ECOLOGIA BALKANICA
2018, Vol. 10, Issue 2 December 2018 pp. 173-184

Microbial Community Structure and its Biofilm Forming Capacity in Wetland Soils, Southern Bulgaria

Mariana Marhova*, Marinela Tsankova, Sonya Kostadinova, Ivan Iliev

Plovdiv University "Paisii Hilendarski", Faculty of Biology, Department of Biochemistry and Microbiology, 24 Tzar Assen St., Plovdiv 4000, BULGARIA
*Corresponding author: mmarhova@yahoo.com

Abstract. The aim of this study was to determine the current microbiological status of the microbial communities and their ability to form biofilms in two protected by Natura 2000 wetlands in Southern Bulgaria. The numbers of heterotrophic bacteria (TVC22 and TVC 37), actinomycetes, fungi, and sanitary state indicators were determined for dry soil samples and sediments collected form Zlato pole wetland and Tsalapitsa rice paddies. The number of heterotrophic microorganisms (TVC22 and TVC 37) and indicators of sanitary status (FS, FC, and *Escherichia coli*) in the two rice paddies near the city of Plovdiv is higher in comparison to the control zone Zlato pole – the maximum was recorded in the rice paddy near Tsalapitsa village (C1 and C2 was $12.6x10^6$ cfu.g^{-1} and $26x10^6$ cfu.g^{-1}, respectively). In the studied samples, the bacterial complex takes a dominant position and it exceeds the number of both fungus and actinomycetes at least 1.5 times. The cluster analysis showed a high similarity between the soils surrounding the paddy fields and separated sediment from Zlato pole (ZP2) because of the lowest organic load. The biofilm formation capability of the soil microbial communities was tested *in vitro* and measured using the dye crystal violet (CV). The development of the biofilm was analyzed for seven days in four different nutrient mediums. Results showed better biofilm growth on R2A media for all stations. Our data showed a good correlation between the structure of microbial communities and biofilm-forming capacity.

Key words: wetlands, TVC, FC, FS, biofilm, microbial community.

Introduction

Wetlands are important ecological territories or aquatories, and their microbial community structure, including biofilms, play a significant role in primary productivity, nutrient cycling, and water pollution. Constructed wetlands are low-cost ecological facilities based on the idea of biological treatment methods for remedying anthropogenic pollution. The microbial biodiversity depends on different environmental parameters such as pH, nutrient source, the size of the zone, macrophyte community etc. (HORNER-DEVINE *et al.*, 2003). The effective removal of coliforms (over 90%), fecal enterococci (over 80%) (KADLEC & KNIGHT, 1996) and other indicators for contamination, such as *Clostridium perfringens*, is due to the long water retention time, the activity of microbial community and natural macroflora (STEER *et al.*, 2002; AGUILAR *et al*, 2008). Most functional wastewater treatment systems, such as constructed wetlands, depend on naturally occurring microorganisms that are responsible for organic carbon degradation and nutrient cycling (DAIMS *et al.*, 2006). Although

© Ecologia Balkanica

these microorganisms are considered to be in the planktonic, now it is known that the most active microorganisms are located in biofilm consortium (WUERTZ *et al.*, 2003). The soil is a natural habitat with multiple variables, which affects the growth of microorganisms and in general is nutrient poor. The amount of organic matter varies widely with the bulk of the carbon in recalcitrant forms, such as humic acids. Therefore, soil bacteria often are forced to cope with nutrient deprivation. Microorganisms can exist freely in nature, but most often, they proliferate more effectively by interacting and forming communities (DAVEY & O'TOOLE, 2000). The initial definition of biofilms as structured communities included in the polymer matrix attached to biotic or abiotic surfaces have undergone evolution in recent years. Today, the authors note the irreversible adhesion and the different phenotype of microorganisms in biofilms as a reflection of altered gene expression and metabolic activities (HALL-STOODLEY *et al.*, 2004). The matrix is the predominant part of the mass of biofilms. Its composition is highly hydrated and includes exo-polysaccharides produced by the cells in the community, as well as nucleic acids, proteins, lipids. The functions of biofilms are always determined by the biodiversity, environmental conditions, attachment surface and nutrient availability (WATNICK & KOLTER, 2000). Microbial cells attached to the wetland matrix (solid particles and plant roots) are responsible for effective biodegradation of waterborne pollution. Wetlands are dynamic and variable systems and any change reshape the structure and diversity of the microbial community. There are many data from *in vitro* studies for monospecies biofilms, but there are differences in composition of natural biofilms as they are found mostly as multispecies and may include bacteria as well as fungi, algae, and protozoa (BURMØLLE *et al.*, 2014). Multispecies composition of natural biofilms leads to search for a convenient model for investigation of such complex systems *in situ* and *in vitro* (RØDER *et al.*, 2016). In addition, most of the microorganisms in natural habitats are "unculturable", and their roles in natural systems are unclear (TYSON *at al.*, 2004). Microbiologists have been developing new strategies for culturing "unculturable" bacteria. Such strategies include the use of dilute nutrient media (particularly for oligotrophs) and attempting to simulate natural environmental conditions (VARTOUKIAN *et al.*, 2010).

The aim of the study was to determine the current microbial status of protected areas in Natura 2000 along the Maritza River. The total number and the ratio bacteria: fungi: actinomycetes were used to characterize the condition of soil micriobiocenosis and to evaluate the status of the Tsalapitsa rice paddies and Zlato pole wetland. The analysis of the ability of the natural microbiota to form biofilm gives additional insights into the physiological status of the microbial community and contributes for complete and accurate assessment of wetlands.

