, 1991; JANNICHE et al, 2012; GHIMIRE et al, 2014).

In this study, EcoPlates was used to evaluate the physiological profile of existing microbial communities in samples collected from aquatic habitats.

The average color development (AWCD) of all carbon sources is closely related to the metabolically active cells of the microbial communities. Color change is highly dependent on cell density, as well as their functional diversity (JANNICHE et al, 2012).

The incubation time is very important, as this can significantly affect the results. The change in color as well as the value of the AWCD parameter forms an asymptotic sigmoidal curve with time, and the response of each well varies with time, some having a lag phase longer than others. Thus, if the incubation time is too short, color development in some wells may be lacking, and if the incubation time is too long, saturation levels will be reached in some wells. In addition, since the relationship between time and color may vary between wells, it is highly likely that a single optimum reading time cannot be determined, so repeated monitoring is essential.

For the analyzed water samples, the following parameters were determined using a HQ30D Portable Multi Meter: dissolved oxygen, water temperature and pH; regarding the sludge samples, only atmospheric temperatures were recorded. Parameters can be found in Table 1.

Table 1. Physical parameters

Sample	Dissolved oxygen	Temperature	pH
Lake Amara	7,35 mg/L	26°C	7,60
Lake Balta Albă	6,75 mg/L	27°C	8,70
Sample 1	-	30°C	-
Sample 2	-	30°C	-

The AWCD values of the water samples reached a maximum of 0.6816 at 240 h for Lake Amara, from 0.0424 at 264 h for Lake Balta Albă (Fig. 1). For Lake Amara, an exponential growth phase can be observed between 24 h and 240 h (0.0068-0.6816), followed by a slight decline. As regards Lake Balta Albă, a lag phase can be observed between 24 h and 72 h (0.0034-0.0068), followed by an exponential increase between 72 h and 264 h (0.0068-0.0424) (Fig. 1). For Sample 1 and Sample 2, we could not observe an exponential growth phase, which is due to a low microbial biomass, but also a small microbial functional diversity.

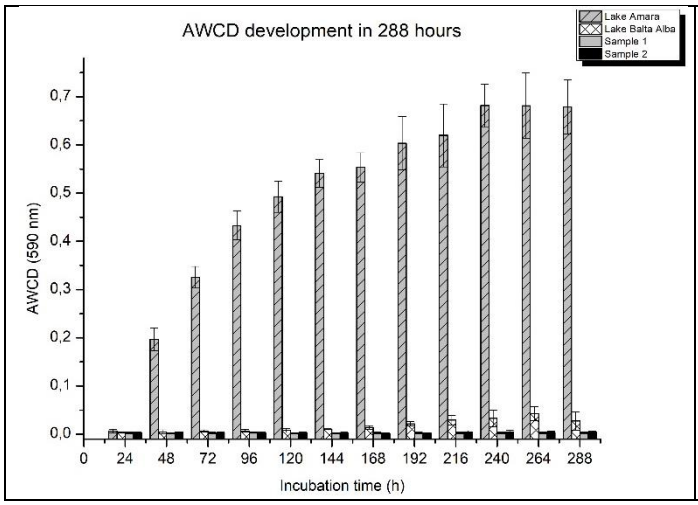

Figure 1. Average color development in the well (AWCD) obtained over time for the microbial communities from the samples: Lake Amara, Lake Balta Albă, Sample 1, Sample 2.

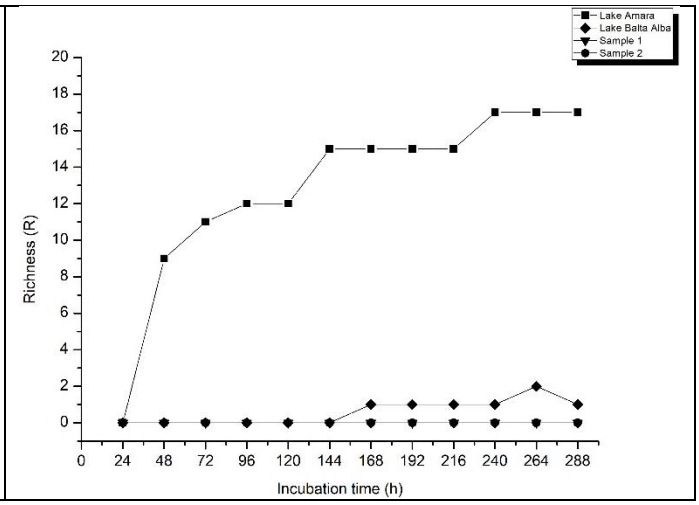

Figure 2. The CMD values obtained over time for the microbial communities from the samples: Lake Amara, Lake Balta Albă, Sample 1, Sample 2.

