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Bacterioplankton of Wetlands along the Lower Danube River (Bulgaria) and Its Relation to Environmental Factors

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Abstract: Several temporary and permanent wetlands of lake type (Peschin, Murtvo Blato and Dulyova Bara), canal type (Brushlen and Kalimok), and a Danube River branch (Belene Island) were sampled seasonally during the vegetation period from summer 2009 until spring 2012. The bacterioplankton is the most abundant component of plankton communities and participates in the biogeochemical processes. We determined its abundance, biomass, morphological and size structure. The environmental variables measured simultaneously encompassed temperature, pH and dissolved oxygen, as well as nutrients (N, P and Si) and chlorophyll-a. Spatial and temporal variability of bacterioplankton and its relations to environmental variables were tested using statistical analyses. Bacterioplankton numbers were the highest in the river and higher in the marshes than in the canals. The displayed spring maximum increased towards 2011 during the lower water level of the Danube River and decreased again in 2012 during the new flooding after the total dry up. In 2011 we recorded phototrophic anaerobic purple sulfur bacteria from the genera *Chromatium* and *Thiopedia* in the canals, and filamentous phototrophic green nonsulfur bacteria from the genus *Chloroflexus,* both in the marshes and canal types of wetlands. Turbidity, chemical oxygen demand, total nitrogen and the depth were positively related to the morphotypes and size dynamics of bacteria, while ammonium ions and the distance of the wetlands from the Danube River were negatively related.

Keywords: Bacterioplankton, abundance, wetlands, nutrients, chlorophyll-a, relations

Introduction

Wetlands are extremely vulnerable ecosystems. They are highly sensitive to pollution and influences causing changes in the water level. The wetlands in Bulgaria have decreased significantly from 2% to 0.1% of the total area of our country over the last century (Kalcheva 2011). Most seriously are affected the wetlands along the Danube River and some of them are an object to comprehensive study, where the determination of bacterioplankton is a precondition to ensure their good ecological status and development. The rise in global temperature clearly influences and will continue to have an effect on the temperature and biogeochemical processes in aquatic ecosystems (Häder *et al.* 2007). Bacteria, as a major component of plankton communities, play an important role in the microbial food web as a food source, in the utilisation of DOC, in the decomposition of the dead organic matter and in the remineralisation of nutrients in the aquatic ecosystems (Cole 1999, Vadstein *et al.* 2003). Factors controlling their abundance, size and morphology are of major importance for the prognosis of organic matter removal from the aquatic systems or the so-called self-purification potential (Freese *et al.* 2007). Therefore, the need of studying bacterioplankton and establishing the influence of environmental factors on it is the key in understanding the effects of global warming (like flooding or drought) on biogeochemical processes in aquatic ecosystems (Hall 2006, Häder *et al.* 2007). Only a few studies on bacterioplankton in Bulgarian wetlands have been published recently (Naumova 2007, Kalcheva 2011, Kalcheva *et al.* 2011, Naumova, Kalcheva 2012).

The aim of the study is to determine the seasonal, spatial and annual dynamics in bacterioplankton abundance, biomass, morphological and size structure, and to establish its relation to environmental factors in wetlands on Belene Island and Kalimok-Brushlen protected area during their restoration after reconnecting them to the Danube River.

Material and Methods

A total of 62 water samples were taken 0.2-0.3 m below the surface from three marshes on Belene Island, from the branch of the Danube River flowing between the island and the town of Belene, from the canals Kalimok (from 0.3 m above the bottom as well) and Brushlen and from Kalimok Marsh, close to Kalimok canal (Fig. 1). The sampling included spring, summer and autumn samples, beginning from the summer of 2009 until the spring of 2012.