Materials and Methods

Study sites and sample collection

The study was carried out in two wetlands along the Maritza River included in the Natura 2000. Zlato pole is the largest natural wetland along the river. Tsalapitsa rice paddies are constructed wetlands. Both protected under the Birds Directive 2009/147/EC (EC, 2009). Dry soils (ZP1, C2, P2) and sediment samples (ZP2, C1, P1) were collected from three sampling sites situated as follows: Zlato pole – ZP1 (42° 2.241', 25° 42.944'), ZP2 (42°2.207', 25°42.938'); Tsalapitsa rice fields - P1 (42° 10.436', 24° 40.403'), P2 (42°10.307', 24°40.502'), C1 (42°13.600', 24° 33.804'), C2 (13.396', 24° 33.202').

Bulk samples from the upper soil layer (5 to 10 cm) were collected in sterile containers, each consisting of at least four single samples to reduce spatial variability. They were stored at 4°C in the dark until microbiological analysis for no longer than 24 h.

Microbiological analysis

Prior to the analysis, the samples were diluted x100 in sterile physiological solution and homogenized for 30 minutes at 200 rpm, then left 10 minutes for precipitation. The soil suspension was used for bacterial enumeration

according to ISO 6222:2002 (TVC at 22°C and 37°C), ISO 9308-1:2014 (*Escherichia coli* and coliforms), ISO 9308-1:2004 (fecal enterococci). Actinomycetes were analyzed according to ZHANG (2011). In brief, 5g sieved soil sample (particle size <1 mm) were dried for 7 days at room temperature and heated for 10 minutes at 110°C. The treated soils were diluted 1:9 with sterile physiological solution and homogenized at the same conditions. A hundred microliters were plated on Actinomyces Isolation Agar (HiMedia, India) supplemented with 50mg/L nalidixic acid (Sigma-Aldrich, USA) and 100mg/L nystatin (Sigma-Aldrich, USA) and cultivated for 14 days at 28°C. the total number of culturable fungi was determined by colony enumeration after incubation at 22°C for 14 days on Sabouraud dextrose agar (HiMedia, India) supplemented with chloramphenicol (PITT & HOCKING, 2009).

Biofilm formation assay

Biofilm-forming capabilities of microbial communities were quantified after cultivation in microtiter plates with crystal violet (CV) assay as described by DEMETER *et al.* (2015). Samples for analysis were prepared by dilution x100 of 1g in four different media - TSB (National Center of Infectious and Parasitic Diseases, Sofia), AB minimal medium (CLARK & MAALÙE, 1967), M63 minimal medium and R2A (Merck, USA) to obtain soil/sediment slurry and homogenized on rotary shaker for 30 min at 200 rpm. After 10 min for precipitation 200 µl samples with three replicates were cultured in flat bottomed 96-well cell culture plates (Costar) at 22°C for 7 days. Sterilized (autoclaved) samples and sterile media were used as a negative control. The development of biofilm was measured each day after staining with 0.1% crystal violet, dissolution of attached CV with 95% ethanol and measurement of the absorbance at 630 nm using a microtiter plate reader Bio Tek ELX800G.

Statistical analysis

Statistical analysis was performed with SPSS v. 23 (IBM analytical). A bivariate Pearson correlation was used for interconnection analysis between the microbiological parameters and biofilm forming capability of the microbial communities. Hierarchical cluster analysis (CA) was applied for the determination of the similarities between the sampling sites based on the studied variables.

Results and Discussion

Soil quality is defined by its compositional structures, functional characteristics and natural functions (FILIP, 2002). The assessment of the ecological status of territories that have long been under the influence of anthropogenic pressure is often based on microbiological and sanitary state research. The assessment of the total microbial count (TVC) at 22 ° C / 37 ° C, fungi and actinomycetes, makes it possible to form an idea of the general status of the soil microbiota, which is a reliable indicator for monitoring the soil response and, more generally, the quality of the soil itself (SHARMA *et al.*, 2005; KENNEDY *et al.*, 2006).

The TVC22 in the dry soil samples varies from $8.4x10^6$ cfu.g^{-1} at ZP1 to $26x10^6$ cfu.g^{-1} at C2 (Figure 1a), in the sediments TVC22 are in range of $5x10^6$ to $12.6x10^6$ cfu.g^{-1}. The highest values for TVC22 were established for the rice field samples from C1 and C2 stations, and the lowest – in the Zlato pole control zone. On average, their numbers are higher in dry soils than sediments. According to KUZNETZOVA (2007), the fluctuations of the microbial count in the soils are often related to the increase in the amount of bioavailable carbon as a result of the plant material decomposition.

The TVC at 37°C (TVC37) provides information on the presence of organic contamination. The number of TVC37 is in the range of $6x10^5$ - $52x10^5$ cfu.g^{-1} in the dry soils and $4.6x10^4$ - $23x10^5$ cfu.g^{-1} in sediment samples (Fig. 1a). The lowest value was recorded in the sediment from the control zone (ZP2), which is a functional indicator of a good ecosystem potential. The highest load based on the indicators is recorded at station C2, evidencing a higher anthropogenic pressure. The values of both indicators were higher in the sediments compared to the dry soils, but the differences are not statistically significant, due to higher levels of variation of the TVC22 indicators in the dry

soils and TVC37 in sediments (Fig. 1a). TVC22 and TVC37 showed high levels of correlation, which coincides with the results of the BATH & ARNEBRANT (1994). Higher TVC values in the rice fields suggest an intense bacterial mineralization activity in the soils, rich in accessible organic substances (ONET & ONET, 2013).

