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THE USE OF FUNCTIONAL APPROACHES TO ANALYZE THE DIFFERENCES IN LAKE WATER MICROBIOME ASSEMBLAGES

Paula POP, Vlad STOIAN, Bianca POP, Alexandra GHEORGHIȚĂ, Roxana VIDICAN

Department of Microbiology, Faculty of Agriculture, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 3-5 Calea Mănăștur, Cluj-Napoca, Romania

Corresponding author email: vlad.stoian@usamvcluj.ro

Abstract

Clean water and sanitation are one of the Sustainable development goals of the United Nations, and this resource needs constant monitoring due to its importance for human health. The aim of this paper was to assess the potential use of functional microbiome method to identify the geographical differences in functional microbiome in Lake Vârșolț, from Sălaj county. Basal community showed small-daily changes due to incubation, which represents a constant activity for the general microbial population. Guild activities presented a 50-100% increase each 24 hours, which sustain both the phenomenon of adaptation to substrate and proliferation due to favorable conditions. In terms of separate activities for functional groups, a different pattern of activity increases was observed for the entire 96 period of incubation. In terms of total microbial activity, the growth curves were 4-5 times in 96 hours, which represents an efficient indicator for short-term monitoring potential microbial development. The analysis of the entire database showed that functional profile is a suitable tool that enables the detection of difference in the microbiome assemblage and the forecasting of functional shifts in the community.

Key words*: activity patterns, functional microbiome, geographical distribution, guild and group assemblage, water monitoring.*

INTRODUCTION

Microbial communities are essential components of aquatic ecosystems, playing a major role in global energy fluxes and biogeochemical cycles (Falkowski et al., 2008). They exhibit high species and genetic diversity, which makes them sensitive to environmental disturbances (Amann & Rossello-Mora, 2016; Levi et al., 2017; Ren et al., 2017). In lakes, the balance of external nutrient loading influences microbial functional genes and associated metabolic processes (Liu et al., 2020).

Microorganisms populate nearly all aquatic ecosystems and are usually among the first to respond to physical and chemical disturbances. As a result, changes in the microbial community structure frequently led to broader changes in the overall health and stability of the environment.

One of the most important services performed by microbial communities in aquatic ecosystems, is the regulation of energy flows and biogeochemical cycles, with bacteria playing a central role. The microorganisms in these ecosystems exhibit high diversity and considerable biomass (Whitman et al., 1998; Battin et al., 2003; Lennon & Locey, 2016).

In lake ecosystems, most microorganisms are found in benthic biofilms, where they play a key role in organic matter mineralization, nutrient, and contaminant transformation (Schiller et al., 2007; Findlay, 2010; Buchkowski et al., 2015). Microbial communities exhibit high genetic diversity, and their taxonomic and functional changes are visible in the flow of ecosystem processes (Newton et al., 2011; Ylla et al., 2014; Hayden & Beman, 2016; Peter & Sommaruga, 2016).

Community-level physiological profiling (CLPP) is a proven approach for identifying spatial and temporal variations in aquatic microbial communities (Nowak et al., 2025; Torrús-Castillo et al., 2026). In the studies of applied ecology, EcoPlates™ are commonly used to assess the stability of microbial communities and detect functional changes triggered by environmental disturbances.

The aim of this paper is to analyze the structure of the functional microbiome within a lake ecosystem based on the community-level physiological profiling approach. The

hypotheses tested were proposed to answer a series of research questions:
i) Is there a difference between functional microbiomes due to geographical distance within the same ecosystem?
ii) Can continuous monitoring of functional activity reveal changes in the community stability?
iii) Do the dominant functional groups show shifts within the community during the incubation time?

