Microbial Physiological Diversity Assessment in Protected Wetlands: A Case Study in Maritza River Basin, South Bulgaria

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Abstract. This study explored the spatial differences and physiological diversity of soil microbial communities in natural wetland and constructed rice paddies in the Maritsa River Basin, protected under the Birds Directive 2009/147/EC as natural habitats. Community level physiological profiling was used to characterize the metabolic function of the communities. The microbial activity expressed, as average well colour development (AWCD) and substrate richness and diversity was higher in the non-flooded soils with no significant spatial differences. Individual carbon source utilization patterns (CSUPs) segregated the paddy fields profiles from the natural wetland sediments and showed that communities` metabolic functions were strongly influenced by rice cropping and not by the sample location. The CUSPs revealed that easily degradable carbohydrates were the most widely used substrates followed by amino acids, carboxylic acids, and polyols. Natural wetland communities held an isolated position with lower nutrient supply and intensive catabolic activity regarding somewhat biochemically inert substrates polyols, phenolic acids, and amines indicating higher functional diversity. The presented study is evident for the good ecological potential of the Zlato pole wetlands and demonstrates that the extent of carbon uptake is determined by the habitat but also reflects the anthropogenic impact.

Key words: Wetlands, rice paddies, Biolog EcoPlates, AWCD, Community level physiological profile, PCA.

Introduction

Wetlands are found at the interface of aquatic and terrestrial ecosystems. In Bulgaria, they vary in size from large water bodies such as lakes (> 100 ha) to small water complexes (< 0.1ha) (Stoyneva & Michev, 2007). Wetlands play a crucial role in nutrient cycling as they are estimated to contain 20% to 30% of the global soil carbon pool, yet their area worldwide is shrinking as a result of constant urbanization, agricultural pressure and climate change (He et al., 2015). Wetlands located along the Bulgarian part of the Maritsa River basin are part of the Ramsar Convention and represent specific types of ecosystems rare for the state and with international importance. They include natural wetlands and periodically flooded rice paddy fields located in a densely populated area and are therefore very sensitive to human activities related to water management and forestry. The studied wetlands play a crucial role in biodiversity support of many protected animal and plant species. According to Weber (2016), rice paddies can also be

classified as "treatment wetlands" (TW) due to their role of catchment areas for water pollution and water quality improvement.

Monitoring wetland health and functioning has been an ongoing goal of their management. Over the years the studies have been focused on microbial communities` structure and diversity as biological indicators for wetlands ecosystem stability due to the relative ease of identifying and counting them (Urakawa & Bernhard, 2017). Microbial communities are considered as functional units that consist of the sum of the metabolic properties of individual bacteria (Garland, 1997). They play fundamental role in wetland biogeochemistry, and are directly related to the rate of decomposition of organic matter and, in turn, depend on the factors of the environment, such as quantity and quality of organic matter, pH, moisture, etc. (Boivin et al., 2006; Kenarova et al., 2014). In this regard, rice paddy fields are considered unique model ecosystems to study wetland soil microbial ecology (Hussain et al., 2012).

Intensive rice cultivation relates to the usage of organic and chemical fertilizers. This practice could have a negative impact on the soil ecosystem (Luo et al., 2016) affecting the microbial composition and function. The extreme sensitivity of the microorganisms to even small fluxes in the environment renders their metabolic diversity arguably more relevant measures for detection of early signs of degradation in wetland ecosystems (Merkley et al., 2004). The functional diversity and catabolic potential of the communities can be evaluated using culture-based methods such as community level physiological profiling (CLPP) (Garland, 1997; Liu et al., 2013). Biolog EcoPlateTM technique is designed to estimate CLPPs of bacteria by measuring the relative utilization of various ecologically relevant organic substrates. Despite its limitations (Garland, 1999), it has proven to be a useful tool for the detection of differences in CLPPs of bacteria in wetlands (Button et al., 2015; Luo et al., 2016; Zhang et al., 2010).

The objective of the present study was to explore the spatial differences in the metabolic activity of bacterial communities in natural and constructed wetlands in the Maritsa River Basin. The effect of rice cropping as a major factor affecting the soil chemical composition and differentiating bacterial community physiological profiles was tested in rice planted soil versus naturally flooded wetland.

Materials and Methods
Site description and sampling
Two wetlands along the Bulgarian part of Maritsa River were studied – Zlato Pole wetland and Tsalapitsa rice paddies ("Orizishta Tsalapitsa"), both protected under the Birds Directive (79/409/EEC). Protected zone Zlato Pole (42°2.207', 25°42.938') is the largest natural wetland (BG0002103) along the Bulgarian part of the Maritsa River. It is located several kilometers away from the town of Dimitrovgrad, south of the Zlato pole village. Protected zone "Orizishta Tsalapitsa" (BG0002086) is a complex of rice paddies situated between the village of Tsalapitsa (42°13.600', 24° 33.804') and the city of Plovdiv (42°10.307', 24°40.502').