The fecal coliforms ($7x10^3$ cfu.g^{-1} to $12x10^3$ cfu.g^{-1}) were the predominant group of sanitary state indicators, detected in the studied samples (Fig. 1b). At all stations, the numbers of *Escherichia coli* and fecal streptococci were relatively low and with the exception of C2 station did not exceed $5x10^2$ cfu.g^{-1}. The lowest values of under 20 cfu.g^{-1} were established for the Zlato pole sediments, evidence of the lack of contamination with human or animal origin. The FC: FS ratio in the other samples range from 0.9 to 1.3, which indicates the presence of a non-point source of pollution. In general, the number of indicators in sediments is lower than in the soil layer.

The point and non-point sources of organic pollution within the river system are the major sources of sanitary indicators in the wetlands. Discharges from combined sewage systems are considered as the main source of bacteria in streams in many urban areas (MURRAY *et al.*, 2001). The lower numbers in the sediments stress the importance of the artificially created wetlands in the environmental pollution control. The natural sedimentation processes and the combination of environmental factors in the wetlands facilitate the removal of fecal and pathogenic microorganisms from the water layer (HENCH *et al.*, 2003; STOTT *et al.*, 2003).

The data analysis showed that fungi form a significant component of the soil microbiota in the studied area (Fig. 2a). Their numbers range from $16x10^4$ to $101x10^4$ cfu.g^{-1} in the dry soils, and from $2x10^4$ to $78x10^4$ cfu.g^{-1} in the sediments. Fungi play a crucial role in the soil biogeochemical cycles (DE RUITER *et al.*, 1993). The anthropogenic pressure can result in a shift of the microbial composition in the direction of increasing or decreasing the proportion of one or another type of fungi and the conditions of permanent technogenic soil contamination may cause the replacement of "safe" species with potentially pathogenic and epidemiologically unsafe for humans and animals' species (MARFENINA, 2015).

The higher values at ZP1, suggest better degradation capability and organic matter processing efficiency of the microbiota, as 85% of the organic matter is usually degraded by the combined effect of fungi and bacteria (STUR *et al.*, 2015). The reduction of their number at P2 and C2 station in our study suggests a decrease in the soil quality, as the increase of the anthropogenic pressure on the soil communities, follows a significant reduction of the fungal component in the ratio of fungi: bacteria (BARDGETT & LEEMANS 1995; IVASHTENKO, 2017).

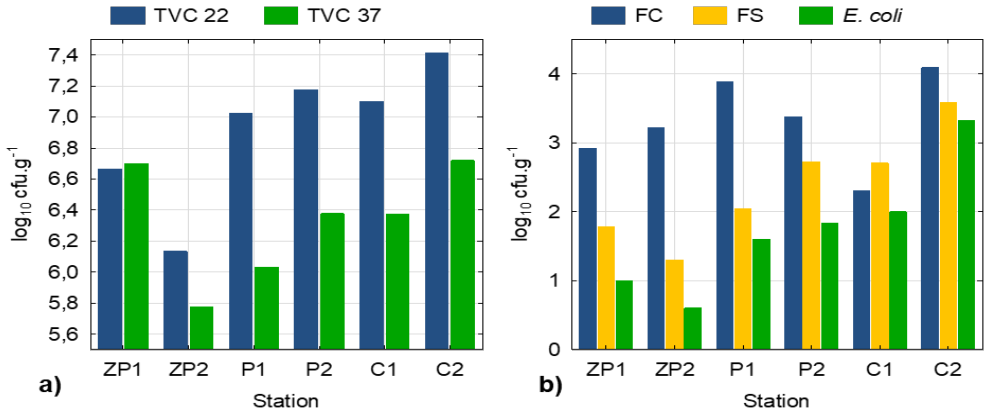

Fig. 1. Average values by station for: a) total number of heterotrophic bacteria; b) sanitary state indicators.

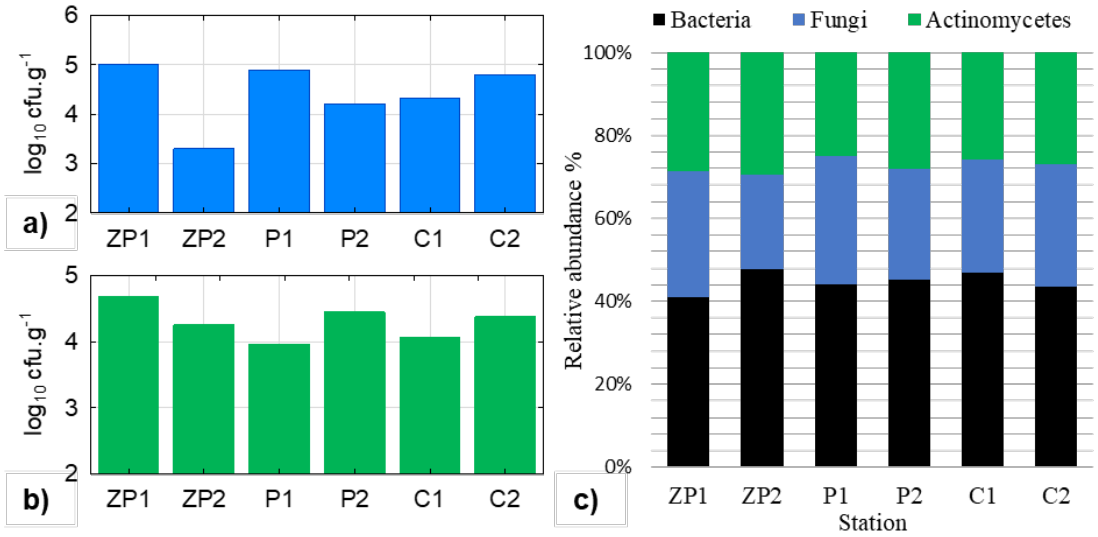

Fig. 2. Average values by station for: a) Fungi; b) Actinomycetes; c) Relative abundance of the studied taxa

Actinomycetes are the third major group forming the microbial communities in the studied samples (Fig. 2b, 2c). Their abundance in the dry soils ($238x10^2$ to $480x10^2$ cfu.g^{-1}) was significantly higher than in the sediments ($92x10^2$ to $181x10^2$ cfu.g^{-1}) (ANOVA, $p<0.05$).