Belene Island, with the reserve "Persina Marshes", is situated between rkm 560 and 576 of the Danube River, opposite to the town of Belene (43°40'N, 25°10'E, Fig.1). The three largest marshes there were studied. The area of Murtvo Blato is 1.23 km^2, Peschin is 1.82 km^2 and Dulyova Bara is 0.49 km^2 (Kalcheva *et al.* 2011). The water level in the marshes depends on the level of the Danube River. The marshes are 1-2.5 m deep during spring, while in summer parts of them or all of them dry up completely. We recorded depths of 1 m in the summer and 0.5-0.7 m in the autumn of 2009. In the spring of 2010, after heavy rainfall, the water levels were from 1.60 to 3.00 m. In the summer of 2011, owing to droughts and very low levels of the Danube River, the marshes began to dry up. Dulyova Bara was presented by 2-3 puddles, Murtvo Blato had a water area of 15-20 m^2, while Peschin was with considerably larger water area, and its average depth of 0.2 m showed the best status. In the autumn of 2011 they all dried up completely, but in the spring of 2012 they were flooded again reaching depths of 0.7-1 m, increasing from Dulyova Bara to Peschin Marsh. Kalimok-Brushlen (or Brashlen) protected site (Fig. 1) is located between Babovo village and Tutrakan town, between rkm 440 and 465. It includes two drainage canals and marshes in its restoration area (with depths between 0.5 and 3.5 m).

The trophic state of the studied wetlands was meso- to hypereutrophic (according to the measured chlorophyll-a). The studied sites belonged to different types of ecosystems: the marshes and Kalimok Canal are lentic, while the Brushlen canal seems to be semi-lotic and the river is lotic.

Bacterioplankton samples were prefixed with 2% formalin, as a final concentration, then filtered through 0.2 μm pore-sized membrane filters and stained with erythrosine (Naumova 1999, Kalcheva *et al.* 2008). The number of bacteria was determined

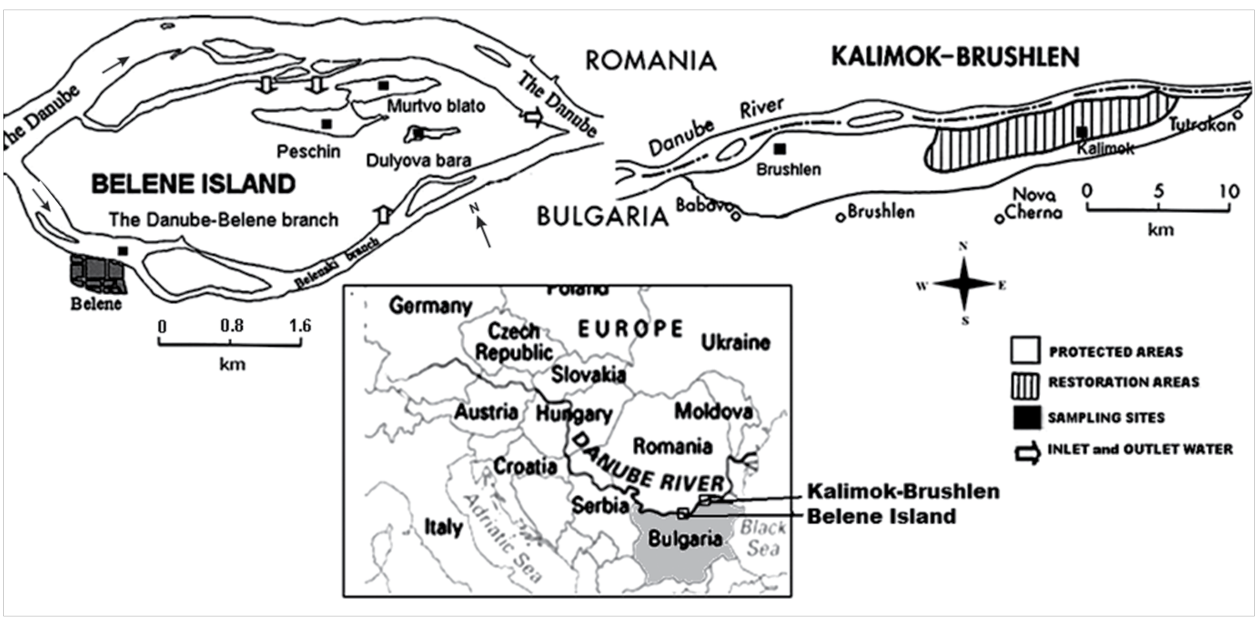

Fig. 1. A map of Europe, shown in the inset, with the Danube River passage through the countries. The location of the studied areas in Bulgaria: Belene Island and Kalimok-Brushlen system, with the sampling sites is shown