Soil samples from the topsoil 0-10 cm were collected in triplicates in July 2017 and July 2018 during the rice maturity stage (after application of all fertilizers), from flooded and non-flooded rice paddies and from the sediments and the non-flooded area at Zlato Pole. Nine random soil cores within each site were mixed to provide one sample (Luo et al., 2016). Each sample was partitioned into two subsamples: one was partially air-dried and sieved through a 1 mm sieve for chemical analysis, and one was placed in sterile 50 ml containers and stored at 4°C in the dark for microbiological analysis for no longer than 24 h.
Soil parameters
The sieved samples were used to determine soil moisture, pH, $N-NH_4^+$ (ammonia), $N-NO_3^-$ (nitrate), ON (organic nitrogen), OM (organic matter), AP

(available phosphorus). Soil moisture was determined by calculating the weight of lost water after drying the sample at 105°C for 24 h. Soil pH was determined in a 1:2.5 soil:deionized water suspension with a pH meter (WTW/SET). Inorganic N-NO$_3^-$ and NH$_4^+$ were analysed after soil extraction with 0.1 N KCl according to (Motsara & Roy, 2008). Organic nitrogen was determined by the Kjeldahl method after mineralization with selenium in DK-6 (VELP-Scientifica) module for decomposition and a semi-automatic analysis system for distillation UDK-132 (VELP-Scientifica). OM was measured by calculating the loss of weight on ignition at 600°C. AP was determined spectrophotometrically by the Olsen's method (Motsara & Roy, 2008).

Microbial community profiles

The pattern of utilization of individual carbon sources by the soil microbial communities was assessed using BIOLOG-EcoPlates (Biolog Inc., Hayward CA, USA), containing 31 different C sources in three replicates. Tetrazolium redox dye that changes from colourless to purple was used as an indicator of the ability of microorganisms to metabolize the organic carbon. Cell suspensions were prepared from 1 g soil suspended in 99 ml sterile saline solution (0.85% w/v NaCl) on a rotary shaker at 200 rpm for 30 min and then left to settle for 10 min. The supernatant was filtered subsequently through 8.0 and 3.0 µm nucleopore membranes (Whatman). Each well in the plates was inoculated with 150 µl of the cell suspension. The plates were incubated at 22°C in dark for 14 days. Colour development was measured as described by Garland (1997). Optical density (OD) at 590 nm of each well was recorded with MULTISKAN FC microplate reader (Thermo Fisher Scientific, Shanghai, China) every 24 h.

Prior to analysis, the OD value of the control wells was subtracted from the individual substrate absorbances and the negative readings (OD < 0) were excluded. Data were represented by the mean (n = 3). The microbial activity was expressed by average well color development (AWCD) according to Garland and Milles (1991):

$$AWCD = \sum \frac{(n_i - c)}{31}$$

where n$_i$ and c were the average absorptions of the three wells of the substrate and the control wells (without a C source), respectively. AWCD were calculated daily throughout the incubation period to reveal the kinetic profiles. EcoPlate substrata were grouped into six biochemical categories including carbohydrates, carboxylic acids, amino acids, amines, polyols, and phenolic acids following Kenarova et al. (2014).

Data Analysis

Pearson correlation, analysis of variance (ANOVA) and cluster analysis were carried out with Statistica software (StatSoft, version 10). Principal component analysis was conducted with PRIMER 6 (Clarke & Gorley, 2005). The AWCD 0.75 value (between 0.25 and 1) was chosen as a reference point for all further statistical analysis as recommended by Garland et al. (2001) and standardization of the data was performed to reduce any bias due to inoculum density differences. Calculation of precise OD values at the point of AWCD$_{0.75}$ was achieved by linear interpolation based on the assumption that the relevant part of the curve follows a linear function (Salomo et al., 2009). The normality, homoscedasticity and the number of linear correlations of data set were evaluated, followed by principal component analysis (PCA) based on AWCD for reduction of the dimensionality of the n-dimensional data set and extraction of an orthogonal set of principal components (PCs) made up of linear subsets of the original ordinates. Subsequent cluster analysis (CA) based on the squared Euclidian distances was performed to validate the results obtained from the PCA, and to group and visualize

the similar bacterial CLPPs in a dendrogram (Weber & Legge, 2014). Shannon richness, diversity and substrate evenness indices were calculated to assess the functional diversities of the communities. Richness [S] values were calculated as the average number of oxidized C substrates in the three replicates. The substrate diversity (Shannon index [H]) was calculated as follows: $H = -\Sigma p_i(\log_2(p_i))$, where p_i is the ratio of the activity on each substrate (OD_i) to the sum of activities on all substrates (ΣOD_i). The evenness [E] was calculated as $E = H/\log_2(S)$ (Luo et al., 2016).

Results

Environmental parameters

The soil profiles of the two types of samples were highly differentiated, with parameter values varying in a relatively wide range between the stations located in different zones as well as between the samples of the non-flooded and flooded areas (Table 1). The sediment samples from Zlato Pole (ZP1) and the samples from the surrounding non-flooded area (ZP2) were characterized as sandy. The samples taken from the sediments at Plovdiv (P1) and Tsalaptsa (C1) were characterized as clay and silty-clay – for the soils from the adjacent non-flooded zones at Plovdiv (P2) and Tsalapitsa (C2) rice paddies. The soil water content (SM) of the sediment was at 25-47%, with significantly higher values in the clay soils (p < 0.05). For ZP2, P2, and C2 the moisture was at 7-9% with no significant differences. The soil pH was neutral to slightly alkaline (6.9-7.3) with the exception

of C1 and C2 (5.12-5.25) which were defined as acidic. The distribution of nitrogen forms, AP and organic matter was site-specific with higher values in the paddy filed samples, compared to ZP. In general, the sediments were less abundant in ammonium (N-NH$_4^+$), nitrate (N-NO$_3^-$), organic nitrogen and organic matter than the non-flooded soils (P2 and C2) and ZP2 (p = 0.039), and were more abundant in ammonium nitrogen (N-NH$_4^+$). A significant positive correlation was established for N-NO$_3^-$ with organic matter (r = 0.892) (Table 2). A high concentration of available phosphorus was found at all stations, explaining the lack of correlation between AP and the substrate utilization rates by the microbial communities.