According to KOLESNIKOV (2006), the main reason for the increase in their number is the entry of additional amounts of organic matter into the habitat. Similar results have been reported by ABROSIMOVA *et al.* (2012), showing a clear trend of increase of the actinomycetes number in soil samples under high anthropogenic impact in the urban areas, exceeding more than twice those in the control samples. Hence, the change observed in the species diversity of microorganisms, such as the increase in the abundance of actinomycetes and fungi, and the decrease in the abundance and biodiversity of the bacteria in the studied samples has a strong impact on the condition of soils and, respectively, ecosystems. In the studies of NAZARKO & LOBANOV (2007) an increased number of actinomycetes was recorded in the agrocenoses, regardless of the type of soil, which reflects another kind of anthropogenic influence on the ecosystems - agricultural and management activities.

The ratio of the different groups of microorganisms characterizes the condition of the microbiocenoses. Comparison of the abundance of the studied taxa in the soil (bacteria: fungi: actinomycetes), is a reliable tool for general characterization of soils from territories under different level anthropogenic impact (BELUCHENKO, 2016).

The comparative analysis of the soil from different stations demonstrates the heterogeneous structure of the communities and shows that the bacterial complex has a dominant position exceeding the number of fungi and the actinomycetes at least 1.5 times (Fig. 2c). In the ZP2, P2 samples fungi are a minor component of the soil microbiota, while in all other samples their number exceeds that of actinomycetes. The control zone at ZP1 station is characterized by the highest reported number and highest fungi: bacteria ratio, which has a significant effect on the organic matter decomposition efficiency (Fig. 2c). On average, higher numbers of all taxonomic units were reported in dry soil samples compared to sediment, which is direct evidence of higher levels of biological activity and a better physicochemical regime.

The current results are in agreement with other authors (LEON *et al.*, 2006) and suggest that the field amendments related to rice

production caused a shift in the microbial population compared to the control zone. The data shows that the constructed wetlands play a crucial role in pollution protection, providing conditions that favor the removal of the sanitary microbiological indicators and the potentially present pathogens.

The assembly of the microbial population in natural biofilm is spontaneous and therefore considered as "black box". A large part of it still remains unraveled (TRUU *et. al.*, 2009). One of the reasons is the extracellular matrix as it contains water and a large range of biopolymers that are difficult to analyze. For this reason, it is known as a "dark matter of biofilm" (FLEMMING *et al.*, 2007). One of the most varying soil parameters is the water content and the biofilm formation for soil microorganisms is a good solution to the problem of drying up. The matrix is a major component of biofilm and its high water content provides protection against desiccation (BURMØLLE *et al.*, 2011).

We investigated the ability of soil microbial communities at different stations to form biofilm using *in vitro* model for static cultivation in flat-bottomed 96-well cell culture plates. Four different culture media were used in order to compare their suitability for multispecies soil and sediment biofilm formation in laboratory conditions. The TSB broth is a nutritious medium that supports the growth of a wide variety of microorganisms, including common aerobic and facultatively anaerobic bacteria. The AB and M63 media are used as defined glucose minimal media. The R2A media was used as dilute nutrient media in an attempt to simulate better the natural environmental conditions in *in vitro* model. Biofilm formation was detected daily in seven days period. When TSB media was used for multispecies biofilms, most biomass was detected at sixth day for all tested samples (Fig. 3a). Similar results we obtained when the soil and sediment samples were tested for biofilm formation in R2A media but the formed biomass was significantly greater (Fig. 3b). Biofilm biomass formed from tested multispecies communities in M63 reaches maximum on the seventh day and was lower from that in R2A (Fig. 3d). Microbial societies in the tested samples showed almost non-biofilm forming capacity in AB medium (Fig. 3c). We observed that the multispecies communities in tested soils and sediment samples formed maximum biofilm biomass in R2A medium (Fig. 4). Our data show better biofilm forming capacity of tested sediment communities at P1 and C1 stations compared to control sediments ZP2. The same ratio is observed for soil samples P2 and C2 and control ZP1 but with less biofilm biomass formed *in vitro*.

In multispecies biofilms, complex relationships exist that include the whole array of symbiotic interactions between cooperation and competition such as mutualism, commensalism, synergy, antagonism (ELIAS & BANIN, 2012; RENDUELES & GHIGO, 2012; BURMØLLE *et al.*, 2014). There is evidence of the establishment of complex metabolic relationships in multi-species communities reflecting the amount and accessibility of nutrients in certain ecological niches. Researchers support the view that the co-presence of many species in biofilm structures is an advantage for the microbial communities at different ecological and clinical environments; one can assume that synergistic interactions between species predominate over antagonistic ones, particularly synergies that facilitate a robust coexistence (ELIAS & BANIN, 2012). Data from different models for *in vitro* biofilm formation experiments suggest more often cooperative and synergistic relationships than competitive or antagonistic for different bacterial soil isolates obtained from one specific soil environment simultaneously (REN *at al.*, 2015). It is more likely that in natural multispecies biofilms, adhesion and initial stages of formation are part of the activity of naturally occurring biofilm "formers", which

then facilitates the attachment of non-biofilm forming species (CHRISTENSEN *et al.*, 2002; REN *et al.*, 2015). We concluded that minimal composition of AB media probably leads to slower development and synchronization of species present in the sample and so restrict their opportunities for development step by step of complex metabolic network and biofilm and so was not suitable for the further analysis.