MATERIALS AND METHODS

EcoPlate™ contains 31 organic carbon substrates and a control well, each in three replicates, with redox-sensitive tetrazolium dye, used for community-level physiological profiling (CLPP) of metabolically active heterotrophic bacteria (Stefanowicz et al., 2006). Key CLPP indicators include average well color development (AWCD), which reflects total metabolic activity, and the Sum of activities, which shows both the physiological diversity of microbial communities and the ability of a microbiome to metabolize different substrates (Kenarova et al., 2014; Muniz et al., 2014). Three sampling points were chosen in the lake and analyzed with the EcoPlate tool to score the difference between functional microbiomes recorded on a 96 hour incubation period.
The guilds used in the description of microbial metabolic activity follow the proposal of Stoian et al. (2022). The list of them is separated in 5 large guilds; CX - Carboxylic & acetic acids; AA - Amino acids; CH - Carbohydrates; PX - Polymers; AM - Amines/amides to which is added the SUM, as total metabolic activity; Water - bazal respiration; AWCD-average well color development.
All data were analysed with RStudio (R Core Team, 2024), with a flow of tests created to explore the trends of activity and community assemblage from a general view to an in-depth one. Data from 24, 48, 72 and 96 hours were supposed to a Principal Component Analysis (PCA), in "vegan" package, for the assessment of community stability and differences between sampling points (Oksanen et al., 2022). The ordinations of microbiome and environmental fitting of guild vectors were projected to determine the links between guilds and community stability. A second step for data analysis was performed with "agricolae" package, from which ANOVA and LSD tests were used to record the significant differences between sampling points in terms of guild activity and the total activity (de Mendiburu, 2023). The plate was read continuously every 24 hours up to 120 hours using a BioTek Epoch plate reader at an optical density of 590 nm.
The third step in data analysis was to extract the group with the highest activity within each guild, separately for each sampling point and incubation period. This procedure was performed to create a pattern analysis that can be developed further into a screening for indicator groups at different incubation time.
Biolog EcoPlates were selected for their power to characterize the entire microbial community in environmental matrices and providing insights on spatio-temporal changes and the kinetics of microbiome response to a set of standardized substrates (Zhao et al., 2021; Angelova et al., 2025).

RESULTS AND DISCUSSIONS

Principal Component Analysis (PCA) highlighted a clear functional differentiation between the three sampling points (W1, W2, and W3), demonstrating the ability of functional microbiome profiling to differentiate geographical variations within the lake (Figure 1, Table 1). The first principal component (PC1) explained most of the variability and distinctly separated the samples from W3 from those from W1 and W2, suggesting the existence of a functional ensemble specific to this area and a different pattern of substrate use. W1 and W2 showed relatively similar profiles, being positioned in the negative region of PC1, but a moderate separation on PC2 indicates subtle differences in the dynamics of microbial activities. The more pronounced dispersion of samples from W1 suggests higher functional variability, while the compact grouping of samples from W2 indicates a more stable structure. The vectors associated with the model highlight the differentiated contribution of certain metabolic activities to the total explained variance (95%). Both Sum and CH vectors were oriented parallel with Axis 1, showing a high degree of importance of CH guild within the

entire community activities. At the opposite, AA vector associated with W3 show an angle toward PC1, which indicate the potential of this guild to reshape the entire microbiome and gain a higher importance within community.

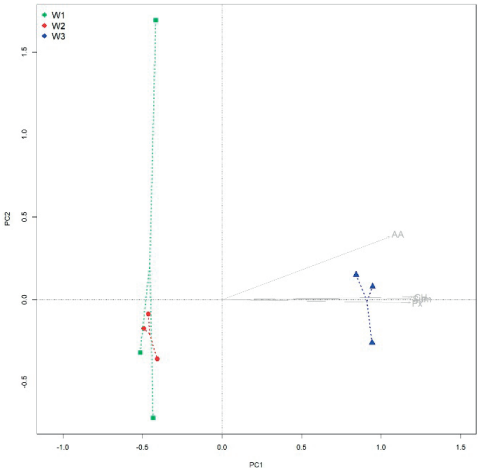


Figure 1. PCA projection of microbial communities and guild vectors at 24 h of incubation

Table 1. Vector fitting and importance on PCA projection (24 h)

	PC1	PC2	r2	p.val	
Sum	0.99999	0.00518	0.9795	0.002	**
PX	0.99987	-0.01593	0.974	0.005	**
CH	0.99992	0.01284	0.9973	0.001	***
CX	0.64094	-0.76759	0.05	0.887	
AA	0.94095	0.33854	0.875	0.002	**
AM	-0.58727	-0.80939	0.6007	0.066	.