The highest functional richness among the analized microbial communities was detected for the sample collected from the Lake Amara. Bacteria were able to metabolize 17 of the 31 substrates of Biolog EcoPlates (Fig. 2), including many complex carbon sources represented by polymers (Tween 40, Tween 80, Glycogen) and

carbohydrates (Table 2). For the microbial communities in Lake Amara, the capacity of use of the six types of carbon sources was different. AWCD for polymers was the highest (1.2068), and the lowest was for phenols (0.0077), thus illustrating that polymers were the carbon sources with the highest degree of metabolic use, and the lowest the degree of metabolic use had phenols (Fig. 4). As expected, the sources that provided the best growth were represented by sugars and polymers, and the smallest bacterial growths were represented among the sources that contain in their structure aromatic nuclei (GARLAND et al, 2010).

The CMD / Richness (R) values of the aquatic microbial communities at 264 hours can be determined in Fig. 3.

The microbial communities in the water samples collected from Lake Balta Albă metabolized a smaller number of carbon sources compared to those in Lake Amara. It can also be observed that the samples from Sample 1 and Sample 2 did not metabolize any substrate. β-methyl-D-glucosidase and L-arginine were the two substrates consumed by the bacterial communities in the Lake Balta Albă water samples (Table 2).

The evolution in time of the use of the 6 types of substrates by the aquatic microbial communities from Lake Amara, Lake Balta Albă, Sample 1, Sample 2, can be observed in Fig. 5, 6, 7 and 8.

The functional diversity of the microbial communities was analyzed by calculating the statistical parameters: Shannon's-Wiener index, Simpson's index and substrate evenness (E) during the incubation time of 264 hours. The results are presented in Table 3.

Table 2. Use of Biolog ™ EcoPlates substrates by microbial communities. An absorption threshold value of 0.25 was used as a positive growth response, after 264 hours incubation, at 25°C, in the dark. (AA): amino acids; (AM): amines / amides; (CAA): carboxylic acids and keto acids; (CH): carbohydrates; (POL): polymers; (PHE): Phenolic compounds.

Carbon source	Compound group	Lake Amara	Lake Balta Albă	Sample 1	Sample 2
Water		-	-	-	-
β-Methyl-D-Glucosidase	CH	+	+	-	-
D-galactonic Acid γ Lactone	CAA	+	-	-	-
L-Arginine	AA	+	+	-	-
Pyruvic Acid Methyl Ester	CH	-	-	-	-
D-Xylose	CH	+	-	-	-
D-Galacturonic Acid	CAA	+	-	-	-
L-Asparagine	AA	-	-	-	-
Tween 40	POL	+	-	-	-
i-Erythritol	CH	-	-	-	-
2-Hydroxy Benzoic Acid	PHE	-	-	-	-
L-Phenylalanine	AA	+	-	-	-
Tween 80	POL	+	-	-	-
D-Manitol	CH	-	-	-	-
4-Hydroxy Benzoic Acid	PHE	-	-	-	-
L-Serine	AA	+	-	-	-
α-Cyclodextrin	POL	-	-	-	-
N-Acetyl-D-Glucosamine	CH	+	-	-	-
γ-Hydroxybutyric Acid	CAA	-	-	-	-
L-Threonine	AA	-	-	-	-
Glycogen	POL	+	-	-	-
D-Glucosaminic Acid	CAA	-	-	-	-
Itaconic Acid	CAA	-	-	-	-
Glycyl-L-Glutamic Acid	AA	-	-	-	-
D-Cellobiose	CH	+	-	-	-
Glucose-1-Phosphate	CH	+	-	-	-
α-Ketobutyric Acid	CAA	+	-	-	-
Phenylethyil-amine	AM	+	-	-	-
α-D-Lactose	CH	-	-	-	-
D, L-α-GlycerolPhosphate	CH	+	-	-	-
D-Malic Acid	CAA	-	-	-	-
Putresceine	AM	+	-	-	-
Richness (R)		**17**	**2**	**0**	**0**

Tabel 3. Microbial diversity at 264 hours

Water samples	Shannon's index (H)	Simpson's index (D)	Shannon Evenness (E)
Lake Amara	2,81	0,932	0,992
Lake Balta Albă	1,416	0,621	2,043
Sample 1	2,414	0,858	-
Sample 2	2,108	0,807	-