Comparative analysis of the sampling sites performed by PCA (Fig. 1A) and cluster analysis (Fig. 1B) suggested distinct physicochemical characteristics between the flooded and the non-flooded areas as well as significant spatial differences between stations. The PCA generated two principal components accounting 80.32% of the total data variance, separating the samples in four groups - of acidic sediment C1 (quadrant 1) and non-flooded soil C2 (quadrant 2), ON and OM depleted sediments ZP1 and P1 (quadrant 3), and nutrient-rich dry soils P2 and ZP2 (quadrant 4). With high loadings in PC1 were SM (0.904), N-NH$_4^+$ (0.737), N-NO$_3^-$ (-0.873), ON (0.521) and AP (-0.910), while in PC2 variability was caused by pH (0.843) and OM (-0.775). Cluster analysis showed similar grouping, differentiating sites based on their specific environmental characteristics (Fig. 1B).

Table 1. Average values for the environmental parameters from the sampling sites at the studied wetlands.

Station	SM %	pH (H$_2$O)	N-NH$_4^+$ mg.kg^{-1}	N-NO$_3^-$ mg.kg^{-1}	ON %	OM %	AP mg.kg^{-1}
ZP1	25.36	7.55	86.6	5.7	1.18	3.53	83.5
ZP2	9.11	7.21	18.8	14.4	10.1	44.64	72.5
P1	37.51	7.24	68.8	10.3	3.98	12.02	264.0
P2	7.18	7.33	26.9	39.9	12.2	59.90	99.5
C1	44.20	5.12	70.0	7.5	3.76	9.40	175.0
C2	7.60	5.25	48.7	20.8	6.05	86.07	78.0

Table 2. Pearson correlation matrix of the environmental parameters, AWCD and substrate classes utilization by the bacterial communities inhabiting the studied wetlands. Legend: * - Correlation is significant at the 0.05 level (2-tailed). ** - Correlation is significant at the 0.01 level (2-tailed).

	SM	pH	NH$_4^+$	NO$_3^-$	ON	OM	AP	AWCD	PM	CH	CA	PA	AA	AM
SM	1													
pH	-.140	1												
NH$_4^+$	**.855***	-.116	1											
NO$_3^-$	**-.818***	.128	-.693	1										
ON	-.684	.188	**-.962****	**.835***	1									
OM	-.809	-.280	-.694	**.892***	.651	1								
AP	**.903***	-.028	.425	-.320	-.360	-.533	1							
AWCD	**-.726***	-.282	**-.875***	**.845***	**.827***	**.900***	-.395	1						
PM	.504	.234	**.871***	-.557	-.802	-.717	.097	**-.936****	1					
CH	-.071	-.431	-.607	.433	.603	.440	.278	**.825***	**-.871***	1				
CA	**-.899***	.045	-.524	.348	.393	.782	-.775	.641	-.376	-.092	1			
PA	**-.668***	.668	-.404	.486	.477	.195	-.704	.081	.084	-.403	**.749***	1		
AA	.644	.333	**.858***	-.680	-.808	**-.875***	.291	**-.985****	**.954****	**-.792***	-.516	-.014	1	
AM	.125	.110	.663	-.644	-.767	-.296	.839	-.535	.710	**-.853***	.226	.002	.649	1
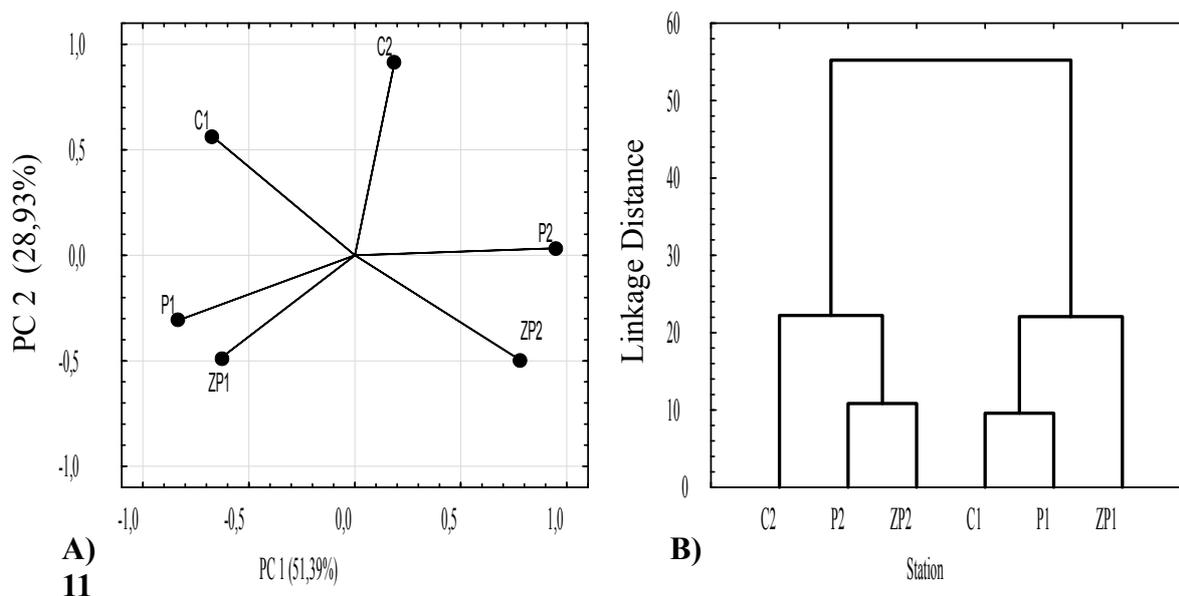
Fig. 1. Soil plots based on the environmental parameters by principal component analysis(**A**) and cluster analysis (**B**).