Note: * - significant; ** - distinct significant; *** - very significant. Variance explained by Axis 1=92.64% and Axis 2=2.76%.

PCA analysis highlights a clear separation of the three sampling points along the PC1 axis (Figure 2, Table 2). W3 is positioned in the positive region of PC1 and is associated with variables oriented in the same direction (particularly AM, AA, PX, and Sum), suggesting increased intensity of metabolic activities corresponding to these functional groups. In contrast, W1 and W2 are located in the negative region of PC1, indicating functional profiles distinct from W3. The separation on PC2 further differentiates W1 (negative values) from W2 (positive values), suggesting differences in the dynamics of microbial activities between these two locations. Longer vectors indicate a significant contribution of certain metabolic activities to the total variation explained by the model, confirmed by the existence of pronounced functional heterogeneity between the analyzed points.

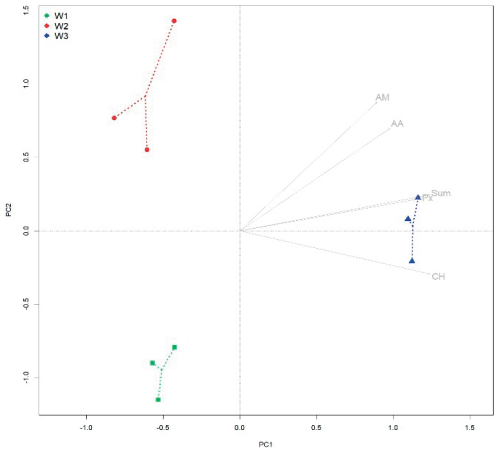


Figure 2. PCA projection of microbial communities and guild vectors at 48 h of incubation

Table 2. Vector fitting and importance on PCA projection (48 h)

	PC1	PC2	r2	p.val	
Sum	0.98097	0.19416	0.9571	0.001	***
PX	0.983	0.18362	0.8653	0.011	*
CH	0.97276	-0.23183	0.9766	0.001	***
CX	0.63794	0.77009	0.5259	0.103	
AA	0.81389	0.58102	0.8735	0.001	***
AM	0.7142	0.69994	0.9454	0.001	***

Note: * - significant; ** - distinct significant; *** - very significant. Variance explained by Axis 1=77.68% and Axis 2=11.62%.

The variance explained by the two axis is 89%, indicating both the dispersion of data in the ordination plan and the amplification of differences between communities. The Sum of activities and PX guild have coupled vectors, indicating the potential perturbation role of this guild within community. AM and AA vectors are oriented toward the higher part of the ordination and their opposite direction against W2 and W1 indicate the reduced activity within community. CH vector makes an angle with these two guilds, with a lower trend of direction. This guild is associated with one of the W3 points, indicating the potential arise of a different assembled community. Both W1 and

W3 show community similarity and stability, due to the low dispersion of points, and only small differences due to the group-activity pattern.

In this analysis, the separation on PC1 is even more pronounced, with W3 showing a clear separation in the positive area of the axis and correlated with the variables AA, AM, and PX, indicating a specific functional profile characterized by metabolic activity oriented to these substrates. W1 and W2 are grouped in the negative area of PCA but show a clear separation through PC1 and a corner position (Figure 3, Table 3).