using the method of a direct count with a phase-contrast microscope at a magnification of 1600x. The biomass of the bacteria was calculated in carbon content using Norland's formula (Straškrabová *et al.* 1999), after determining the mean (average) cell volume during the counting and measuring of a total of 250-400 bacteria of different morphological groups. Bacterioplankton morphological groups, and namely cocci and rod-shaped cells, were distributed into free-living and attached to detritus particles representing four groups. Cell sizes were presented in five size classes: 0.2-0.5 (<0.5), 0.5-0.9, 0.9-1.6, 1.6-2.5 and 2.5-4.2 (>2.5) µm, in order to assess the bottom-up effect on bacteria by resource availability and the top-down control by bacterivores (Pernthaler *et al.* 1996, Jürgens, Matz 2002, Chróst *et al.* 2009). Detritus particles with attached bacteria (5-60 µm in length) were counted as well.

Water temperature, pH, conductivity and dissolved oxygen were measured *in situ* using WTW-Multi 1970i, Greisinger electronic and Winkler titration, correspondingly. Water depth and distance of the wetlands from the main river were also measured. The NH_4-N, NO_3-N, NO_2–N, SiO_2, PO_4-P and Fe, all from filtered samples, and TN, TP, COD (chemical oxygen demand), TOC (total organic carbon), turbidity at 550 nm as absorbance, all from non-filtered samples, were determined colorimetrically by photometer Nova 60 and kits of Merck. The chlorophyll-a samples were collected by filtering through 0.7 µm glass fiber filters and then stored in liquid nitrogen. They were analysed in the laboratory following ISO 10260 standard. Environmental factors, measured simultaneously during the bacterioplankton sampling, were used in the statistical analyses (Redundancy analysis, RDA) using Canoco 4.5 software (Ter Braak, Smilauer 2002) in order to explore their relation to bacterioplankton. Additionally, one-way ANOVA (Sokal, Rohlf 1997) was applied to identify temporal and spatial differences in bacterioplankton abundance, morphology and cell sizes (with the level of significance "p", given in the text).

Results and Discussion

Bacterioplankton dynamics

The total number of bacterioplankton in the period 2009-2012 ranged from 4.40×10^4 to 1.11×10^6 cells. ml^{-1} (Fig. 2). The minimum number was observed in the Brushlen Canal in the summer of 2009, while the maximum was recorded in the Belene branch of the Danube River, in the spring of 2010, most probably as a result of a nutrient and organic matter input from the local runoff owing to the heavy rainfall. The bacterioplankton from the wetlands reached its maximum in Peschin in the spring of 2011 (Fig. 2), while the overall average value for the whole sampling period was maximal in Murtvo Blato (Fig. 3A). In Kalimok and Brushlen the differences between years were more pronounced, rather than between seasons. Bacteria in the deeper horizon in Kalimok Canal increased in comparison to the surface layer and in Kalimok Marsh (measured in years of high water level) numbers were always lower than in the canal (Fig. 3A). The lower values most likely are owing to the better oxygen conditions, bacterivore pressure and an eventual viral impact, as well as the presence of macrophytes (competition for nutrients) or periodic drying, mineralisation of organic matter and subsequent better aeration of the contact layer at new flooding. Similar results were found for the marshes on Belene Island in 2012 after their total drying in the autumn of 2011.