AWCD and CLPP

The rate of the average well color development (AWCD) over time for all six samples followed a sigmoidal curve, with short lag phase (< 24 h) nearly equal in all soil samples (Fig. 2). The kinetic curves demonstrated differences in the substrate utilization pattern. The non-flooded soil bacteria (ZP2, P2, and C2) were generally more active on the EcoPlates, with higher utilization rates and steeper slopes than the flooded communities indicating microbial activity from fast-growing heterotrophs. The AWCD values in the rice paddies sediments were greater than the ZP1, which suggests that rice cropping also stimulated

the growth of fast-growing heterotrophs. In the ZP1 samples, carbon sources were by trend slowest metabolized, differing significantly from the other samples ($p < 0.05$). The $AWCD_{0.2}$ value for all samples was reached over a relatively large time period 24-40 h, whereas the evaluation point, chosen for further analysis $AWCD_{0.75}$ occurred after 48-96 h showing clear differences based on sample types. Such differentiation is based on the physicochemical soil properties expressed as a strong positive correlation of the AWCD with OM ($r = 0.900$), nitrate ($r = 0.827$) and organic nitrogen ($r = 0.845$).

After 14 days of incubation with the exception of ZP1, the studied microbial communities were able to utilize 26 to 30 of the 31 carbon sources in the EcoPlates. None of the analyzed communities metabolized the γ-hydroxybutyric acid. ZP1 bacteria showed a reduced catabolic diversity, being unable to oxidase 50% of the tested carbohydrates: α-cyclodextrin, glycogen, β-methyl-D-glucoside, α-D-lactose, Glucose-1-phosphate and D, L,-α-glycerol phosphate. The rates of substrates utilization by categories are shown in Fig. 3A. The profiles were biochemically dependent and corresponded to the habitat type. With the exception of ZP1, carbohydrates (CH) were the most widely used substrates by the studied communities followed by amino acids (AA), carboxylic acids (CA), and polyols (PM).

The CLPP profiles based on the PCA showed obvious similarities between rice paddies sediments plots (P1 and C1) as well as between the non-flooded samples at all sites (Fig. 3B). ZP1 was characterized by lower CH and higher AA, PM and AM utilization rate and its CLPP apparently held an isolated position. PC1 accounted for 57% of the total variance. It demonstrated that substrate classes of CH, PM, and AA had the highest factor loadings and are responsible for the similarities in C1 and C2 plots regarding carbon utilization rates. The plotted vectors of CA and PA are orientated