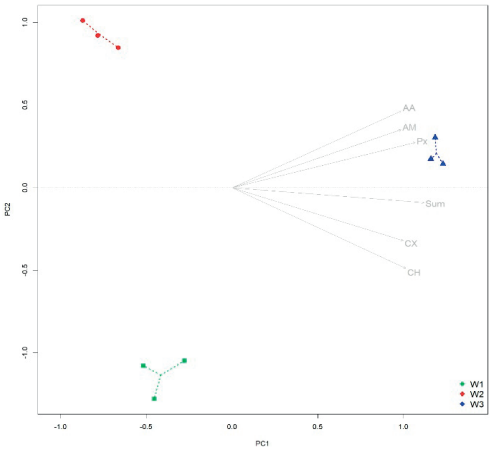


Figure 3. PCA projection of microbial communities and guild vectors at 72 h of incubation

Table 3. Vector fitting and importance on PCA projection (72 h)

	PC1	PC2	r2	p.val	
Sum	0.9968	-0.07997	0.9949	0.001	***
PX	0.96861	0.24857	0.9584	0.012	*
CH	0.90068	-0.43448	0.9954	0.001	***
CX	0.95199	-0.30614	0.8663	0.001	***
AA	0.90497	0.42548	0.9366	0.001	***
AM	0.94205	0.33549	0.8627	0.018	*

Note: * - significant; ** - distinct significant; *** - very significant. Variance explained by Axis 1=66.78% and Axis 2=18.58%.

The 85% of variance explained by the entire PCA is associated with the increased general activity (Sum vector), while all three communities became stable. The differences between communities are more visible due to their position in different quadrants. In the upper part of the ordination, AA, AM and PX guilds are associated within the same community, and their position makes them the candidates for W3 shift. All the guild vectors have an orientation against W1 and W2, indicating that a mix of group activities are responsible for the community specific assemblage.

PC1 represents the central axis of geographical differentiation, clearly separating W3 from W1 and W2 (Figure 4, Table 4). The samples from W3 are strongly positively correlated with the AA and AM, suggesting a functional profile characterized by increased metabolic activity. W1 and W2 are in the negative section of PC1, but they're different on PC2, showing extra variation in functional dynamics. W1 shows more negative values on PC2, while W2 is in the positive area, indicating a different structure of microbial activities. The direction of the vectors in the positive area of PC1 confirms the important role of certain functional groups in the spatial separation of microbial communities.

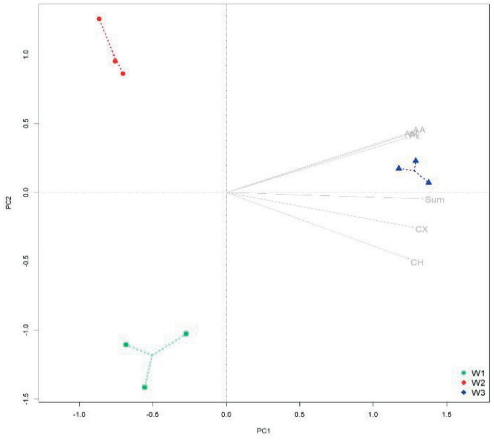


Figure 4. PCA projection of microbial communities and guild vectors at 96 h of incubation

At plateau phase, the variance explained reach 82%, with the Sum vector being the only very significant one. This vector is closed to PC1, in the middle of the angel formed by AA+AM and CH+CX vectors. This indicate that each of the two community types can be observed during the continuous evolution of the microbiome. While AA and AM are closed to each other, indicating a correlated evolution of this community type, in the case of CX and CH, a small angle can be observed, which indicates a direction to one of these guilds.

Table 4. Vector fitting and importance on PCA projection (96 h)

	PC1	PC2	r2	*p* - value	
Sum	0.9994	-0.03458	0.9962	0.001	***
PX	0.95188	0.30647	0.9248	0.005	**
CH	0.93178	-0.36302	0.9817	0.001	***
CX	0.98066	-0.19573	0.9333	0.011	*
AA	0.94472	0.32789	0.98	0.002	**
AM	0.94688	0.3216	0.8845	0.007	**

Note: * - significant; ** - distinct significant; *** - very significant. Variance explained by Axis 1=65.54% and Axis 2=16.51%

In the initial phase (Table 5), all groups (CH, CX, PX, AA, AM) show low values, which are characteristic of the period of substrate adaptation. CH and AA have more significant contributions in W1 and W2, indicating initial activity focused on the use of more easily metabolizable compounds. CX shows moderate variability between samples, with no significant differences, while PX and AM record the lowest values, suggesting limited activity of these guilds in the initial phase. W2 has the highest total value (SUM), which suggests a community with more active initial metabolic potential, but the overall.