The data from comparative analysis of biofilm – forming capabilities of tested soils and sediments multispecies communities suggest that R2A was a better choice for *in vitro* assay creating opportunities to bring experimental conditions closer to the environmental one. We`ve studied the planktonic growth and we`ve established that it reaches its maximum after 24 h of incubation. The cell density remains constant during the experimental period and showed no significant correlation with the biofilm formation.

Our data showed a good correlation between the composition of microbial communities and biofilm-forming capacity (Table 1).

Our studies showed a correlation between the formation of multi-species biofilms *in vitro* from the soil and sediment samples and some of the selected microbiological indicators (Table 1). We believe that the negative correlation between TCV37 and the amount of *in vitro* formed biofilm is suggestive for the importance of anthropogenic impact and organic matter loading on the temporary composition of the microbial communities and the soil quality. To confirm these assumptions, it would be useful to conduct a metagenomic analysis of microbial communities to establish the relative involvement of different species in building a metabolic network and biofilm.

Biofilm model systems are important for better understanding of the mechanisms involved in biofilm development, social behavior of microorganisms in multispecies communities and changed metabolic profile (COENYE & NELIS, 2010; REN *et al.*, 2013). Recently, studies of multi-species biofilms have increased, but there are still difficulties in choosing model systems and approaches.

We`ve performed a cluster analysis based on the studied microbiological indicators in order to assess the similarity between sampling stations (Fig. 5). The analysis grouped the samples into two statistically significant clusters (CA1 and CA2) with a large cluster distance. CA1 include two sub-clusters. The first includes the dry soil samples from the three zones, with the results showing an extremely high degree of similarity between the soils surrounding the paddy fields. The second one combines sediment samples from the rice paddy. CA2 includes only the sample from station ZP2, which is characterized by the lowest anthropogenic impact based on our results. The results confirm the that the Zlato Pole wetland is an area of national significance that has the potential for water retention volume as well as a trap for biogenic elements and pollutants in the Maritsa River (VASSILEV *et al.*, 2013).

We have found the highest biofilm-forming ability for the multispecies communities in the sediments at P1 and C1 relative to the control station ZP2, which correlates with the reported values of TCV37 and indicates the physiological status of the environmental microbiota in these areas. We propose the use of the R2A media in the development of a potential model system for assay of environmental multi-species biofilms *in vitro*. Wetlands are located in areas with a low elevation and a high water table and play an important role in the purification of pollution near urban areas. Studies of environmental multispecies communities and their abilities for development metabolic network and biofilms will be useful for application in bioremediation of environmental contamination.