parallel to the PC2 axis between the ZP1 and the dry soils plots (ZP2, P2, and C2) indicating that they are utilized equally in these samples.
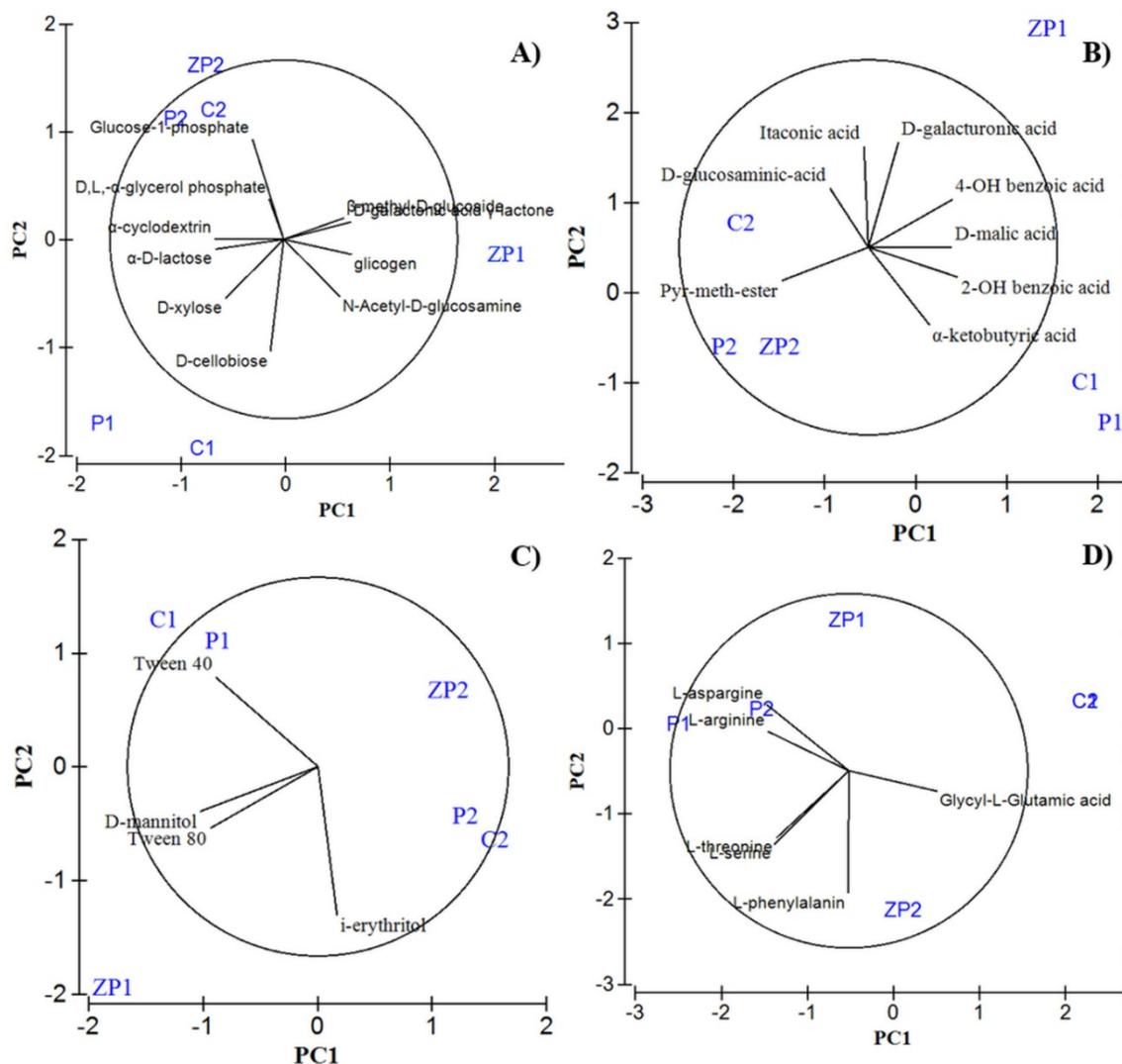
The substrate level utilization patterns of the microbial communities were further analyzed based on the plots of individual carbon sources utilization profiles (CUSPs) (Fig. 4). The non-flooded soils surrounding the wetlands showed extremely high similarity regarding the utilization of the majority of substrates in the Ecoplate, while C1 and P1 differed at substrate level despite their high resemblance regarding substrate classes. The carbohydrates were the most widely used substrates, affecting significantly the overall AWCD ($r = 0.825$) (Table 2). The PCA plot segregated the communities in three separate quadrants (Fig. 4A). The non-flooded soils communities (ZP2, P2, and C2) metabolized easily degradable monosaccharides with a phosphate group, such as glucose-1-P and α-glycerol-P, while sediment communities preferentially oxidized xylose and cellobiose, both products of plant decomposition. The other carbohydrates were evenly metabolized in the different samples. ZP1 showed individual significantly different utilization pattern, where five