Table 5. General and guild activity at 24 h of incubation

Sample	CH	CX	PX	AA	AM	SUM
W1	0.27±0.16 b	0.06±0.03 a	0.14±0.08 b	0.21±0.12 b	0.13±0.07 a	0.51±0.29 b
W2	0.33±0.19 b	0.34±0.19 a	0.17±0.10 b	0.20±0.11 b	0.01±0.00 a	0.86±0.49 b
W3	0.23±0.13 a	0.13±0.07 a	0.12±0.07 a	0.14±0.08 a	0.03±0.02 a	0.53±0.30 a

After 48 hours, all guilds show significant increases, indicating the start of the proliferation phase (Table 6). CH and CX are dominant, especially in W3, where they show the highest values and contribute significantly to the increase in total activity. PX and AA also show a clear intensification of activity, suggesting an expansion of the range of substrates used. The AM guild, although still lower in number than the others, begins to grow more visible, suggesting the activation of additional metabolic pathways. The differences between samples become clearer, with W3 highlighted by a higher functional response.

Table 6. General and guild activity at 48 h of incubation

Sample	CH	CX	PX	AA	AM	SUM
W1	6.52±0.36b	4.56±0.26a	1.96±0.27b	2.74±0.11b	0.49±0.10b	16.2±0.84b
W2	4.43±0.59c	5.47±0.43a	2.38±0.31b	4.07±0.95ab	1.27±0.23a	17.6±2.19b
W3	11.0±0.16a	5.68±0.36a	4.04±0.15a	5.62±0.20a	1.71±0.17a	28.0±0.27a

Note: Different letters after means ± SE indicate significant differences at $p<0.05$ based on LSD test.

CH and CX remain dominant in the functional profile, especially in W3, where values are significantly higher compared to W1 and W2 (Table 7). PX and AA show constant increases, suggests a diversification of metabolism and a more complex use of available substrates. AM becomes better represented, which may indicate microbial succession processes and trophic interactions. W2 shows the lowest values for most groups, suggesting a limited metabolic capacity.

Table 7. General and guild activity at 72 h of incubation

Sample	CH	CX	PX	AA	AM	SUM
W1	8.94±0.34b	7.25±0.13b	2.39±0.28b	3.54±0.09b	0.71±0.20b	22.8±0.84b
W2	4.01±0.55c	6.02±0.29b	2.66±0.25b	4.48±0.21b	1.03±0.19b	18.2±0.85c
W3	12.4±0.13a	8.71±0.52a	5.63±0.15a	6.95±0.46a	2.52±0.25a	36.2±0.76a

At 96 hours, the highest values for all groups are observed, and the differences between samples are most evident at this stage (Table 8). W3 has the highest values for CH, CX, PX, AA, and AM, which indicates a higher capacity to utilize different types of substrates. CH and CX remain the dominant groups, but PX, AA, and especially AM also increase significantly, indicating a more advanced microbial community structure and a more diversified use of resources. W1 has an intermediate level of activity, and W2 has the lowest values for most groups. The evolution of groups over time shows consistent functional differences between sampling locations and confirms the value of group analysis in the determination of variations in the microbial community (Table 9).