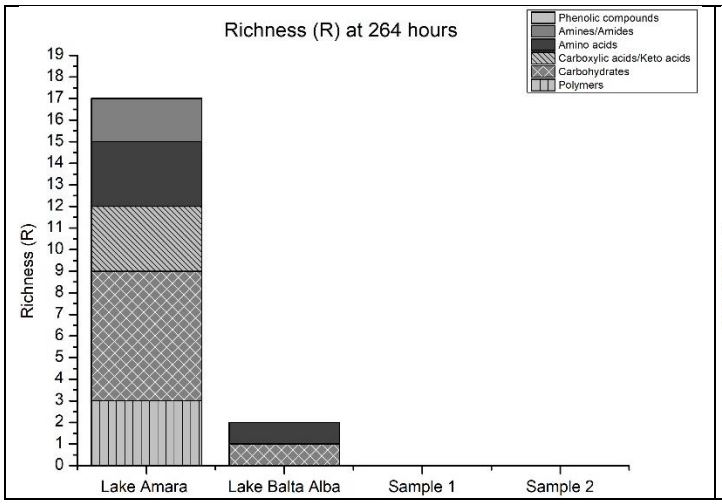

Figure 3. CMD / Richness (R) values of aquatic microbial communities, measured by summing the number of oxidized carbon sources (O.D. values ≥ 0.25) at 264 hours, in each group (data are shown in Table 2).

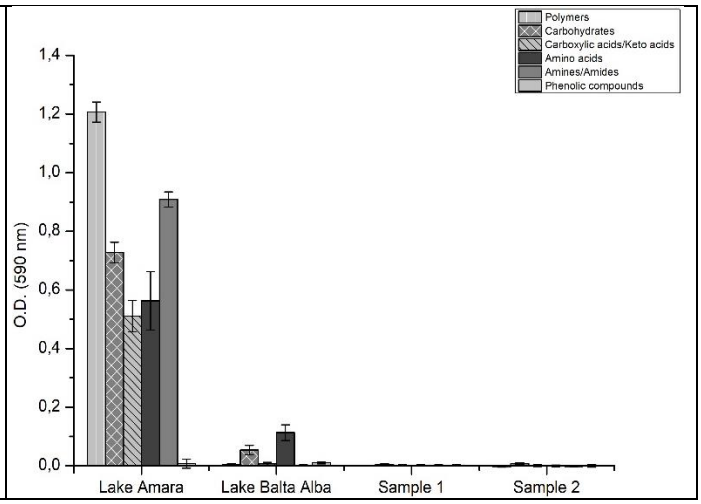

Figure 4. Comparison of the types of substrates consumed by the microbial communities in the four water samples on 11 day.

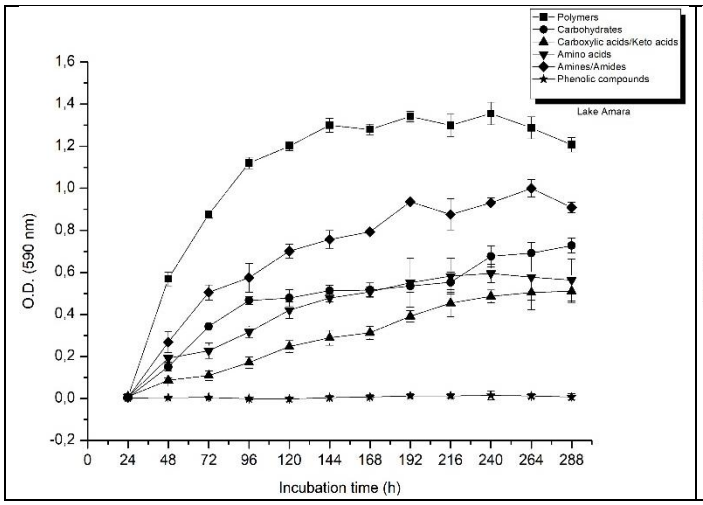

Figure 5. The evolution in time of the use of the 6 types of substrates by the aquatic microbial communities in Lake Amara.