The biomass varied from 0.70 to 24.93 µg $C.L^{-1}$ and its dynamics followed the abundance's dynamics (Fig. 3B). Mean cell volume (MCV) ranged from 0.04 to 0.12 $µm^3$ with higher values in the marshes and in the river near the town of Belene in 2011 (Fig. 3B). This relatively small MCV, most likely was re-

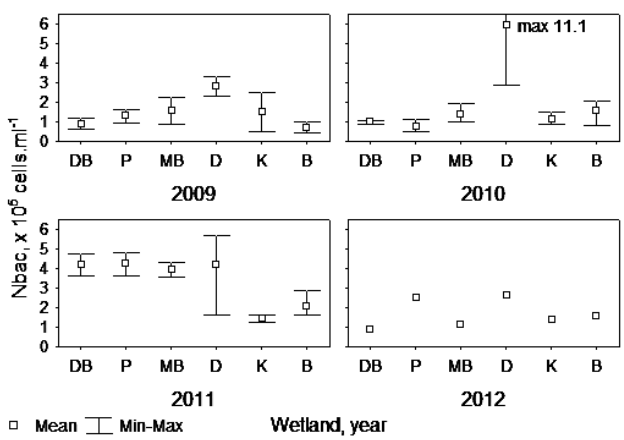

Fig. 2. Annual dynamics of bacterioplankton total number (Nbac x 10^5 $cells.ml^{-1}$, mean and min-max values during the three seasons between 2009 and 2011 and only spring values for 2012) in the marshes Dulyova Bara (DB), Peschin (P) and Murtvo Blato (MB); in the canals Kalimok (K) and Brushlen (B) and in the Danube River Belene branch (D) in 2009-2012

corded because of the prevalence of bacteria from the smallest size class, which was the group 0.2-0.5 µm (< 0.5 µm). Similar volume has been established in eutrophic waters and with Image analysis by Šimek *et al.* (1997). Moreover, domination of bacteria from the smallest size class has been found in freshwater ecosystems with different trophic state (Pernthaler *et al.* 1996, Šimek *et al.* 1997, Cole 1999, Jürgens, Matz 2002, Pernthaler 2005, Chróst *et al.* 2009) and is a normal phenomenon owing to abiotic factors outside of the optimum (temperature, pH, etc.), nutrient limitation (mainly of organic C or inorganic P), increased number of bacterivores or the presence of an inactive state. The largest bacteria (>2.5 µm) were represented better in the river, Murtvo Blato and Brushlen Canal. The cell size group of 0.9-1.6 µm, which includes the most actively dividing bacterial cells and the most vulnerable to being eaten by phagotrophic nanoflagelates (Cotner, Biddanda 2002) and Cladocera filtrators (Jürgens, Matz 2002), indicated significant differences in seasonal ($p = 0.018$, spring maximum) and inter-annual aspect ($p = 0.007$, maximum in 2011 in the wetlands), while the smallest size group differed significantly only between wetlands ($p = 0.022$). The number, biomass and MCV of bacteria showed significant differences between the years ($p < 0.05$, ANOVA, Fig. 3B) and increased towards 2011. Seasonal dynamics indicated spring maximum for the total bacterioplnkton number ($p = 0.05$) and summer maximum for MCV ($p = 0.004$). Palijan *et al.* (2007) found spring maximum of bacterial abundance in Kopački Rit, Croatia during high water level of the Danube River, as observed in our study. Free-living cocci prevailed over the other three groups (39-70%). The average ratio free/attached bacteria in % was 70:30, indicating that a significant part of the bacterioplankton community in the water column includes detritus particles with attached bacteria from the bottom (sediment) during continuous processes of resuspension and sedimentation of organics which is typical for shallow water bodies.

Morphological index M (% rods/cocci %) was with significant annual differences ($p < 0.001$) and less than 1, which was an indication of easily degradable organic matter in the water column. The index reached its maximum in Brushlen canal in 2011 ($M = 0.71$). It was with higher values in the Danube River and Murtvo Blato. Detritus temporally had spring maximum, but spatially the higher numbers were in the river (2-3 times higher), in the canals and in Dulyova Bara ($p = 0.01$). However, there were no significant differences between the years.