of the carbohydrates were not utilized at all. All communities were able to degrade the tested carboxylic acids, expect the biochemically inert γ-hydroxybutyric acid, with an equal rate for the majority of them (Fig. 4B). The utilization of phenolic acids was rather site-specific. They were predominantly oxidized by the flooded soils communities. The degradation pattern also revealed a remarkably high similarity between rice paddies sediment communities. In contrast to carbohydrates, the Zlato Pole sediments were characterized by a broader spectrum and utilization rates compared to the other sites. ZP1 communities showed the best growth on hydrogenated carbon sources (polyols) (Fig. 4C).
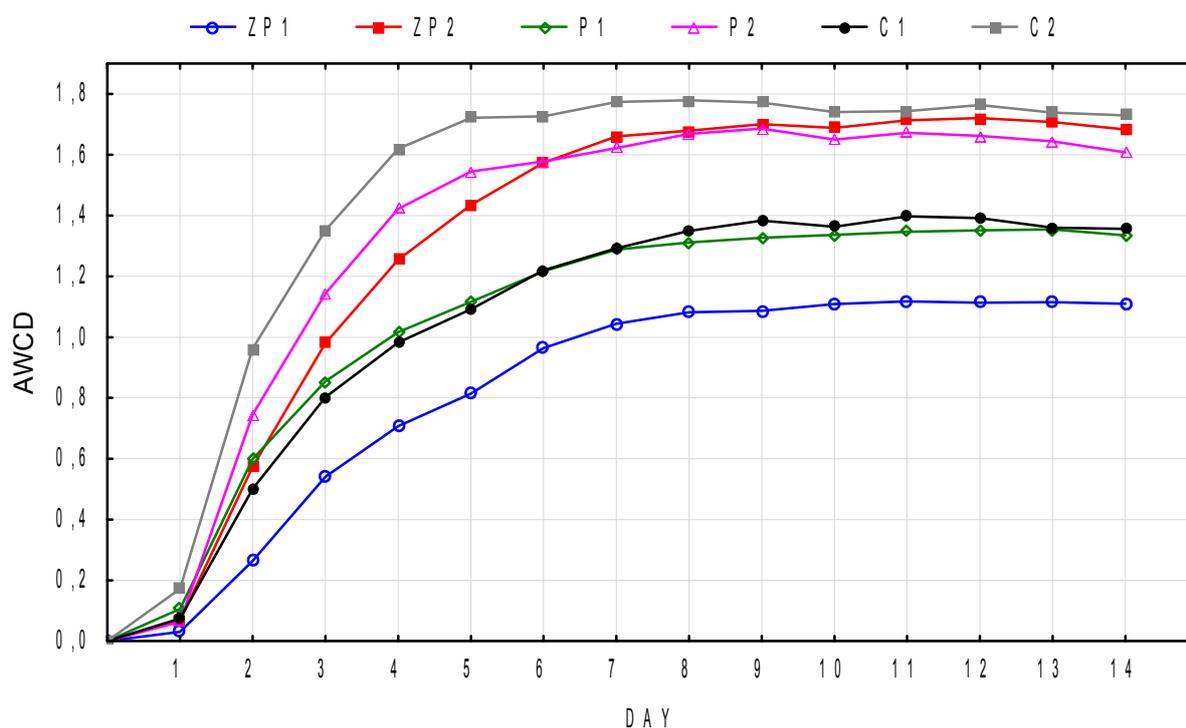
Fig. 2. Kinetic curve of carbon sources utilization by the communities inhabiting wetlands in the Maritza River Basin expressed as AWCD.
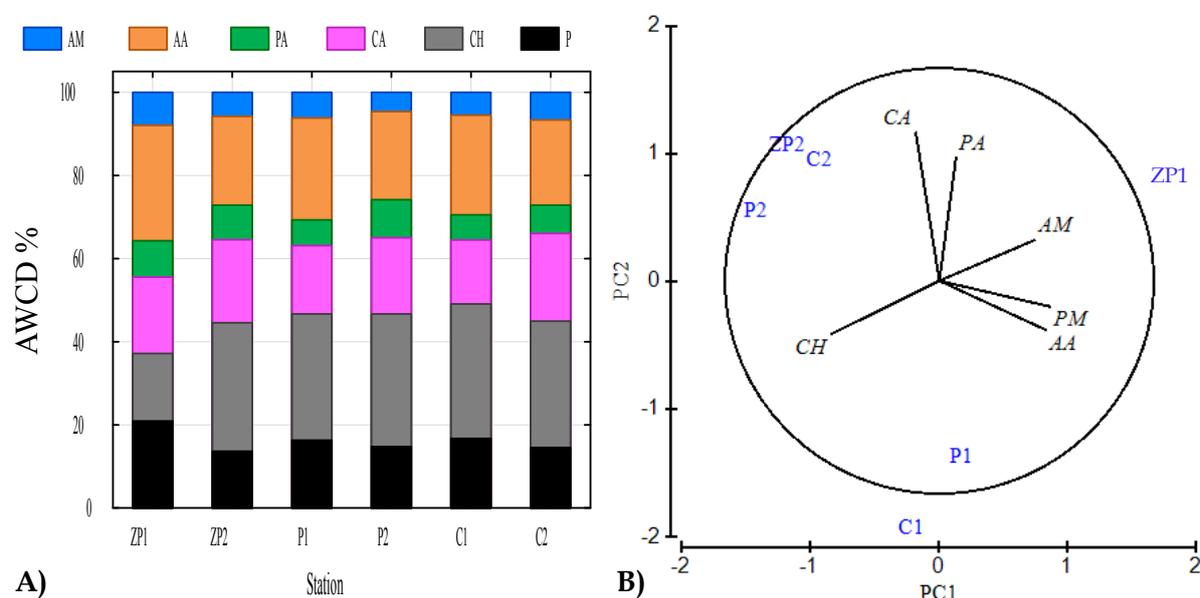
Fig. 3. Bacterial activity (**A**) expressed as a percentage of the total substrate utilization by categories: amines (A); amino acids (AA); phenolic acids (PA); carboxylic acids (CA); carbohydrates (CH); polyols (PM) and PCA (**B**) score plot (PC1 – 57.8%; PC2 – 28.4%) of the community level physiological profiles (CLPPs).