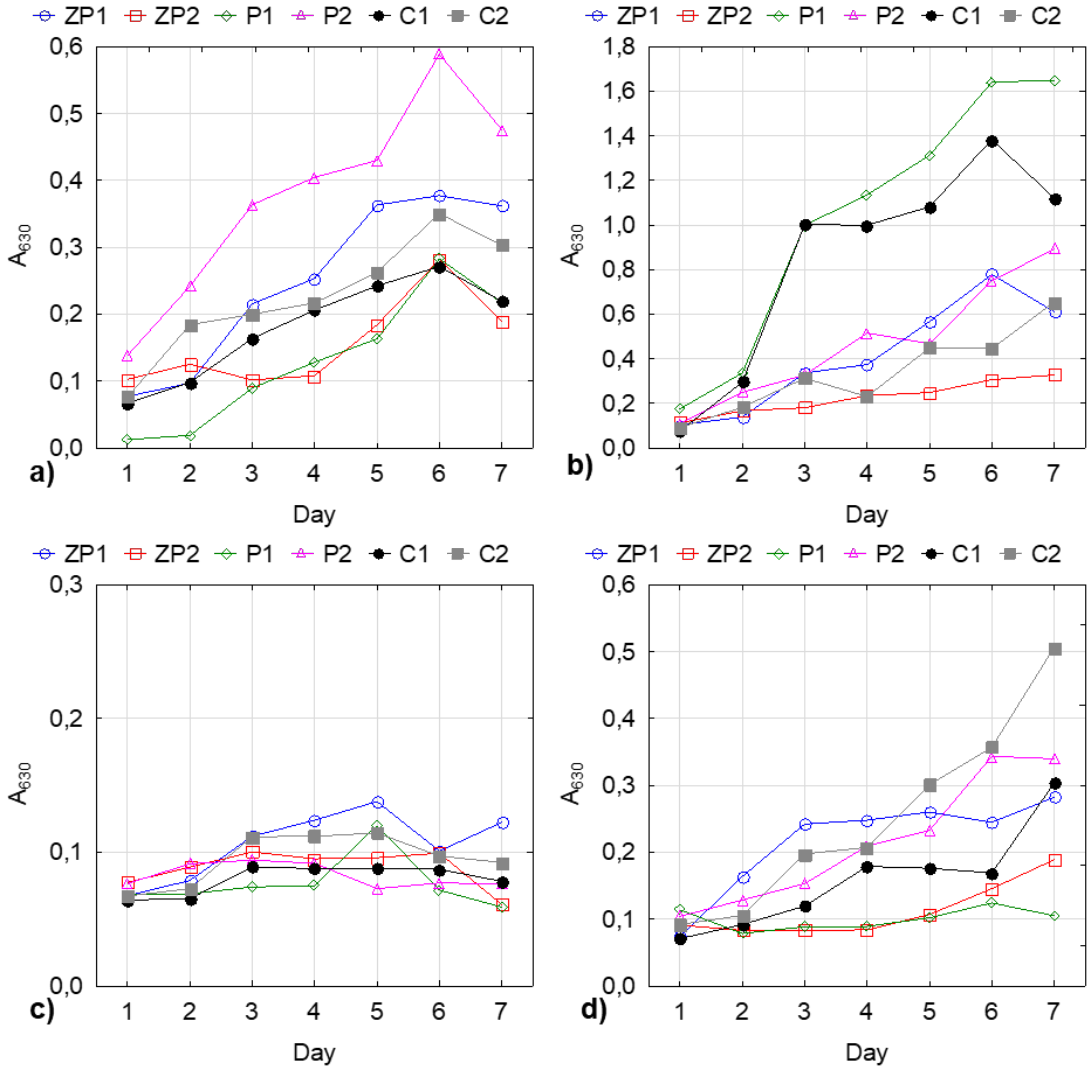

Fig. 3. The biofilm formation of multispecies communities in four different media:
a) TSB; b) R2A; c) AB; d) M63

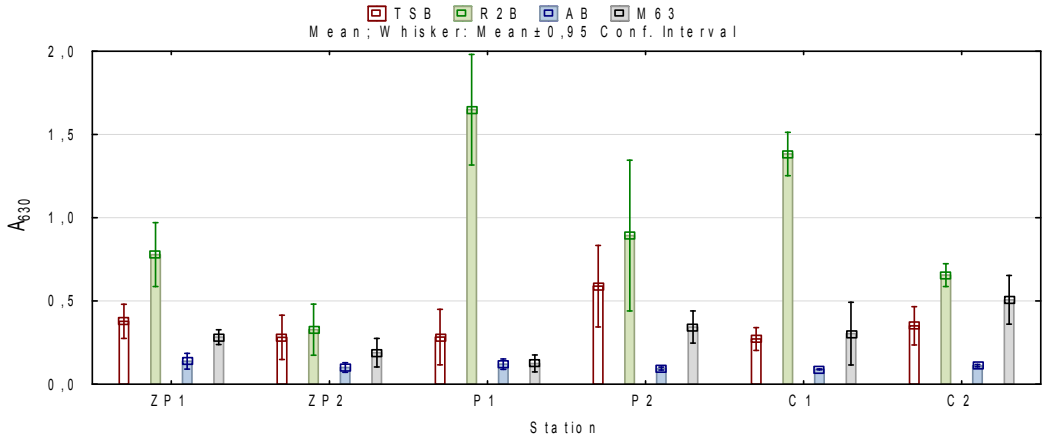

Fig. 4. Comparative analysis of maximum values of biofilm biomass formed from multispecies communities in four different media.

Table 1. Pearson correlation matrix of the microbiological parameters and biofilm formation capability on different media.

	TVC22	TVC37	FC	FS	E. coli	Fungi	Actino	TSB	R2A	AB	M63
TVC22	1										
TVC37	**.777***	1									
FC	-,242	-,071	1								
FS	,591	-,055	,288	1							
E. coli	,522	-,055	,418		1						
Fungi	-,159	-,347	,252	,405	,445	1					
Actino	-,576	,641	-,039	-,004	-,092	,159	1				
TSB	,014	-,012	,136	,282	,124	,045	,564	1			
R2B	,433	**-,929****	-,095	,154	,166	,508	-,638	-,193	1		
AB	**-,837***	,085	,289	-,273	-,180	,656	,437	-,118	-,010	1	
M63	,267	,465	,176	,842*	,793	,237	,453	,370	-,350	-,138	1

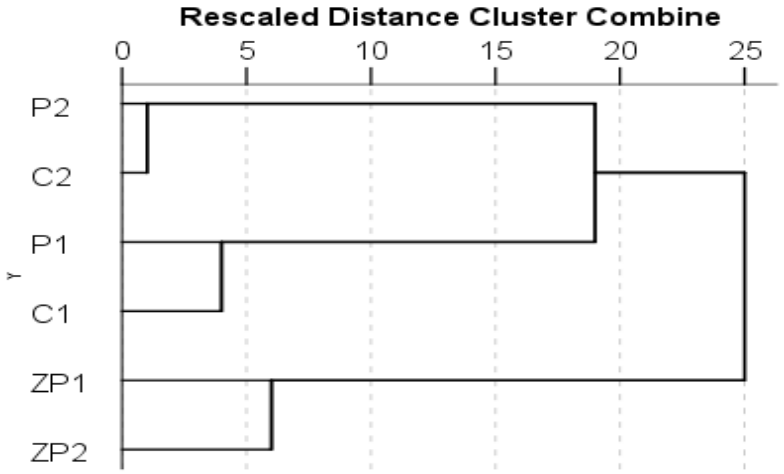

Fig. 5. Cluster analysis using Wards linkage of the similarities between sampling sites based on the studied variables.

Acknowledgements

The research was funded under Project № FP17-BF-001 by the „Scientific research" Fund, Plovdiv University "Paisii Hilendarski", and supported by the National Scientific Program for Young Scientists and Postdoctoral Fellows, Bulgarian Ministry of Education and Science. Special thanks to Dr. Ivelin Mollov, who provided the soil samples for the analysis!