In 2011 in the canals Brushlen and Kalimok for the first time were established anaerobic phototrophic bacteria – purple sulfur bacteria of the genera *Chromatium* and *Thiopedia*, family Chromatiaceae, phylogenetic group Proteobacteria (class

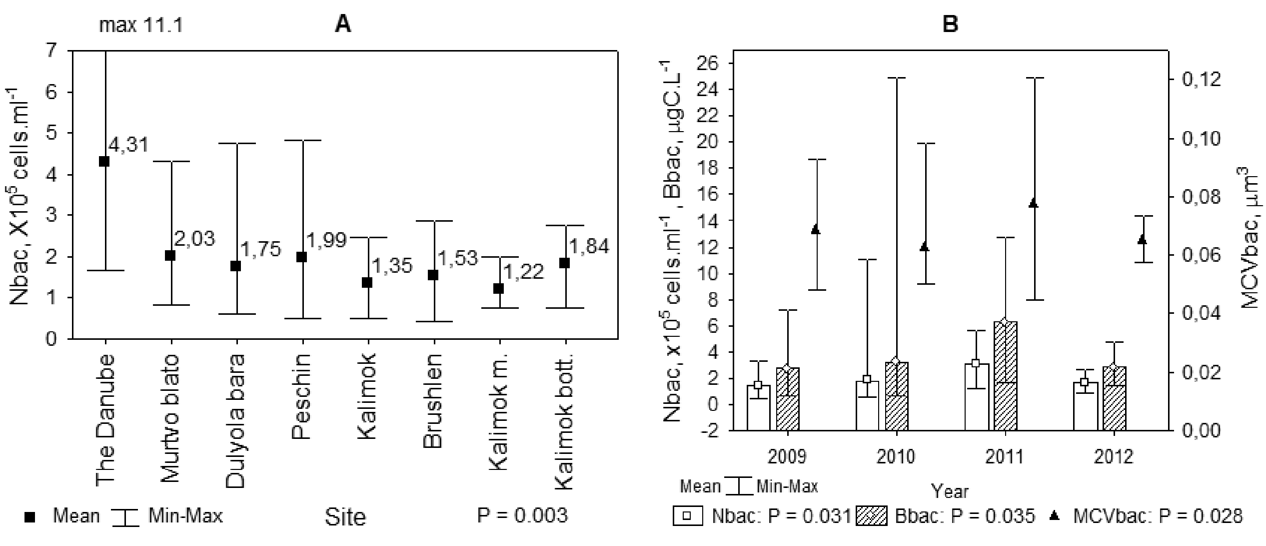

Fig. 3. Total number of bacterioplankton (Nbac x 10^5 cells.ml^{-1}, mean and min-max values for the whole period of investigation 2009-2012) in the Danube River and in the wetlands (A), where Kalimok is presented by 3 sampling sites, in the surface layer (Kalimok), in the bottom layer (Kalimok bott.) and in the nearby marsh (Kalimok m.) and the number, biomass (Bbac) and mean cell volume (MCVbac) of bacterioplankton (mean and min-max values) by years (2009-2012) differing for the presented level of significance "p" after ANOVA f-test (B)