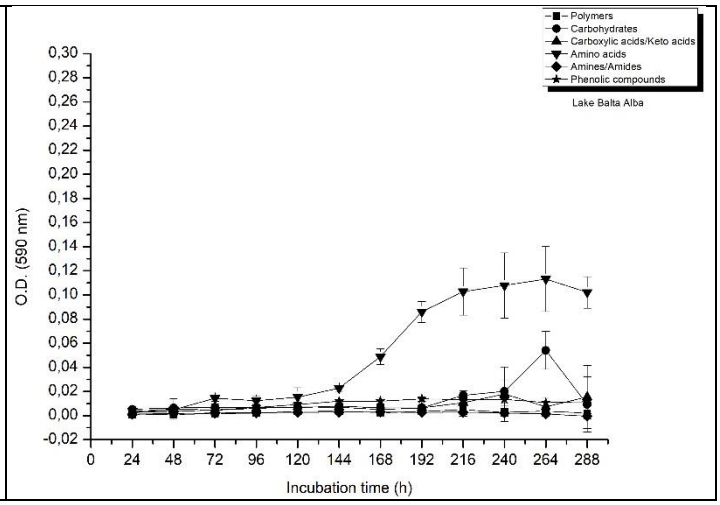

Figure 6. Evolution in time of the use of the 6 types of substrates by the aquatic microbial communities in Lake Balta Albă.

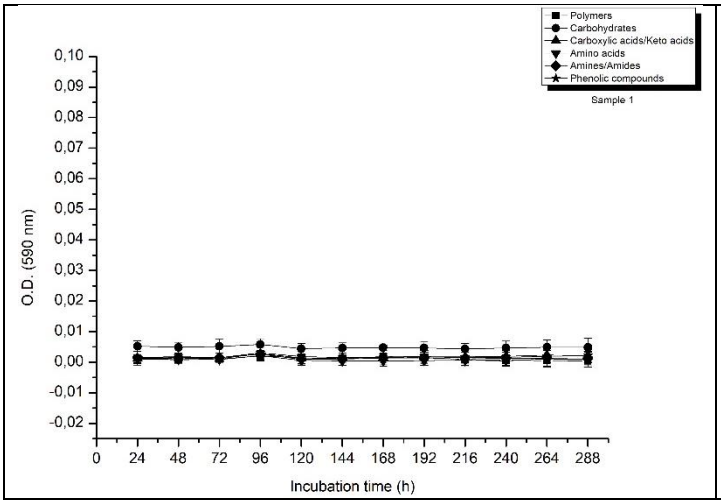

Figure 7. Evolution in time of the use of the 6 types of substrates by the aquatic microbial communities in Sample 1.

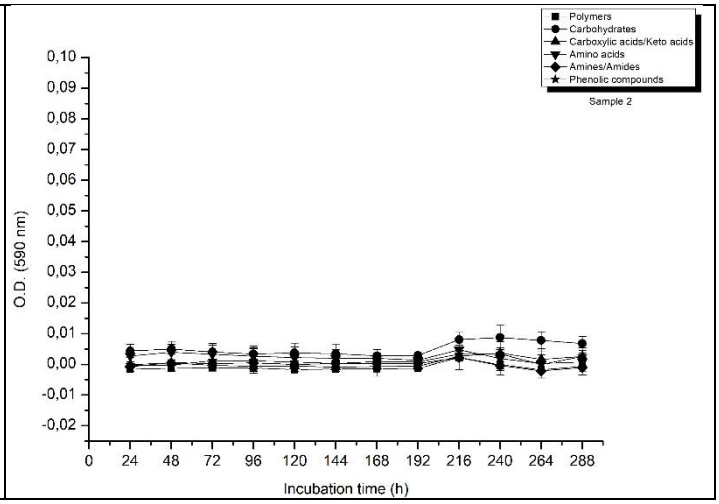

Figure 8. Evolution in time of the use of the 6 types of substrates by the aquatic microbial communities in Sample 2.

Conclusions

AWCD values showed a greater functional diversity of the microbial communities found in water samples from Lakes Amara and Balta Albă compared to the metabolic activity of the microbial communities in the muds of the Muddy Volcanoes from Pâclele Mari and Pâclele Mici. The metabolic diversity of microbial populations in water samples taken from water basins was higher than that of microbial populations in sludge, suggesting a greater diversity of microbial communities in water compared to those in sludge. The greatest functional diversity was detected for the microbial communities in the samples taken from the Amara Lake. The bacteria were able to metabolize 17 substrates of the EcoPlates. Microbial communities in the natural ecosystem (Lake Balta Albă) metabolized fewer carbon sources.

The use of substrates by existing microbial communities in sludge samples has been very low, this being due to the fact that bacteria in these sludges proliferate in different environmental conditions, using different types of substrates, under anaerobic conditions.

Also, the use of EcoPlates Biolog has proven to be a fast and modern method of analysis that can provide useful information about structural or physiological changes in the microbial communities of aquatic ecosystems caused by various chemical and / or physical factors.

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