References

ABROSIMOVA O., E. TROYANOVSKAYA, M. MERKULOVA, E. TICHOMIROVA. 2012. [Assessment of the ecological state of the soil cover in Saratov]. - *Povoljskii ecologicheskii jurnal*, 4: 376–384. (In Russian).

AGUILAR J., J. CABRIALES, M. VEGA. 2008. Identification and characterization of sulfur-oxidizing bacteria in an artificial wetland that treats wastewater from a tannery. - *International Journal of Phytoremediation,* 10: 359–370.

BARDGETT R., D. LEEMANS. 1995. The short-term effects of cessation of fertiliser applications, liming and grazing on microbial biomass and activity in a reseeded upland grassland soil. - *Biology and Fertility of Soils,* 19: 148-154.

BATH E., K. ARNEBRANT. 1994. Growth rate and response of bacteria communities to pH in limed and ash treated forest soils. *Soil Biology and Biochemistry*, 26: 995-1001.

BELUCHENKO I. 2016. [Pedosphere microorganisms and peculiarities of soil

formation in agrarian landscapes]. - *Politematicheskii setevoy elektronnii nauchnii jurnal Kubanskogo gosudarstvenogo agrarnogo universiteta,* 121: 1016-1036. (In Russian).

BURMØLLE M., D. REN, T. BJARNSHOLT, S. SØRENSEN. 2014. Interactions in multispecies biofilms: do they actually matter? - *Trends in Microbiology*, 22(2): 84–91.

CHRISTENSEN B., J. HAAGENSEN, A. HEYDORN, S. MOLIN. 2002. Metabolic commensalism and competition in a two-species microbial consortium. - *Applied and Environmental Microbiology,* 68(5): 2495–2502.

CLARK J., O. MAALÙE. 1967. DNA replication and division cycle in *Escherichia coli*. - *Journal of Molecular Biology,* 23: 99-103.

COENYE T., H. NELIS. 2010. In vitro and in vivo model systems to study microbial biofilm formation. - *Journal of Microbiological Methods,* 83: 89–105.

COSTERTON J., Z. LEWANDOWSKI, D. CALDWELL, D. KORBER, H. LAPPIN-SCOTT. 1995. Microbial biofilms. - *Annual Review of Microbiology,* 49: 711–745.

DAIMS H., M. TAYLOR, M. WAGNER. 2006. Wastewater treatment: a model system for microbial ecology. - *Trends in Biotechnology*, 24(11): 483-489.

DAVEY M., G. O'TOOLE. 2000. Microbial biofilms: from ecology to molecular genetics. - *Microbiology and molecular biology reviews: MMBR* 64(4): 847-67.

DE RUITER P., J. MOORE, K. ZWART, L. BOUWMAN, J. HASSINK, *et al.* 1993. Simulation of nitrogen mineralization in the below-ground food webs of two winter wheat fields. - *Journal of Applied Ecology,* 30: 95–106.

DEMETER M., J. LEMIRE, S. GOLBY, M. SCHWERING, H. CERI, R. TURNER. 2015. Cultivation of Environmental Bacterial Communities as Multispecies Biofilms. - In: McGenity T., K. Timmis, B. Nogales. (Eds.) Hydrocarbon and Lipid Microbiology Protocols. Springer Protocols Handbooks. Springer, Berlin, Heidelberg.

EC. 2009. Directive 2009/147/EC of the European Parliament and of the Council of 30 November 2009 on the conservation of wild birds. - *Official Journal of the European Union*, L20: 7–25. Available at: [eur-lex.europa.eu].

ELIAS S., E. BANIN. 2012. Multi-species biofilms: living with friendly neighbors. - *FEMS Microbiology Reviews,* 36: 990–1004.

FILIP Z. 2002. International approach to assessing soil quality by ecologically-related biological parameters. - *Agriculture, Ecosystems and Environment,* 88: 169–174.

FLEMMING, H., T. NEU, D. WOZNIAK. 2007. The EPS matrix: The "house of biofilm cells". - *Journal of Bacteriology,* 189: 7945–7947.

HALL-STOODLEY L., J. COSTERTON, P. STOODLEY. 2004. Bacterial biofilms: from the natural environment to infectious diseases. - *Nature Reviews Microbiology,* 2: 95–108.

HENCH K., G. BISSONNETTE, A. SEXSTONE, J. COLEMAN, K. GARBUTT, J. SKOUSEN. 2003. Fate of physical, chemical and microbial contaminants in domestic wastewater following treatment by small constructed wetlands. - *Water Research,* 37: 7-921.

HORNER−DEVINE C., M. LEIBOLD, V. SMITH, B. BOHANNAN. 2003. Bacterial diversity patterns along a gradient of primary productivity. - *Ecology Letters,* 6: 613-622.

IVASHTENKO K. 2017. [Abundance and respiratory activity of the soil microbial community during anthropogenic transformation of terrestrial ecosystems], - *Federalnoe gosudarstvenoe budjetnoe uchrejdenie nauki Institut fiziko-chimicheskih I biologicheskih problem pochvovedenia RAN, Puschino:* 138-205. (In Russian).

KADLEC R., R. KNIGHT. 1996. *Pathogens. Treatment Wetlands*: Lewis Publishers, Boca Raton. pp. 533–544.

KENNEDY A., T. STUBBS. 2006. Soil microbial communities as indicators of soil health. - *Annals of Arrid Zone*, 45(3 & 4): 287-308.

KOLESNIKOV S., K. KAZEEV, M. TAROSYAN. 2006. [The effect of pollution by oil and oil products on the biological condition of ordinary chernozem]. - *Pochvovedenie,* 5: 616–620. (In Russian).