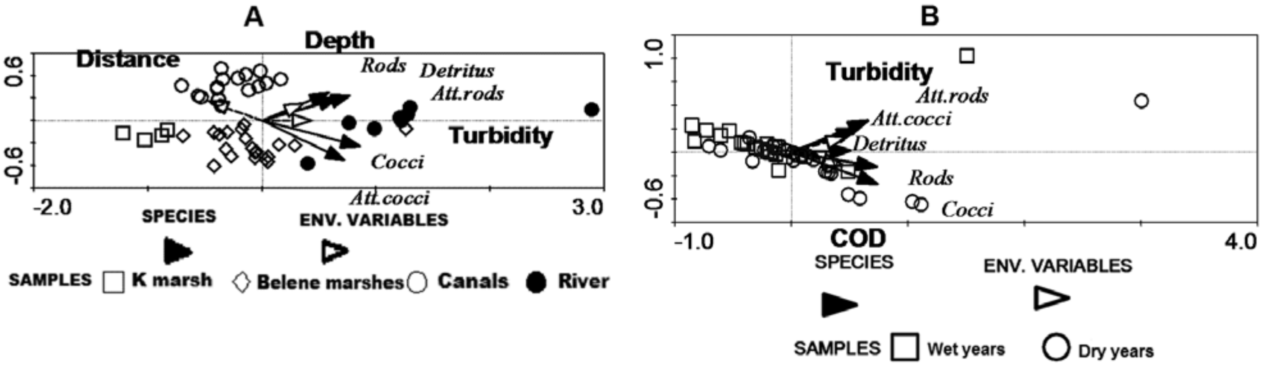

Fig. 4. Partial RDA ordination triplots of bacterioplankton morphological groups by their numbers as response variables (species) and the environmental factors as explanatory (env.) variables in spatial aspect (A) where samples are the sites, given as river, canals and marshes; and in temporal aspect (B) where samples are the years, divided into wet (2010 and 2012) and dry (2009 and 2011)

Gammaproteobacteria), most probably the species *C. okenii* or *C. vinosum* and *T. rosea* (Madigan *et al.* 2003). *Chromatium* was registered in the spring and the summer in Brushlen, and in the deeper layer of Kalimok during the summer, while *Thiopedia* was found only in summer. The number of these bacteria was relatively high (3000-8000 per ml), but was not included in the number of bacterioplankton. In Kalimok Canal release of small pieces of sediment from the benthos into the pelagial (water column) with smell of H_2S was observed periodically. The water in Brushlen Canal was with the same smell, slightly yellowish in colour and the surface layer was completely covered with duckweed during 2011 when the water level was low. At the bottom layers of the canals Brushlen and Kalimok the conditions during the study period, and namely the very low to zero dissolved oxygen and pH = 7, most probably allowed to these anaerobic phototrophic bacteria to develop in the water column (which otherwise usually is aerobic and therefore an atypical for them habitat). Thus, these bacteria established their role in the microbial food web and in the biogeochemical processes (sulfur cycle) under drought conditions. Additionally, for the first time during the summer of 2011 in Peschin, Murtvo Blato and in Brushlen Canal, filamentous phototrophic green nonsulfur thermophilic bacteria from the genus *Chloroflexus* (probably *C. aggregans*) of the group of Chloroflexi (Madigan *et al.* 2003) were observed. These bacteria are easily adaptable as they have several types of feeding depending on the light and oxygen regimes, and on the presence of organics: photoauto-, photohetero- and chemoautotrophy. Perhaps variable environmental conditions in the pelagial of these wetlands are good medium for their development. Quantitative data were not included in our analyses. A more detailed analysis (repeated microscopic analyses and reference) need to be applied in similar studies in the future.

Bacterioplankton interactions with environmental factors

It is known that bacterioplankton number and chlorophyll-a concentrations are the most important indicators of the status of aquatic ecosystems (Kovalova *et al.* 2010). We could not find any significant relation although we observed positive trend between them in their spatial and temporal (inter-annual and seasonal) dynamics. Both types of sources of organic carbon in the ecosystems (autochthonous and allochthonous) are important for bacteria, while higher bacterial abundance is commonly observed in relation to phytoplankton primary production. The lack of that relationship suggests the existence of some source of organic carbon for bacterioplankton, other than phytoplankton. Therefore the prevailing source of organic carbon could have been resuspended sediment in the water column and/or a washout from the floodplain (Palijan *et al.* 2007).