Table 8. General and guild activity at 96 h of incubation

Sample	CH	CX	PX	AA	AM	SUM
W1	10.1±0.57b	8.82±0.12b	2.78±0.39b	4.61±0.28c	0.81±0.28b	27.1±1.31b
W2	5.70±0.79c	7.38±0.27c	3.56±0.46b	5.51±0.14b	1.28±0.37b	23.4±0.85c
W3	15.1±0.49a	11.9±0.36a	6.61±0.15a	8.85±0.24a	3.16±0.25a	45.6±0.22a

Table 9. Group-activity pattern analysis in the 24-96 time range

	W1					W2					W3				
Guild	**CH**	**CX**	**P**	**AA**	**AM**	**CH**	**CX**	**P**	**AA**	**AM**	**CH**	**CX**	**P**	**AA**	**AM**
24 h	CH3	CX9	P1	AA2	AM2	CH3	CX9	P2	AA2	AM2	CH4	CX6	P4	AA4	AM2
48 h	CH8	CX2	P1	AA1	AM2	CH7	CX2	P2	AA2	AM2	CH7	CX3	P4	AA2	AM2
72 h	CH8	CX2	P1	AA1	AM2	CH8	CX3	P2	AA2	AM2	CH7	CX3	P4	AA2	AM1
96 h	CH8	CX2	P2	AA2	AM2	CH8	CX3	P2	AA1	AM2	CH7	CX3	P4	AA2	AM1

Seasonal stratification is a major factor influencing the dynamics of microbial communities, especially in deep lakes, where temperature, oxygen, and nutrient gradients form distinct ecological niches (Winder & Sommer, 2012; Liu & Santamarina, 2024). Microbial communities are influenced by both deterministic, niche-based processes and stochastic mechanisms, including environmental selection and ecological drift (Chase & Myers, 2011; Stegen et al., 2013; Zhang et al., 2021).

Flowing water systems (rivers – lotic ecosystems) and standing water systems (lakes – lentic ecosystems) are essential components of terrestrial aquatic ecosystems, playing a major role in global biogeochemical processes (Maavara et al., 2020; Battin et al., 2023).

Hydrological factors, such as flow rates, water levels, retention times, and precipitation, influence the nutrient cycle and, shaping the structure of bacterial communities, can lead to biodiversity decline (Shabarova et al., 2021; Zhang et al., 2021; Chen et al., 2023).

CONCLUSIONS

The study highlights that functional analysis of the aquatic microbiome is an effective tool for identification of spatial differences between microbial communities in lake ecosystems. Results from CLPP analysis with EcoPlate™ showed distinct metabolic profiles between the sample points (W1, W2, and W3). PCA analysis revealed a distinct separation of samples, especially from W3, which indicates the existence of a functional profile specific to this location and a different pattern of organic substrate utilization. While points W1 and W2 showed relatively similar functional profiles, W3 was characterized by higher metabolic intensity and a greater capacity to utilize different types of substrates.

The functional dynamics of microbial communities showed a progressive increase in metabolic activity during incubation. In the initial stage, the values of all functional groups (CH, CX, PX, AA, and AM) were low, which reflects the period of adaptation of microorganisms to the substrate. After 24 hours, a significant increase in metabolic activity was observed, mainly for the CH and CX groups, which were dominant in the functional profile. As incubation progressed, all groups showed a gradual increase in metabolic activity, suggesting a diversification of metabolism and a more complex use of available resources.

At 96 hours, the highest values were recorded for all functional groups, with differences between sampling points being more evident. W3 showed the highest metabolic activity and substrate utilization capacity, which indicates a more active and diverse microbial community. In contrast, W2 recorded the lowest values for most functional groups, suggesting a lower metabolic capacity or a faster stabilization of microbial activity.

The results show that the analysis of functional groups and substrate utilization can highlight important differences between microbial communities in different areas of the lake. These methods are useful for understanding the metabolic dynamics and adaptation of the microbiome to environmental conditions, confirming the value of functional analyses for monitoring spatial variations and ecological changes in aquatic ecosystems.

The results support the fact that functional analysis is an effective tool for identifying spatial heterogeneity and monitoring potential functional changes in microbial communities in lake ecosystems.

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