KUZNECOVA M., G. OVECHKINA, A. MAKSIMIVA, V. DEMAKOVA. 2007. [Distribution of

nitrile-converting bacteria in the soils of the Perm region]. - *Vestnik Permskogo universiteta*, 5(10): 96-99. (In Russian).

LEON M., A. STONE, R. DICK. 2006. Organic soil amendments: Impacts on snap bean common root rot (*Aphanomyes euteiches*) and soil quality. - *Applied Soil Ecology,* 31: 199–210.

MARFENINA O. 2015. *[Anthropogenic ecology of soil fungi]*. Moscow. 196 p. (In Russian)

MURRAY K., L. FISHER, J. THERRIEN, B. GEORGE, J. GILLESPIE. 2001. Assessment and Use of Indicator Bacteria to Determine Sources of Pollution to an Urban River. - *Journal of Great Lakes Research*, 27(2): 220–229.

NAZARKO M., V. LOBANOV. 2007. [Influence of the composition of microflora on the ecological balance of the soil of the Kuban]. - *Izvestia vusov. Pishtevaia technologia*, 1: 92-93. (In Russian).

O'TOOLE G., H. KAPLAN, R. KOLTER. 2000. Biofilm formation as microbial development. - *Annual Review of Microbiology,* 54: 49–79.

ONET C., A. ONET. 2013. Evaluation of the microbial soil quality indicators in agricultural soils from Crisurilor Plain. - *Agrártudományl Közlemények*, 53: 79-80.

PARSEK M., E. GREENBERG. 2005. Sociomicrobiology: the connections between quorum sensing and biofilms. - *Trends in Microbiology*, 13(1): 27-33.

PEETERS E., H. NELIS, T. COENYE. 2008. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. - *Journal of Microbiological Methods*, 72(2): 157-165.

PHILIP Z. 2002. International approach to assessing soil quality by ecologically-related biological parameters. - *Agriculture, Ecosystems and Environment,* 88: 169–174.

PITT J., A. HOCKING. 2009. *Fungi and Food Spoilage*. Springer, US, 519.

REN D., J. MADSEN S., C. DE LA CRUZ-PERERA, L. BERGMARK, S. SØRENSEN, M. BURMØLLE. 2013. High-throughput screening of multispecies biofilm formation and quantitative PCR-based assessment of individual species proportions, useful for exploring interspecific bacterial interactions. - *Microbial Ecology,* 68(1): 146–154.

REN D., J. MADSEN, S. SØRENSEN, M. BURMØLLE. 2015. High prevalence of biofilm synergy among bacterial soil isolates in cocultures indicates bacterial interspecific cooperation. - *The ISME Journal*, 9: 81–89.

RENDUELES O., J. GHIGO. 2012. Multi-species biofilms: how to avoid unfriendly neighbors. - *FEMS Microbiology Review*, 36(5):972-989.

RØDER H., S. SØRENSEN, M. BURMØLLE. 2016 Studying bacterial multispecies biofilms: where to start? - *Trends in Microbiology*, 24(6): 503–513.

SHARMA K., U. MANDAL, K. SRINIVAS, K. VITTAL, B. MANDAL. 2005. Longterm soil management effects on crop yields and soil quality in dryland Alfisol. *Soil & Tillage Research,* 83: 246–259.

SHTUR A., V. VALKO, D. VINOGRADOV. 2015. [The influence of methods of tillage and fertilization on the number and composition of microorganisms]. - *Vestnik Kurskoi gosudarstvennoi selskohozyaistvennoi akademii,* (3): 41-44. (In Russian).

SOLOVYEV V., E. RUBIN, D. ROKHSAR, J. BANFIELD. 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature*, 428: 37-43.

STEER D., L. FRASER, J. BODDY, B. SEIBERT. 2002. Efficiency of small constructed wetlands for subsurface treatment of single-family domestic effluent. - *Ecological Engineering*, 18: 429–440.

STOTT R., E. MAY, D. MARA. 2003. Parasite removal by natural wastewater treatment systems: performance of waste stabilization ponds and constructed wetlands. - *Water Science & Technology,* 48(2): 97–104.

TRUU M., J. JUHANSON, J. TRUU. 2009. Microbial biomass, activity and community composition in constructed wetlands. - *Science of The Total Environment*, 407(13): 3958-3971.

TYSON G., J. CHAPMAN, PH. HUGENHOLTZ, E. ALLEN, R. RAM, *et al.* 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. - *Nature*, 428: 37-43.

VARTOUKIAN S., R. PALMER, W. WADE. 2010. Strategies for culture of "unculturable" bacteria. - *FEMS Microbiology Letters,* 309: 1–7.

VASSILEV V., R. VASSILEV, P. YANKOV, N. KAMBUROVA, Y. UZUNOV, *et al.* 2013. *National Action Plan for Conservation of Wetlands of High Significance in Bulgaria 2013 – 2022.* Sofia. C print Ltd., Burgas.

WATNICK P., R. KOLTER. 2000. "Biofilm, city of microbes". - *Journal of Bacteriology,* 182(10): 2675-2679.

WINTER J. 2003. Book Review: Biofilms in Wastewater Treatment — an Interdisciplinary Approach by Stefan Wuertz, P. Bishop, P. Wilderer. - *Acta Hydrochimica et Hydrobiologica*, 31: 259-260.

WUERTZ S., S. OKABE, M. HAUSNER. 2004. Microbial communities and their interactions in biofilm systems: an overview. - *Water Science & Technology*, 49(11-12): 327–336.

Zhang J. 2011. Improvement of an isolation medium for actinomycetes. Modern Applied Science, 5(2): 124-127.

Received: 05.11.2018
Accepted: 21.12.2018