Fig. 4. Substrate level utilization pattern of the microbial communities (PC – part of total data variance (%)): **A** – carbohydrates (PC1 – 65.2%; PC2 – 21.4%); **B** – carboxylic and phenolic acids (PC1 – 45.1%; PC2 – 27.8%); **C** – polyols (PC1 – 55.4%; PC2 – 38.6%); **D** – amino acids (PC1 – 56.6%; PC2 – 27.4%).

Amino acids/amines PCA plot was significantly different and revealed a distinct dissimilarity between the two paddy fields (Fig. 4D). The highest OD was measured for arginine and asparagine for all samples, while only the P1 and P2 communities utilized two of the amino acids: threonine and glycyl-L-glutamic acid. An interesting fact was that ZP1 and ZP2 communities were unable to grow on serine and threonine despite their similar chemical structure with asparagine. The growth on amino acids/amines was inversely proportional to the carbohydrates oxidation (r = -0.792/ -0.853). The result suggested that the amino acid utilization pattern was site rather than habitat determined. Both polyols and amino acids were negatively correlated with the AWCD (r = -0.936; r = -0.985).

The data on the substrate utilization was used for calculation of the microbial functional diversity indices (Table 3). $AWCD_{0.75}$ was chosen as a reference point, in order to access the whole community activity and not only that of the fast-growing heterotrophs. The samples differed

significantly in their substrate richness and diversity ($p < 0.05$). Shannon index was high in the range of 3.9-4.4; hence, the bacterial activity in the samples must be quite uniformly distributed with few or no dominant species. The *H* values showed higher functional diversity of the non-flooded soils communities than that in the flooded areas. The Pielou`s evenness index was affected by the sample type segregating flooded and non-flooded soils and detected significant differences between the rice paddies sediments plots (C1 and P1) and natural wetland sediments (ZP1). This indicates that microbial diversity and potential for carbon source utilization was slightly higher in natural wetland than in the paddy fields, despite the relatively similar environmental conditions. Hence, the microbial functional activity and diversity are affected by the studied environmental parameters, but they are also significantly influenced by the anthropogenic pressure expressed as flooding with waters with high organic loads, rice cropping, and other agricultural activities.

Table 3. Substrate richness (S), diversity (H), and evenness (E) indices based on the microbial functional soil bacteria inhabiting flooded and non-flooded wetlands.

Station	Richness [S]	Shannon [H]	Evenness [E]
ZP1	24.7±0.67	4.05±0.02	0.88±0.02
ZP2	30.0±0.0	4.22±0.00	0.80±0.00
P1	26.7±1.5	3.85±0.01	0.79±0.00
P2	30.0±0.0	4.47±0.04	0.90±0.00
C1	28.0±1.0	3.83±0.00	0.77±0.00
C2	30.0±0.0	4.39±0.10	0.90±0.01

Cluster analysis of the CLPP revealed different site grouping compared to that based on the physicochemical parameters and separated the plots into three clusters with site-specific physiological profiles (Figure 5), comprising the rice paddies sediments (CA1), non-flooded substrata (CA2), and Zlato Pole sediments (CA3). The CLPPs clustered in CA2 (P2 and C2) were characterized by lower linkage distance than those of the flooded communities (P1 and C1). The distance between rice paddies CLPPs and that of ZP1 (CA3) was much higher.
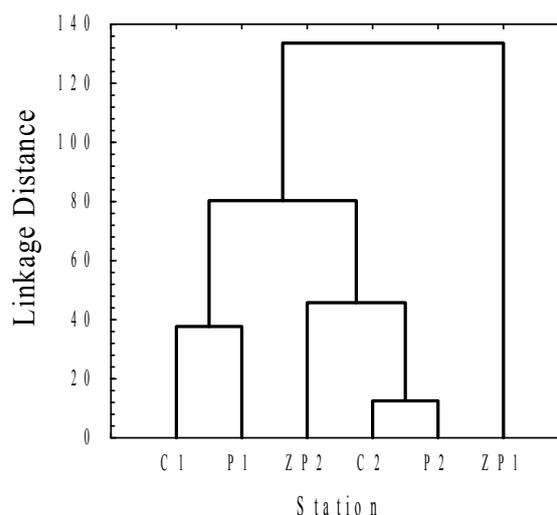
Fig. 5. Dendrogram of the soil plots based on bacterial CLPPs.

Discussion

One of the main purposes of this study was to reveal the metabolic profiles of bacterial communities using CLPP in wetland soil environment and to analyze their activity driven by the environmental factors and agricultural activities. The results indicate that the physiological profiles and the community diversity indices of the flooded and non-flooded areas significantly differed, reflecting the differences in their physicochemical parameters. Certain soil properties were promptly related to the substrate utilization patterns, suggesting their ecological relevance to soil microbial communities.

In particular, soils with high water content, caused by the wetland flooding, were associated with lower AWCD and reduced diversity (*H* values). Our findings

suggest that such soils support lower numbers of microbial species as these zones demonstrate reduced substrate utilization rate. The result confirmed that SM has a negative impact on the soil structure, microbial communities and mineralization rate in general as described by Borowik and Wyszkowska (2016) and Marhova et al. (2018). Water content and water holding capacity of the soils seem to be a major factor influencing the quantitative and qualitative composition of microbial communities in the sediments of wetlands and lake ecosystems (Ma et al., 2018).

Our results indicate a high organic matter and nitrogen concentrations in the non-flooded areas compared to the flooded rice paddies and natural wetland, exceeding several times the values established by other authors for the Plovdiv region (Krasteva-Milanova, 2017, Plovdiv – pers. comm.). The rice paddies were also characterized by a more nutrient-rich environment than the Zlato Pole wetland, due to their regular drainage and flooding leading to the introduction of organic matter with incoming waters (Das & Chakrabarti, 2013), and moreover due to the regular fertilization. However, the significantly lower concentrations of nitrate-nitrogen in rice sediments compared to the adjacent non-flooded areas (p = 0.039), is a result of the higher availability of $N\text{-}NO_3^-$ for plants that can absorb a large quantity of nitrates as described by Farrell et al. (2014). The organic inputs affect food-web interactions and are expected to be one of the primary mechanisms that influence microbial activity (Cartwright et al., 2016). So not surprisingly soil OM and ON were strongly positively related to AWCD (r = 0.900) and H (r = 0.916), suggesting that carbon, nitrogen, and natural vegetation rich non-flooded areas supported more diverse bacterial communities. Marhova at al. (2018) also draws a similar conclusion studying the effect of organic nitrogen content on the quantity and biodiversity of microorganisms in wetland sediments. Our results are consistent with the findings of a significant increase in AWCD, and *H* several months after organic amendment application (Gomez et al., 2006). In the present study, high phosphate levels were found at all stations and the absence of correlation between soil phosphorus and any of the studied microbiological parameters may indicate that AP was not a limiting factor despite its low natural bioavailability and rapid mineralization rates.

The Biolog Eco-plate technique was applied to provide data for the physiological activity of the culturable fraction of the microbial communities (Kirk et al., 2004). The results demonstrated differences in the adaptation period to the artificial environment as well as a significant differentiation regarding the utilization of the carbon sources by the natural wetland and rice paddies communities. The studied groups of energy sources were utilized rather similarly but there were significant differences in the utilization of individual carbon substrates by stations. With the exception of the flooded natural wetland (ZP1), the Biolog data suggests the presence of fast-growing heterotrophs with a relatively higher carbon utilization activity. This shows that soil types and the environmental factors related to and cropping systems have a leading role in the CLPP over the geographical location of the studied areas.

The AWCD highlighted all non-flooded areas as places with the highest number of utilized substrates, confirming the importance of soil water content. The communities showed clear similarities regarding the substrate utilization patterns especially for the group of carbohydrates and carboxylic acids. Easily degradable CH such as glucose-1-P and α-glycerol-P and CA, which are mainly the products of carbohydrate metabolism, were better utilized in comparison with the flooded areas. They represent a preferred energy source for the majority of microorganisms (Button et al., 2015). According to the results,

the communities in these areas have adapted to metabolize accessible carbohydrates, due to their excess in the environment, but also possessed the potential to utilize other diverse energy sources. The observed similarity between non-flooded soils could be explained with the presence of a high variety of plant species transforming the whole land as a rhizosphere. A number of authors (Baudoin et al., 2003; Frac et al., 2012) confirmed that root exudates of in undisturbed natural soils compared to fertilizer-amended soils stimulated bacterial activity and diversity. The catabolic evenness and diversity values are evident for high stress and disturbance resistance of the communities in the non-flooded areas. Such stability is typical for pastures and soils with high vegetation score (Cartwright et al., 2016; Degens et al., 2001). In these ecosystems, OM, cation exchange and microbial biomass are also reported to be greater (Marhova et al., 2018; Sparling et al. 1992). They offer stress resistance to the microbial communities and could enhance the recolonization in the event of environmental disturbances (Degens et al., 2001).

The communities in the flooded Tsalapitsa rice paddies (P1 and C1) were characterized by a preferential growth on α-cyclodextrin, xylose, and cellobiose as well as amino acids and polyols. Such patterns are typical for rice cropping systems where over time the plant residues deposit over the surface layer and cellulose represent a major carbon input, subjected to decomposition by the sediment microbial communities. The microorganisms in the surface sediment layer further metabolize products like xylose and cellobiose (Salomo et al., 2009). The better utilization of α-cyclodextrin and Tween 40 also suggests that the flooded communities receive substances from plant residues and confirms that the communities in such soils are well adapted to such carbon sources (Das & Chakrabarti, 2013), but at the expense of their limited ability to utilize carboxylic and phenolic acids.

The natural flooded wetland was strongly influenced by the lacking availability of organic matter, and nitrogen and always held an isolated position regarding the carbon utilization patterns. The bacterial community of ZP1 area is well adapted to polyols, phenolic acids, amino acids, and amines, suggesting that the bacteria in the natural environment are limited in substrates. Transferring bacteria from such conditions to an artificial medium with high substrate concentrations could lead to "substrate-accelerated death" (Straskrabova´, 1983). In this regard, the polyols seem to be a better substrate than the easily degradable CH, as they pass an initial hydrolysis step and "eliminate sudden exposure" to substrates (Chin et al., 1999). The utilization of a wide variety of substrates is related to higher diversity and catabolic evenness compared to the rice paddies.

Conclusion

Microbial metabolic function in the studied wetlands differed significantly and demonstrated that the substrate utilization profile of the communities is not determined by a single environmental factor. In fact, a broad spectrum of parameters such as soil water content, pH, organic matter, and nitrogen forms in the soils was all dominant. The PCA and cluster analysis clearly revealed that the long-term agricultural exploitation is related to changes in soil properties and bacterial communities, grouping the rice paddies in a separate cluster. The continuous rice cropping in the Tsalapitsa wetland lead to soil acidification and are related to a higher overall metabolic activity but a lower catabolic richness and substrate diversity which makes the microbial communities sensitive to stress and external factors. The higher substrate diversity, along with the sandy river like sediments, the low concentration of organic nitrogen, organic matter, and phosphates, are evident for the good ecological potential of the Zlato Pole wetland. This is also

confirmed by the higher metabolic activity regarding the more difficult for utilization polyols and amino acids due to the lack of easily digestible carbohydrates.

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