The partial RDA analyses indicated that of all the environmental variables measured only turbidity, depth and distance in spatial aspect (Fig. 4A) and turbidity and COD in temporal aspect (Fig. 4B) explained significantly 37% (All EV = 0.770, Canonical EV = 0.285, p = 0.002) and 20% (All EV = 0.748, Can EV = 0.153, p = 0.014), respectively, of the bacterioplankton morphotype abundance variations. The most abundant group of the free-living cocci correlated positively with the turbidity, but decreased in number, when the distance from the

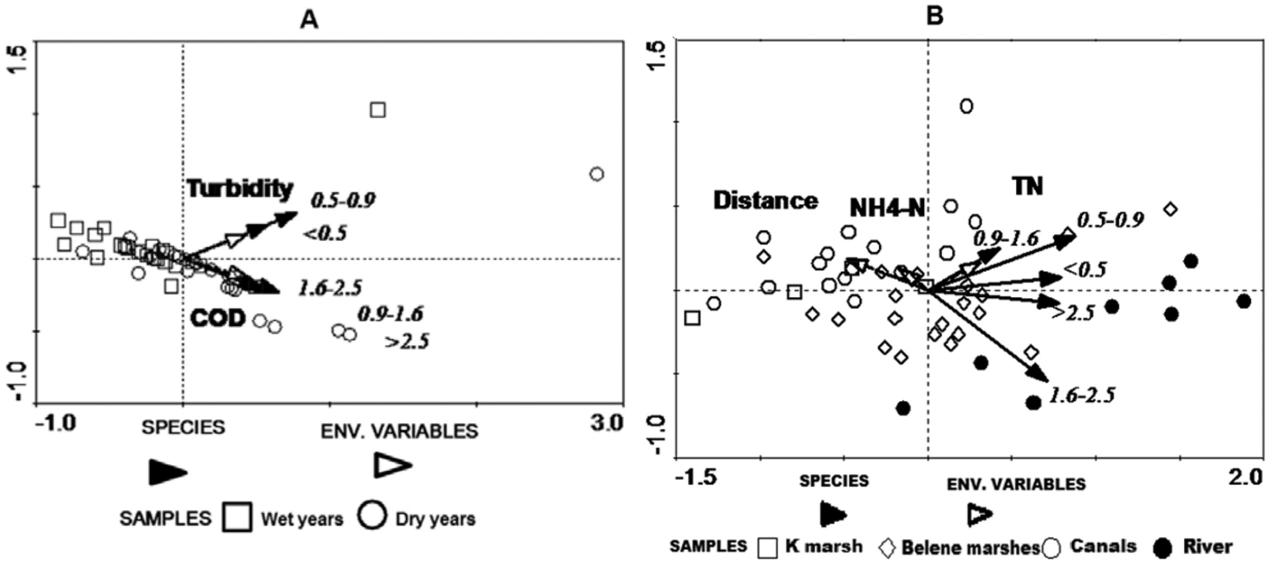

Fig. 5. Partial RDA ordination triplots of bacterioplankton size groups (sizes in µm), presented by their numbers as response (species) variables and the environmental factors as explanatory (env.) variables in temporal aspect (A) and in spatial aspect (B). For coding: see Fig. 4

Danube River increased. Rods and detritus particles were positively correlated with the depth, *i.e.* were encountered in river and canal samples, while the marsh wetlands were rich in cocci forms. Overall, the bacterial morphology delivered a clear separation between marshes, canals and the river sites. On the contrary, in temporal aspect the morphological characteristics showed only indication of weak separation between wet and dry years with tendency to high abundance of all morphological groups in dry years. Kovalova *et al.* (2010) observed decrease in bacterioplankton number further from the Danube River delta and in their opinion; this was caused by a decrease in the distance from the Danube River and from the Danube discharge influence carrying allochthonous organic matter and nutrients.

The partial RDA analyses of the temporal variations (Fig. 5A) by size groups indicated also that the turbidity and COD were statistically significant factors with 57% (All EV = 0.769, Can EV = 0.441, p = 0.004). 38% of the spatial variations (Fig. 5B) were explained (All EV = 0.799, Can EV = 0.302, p = 0.002) with the distance from the river again, but also with NH_4-N, which was negatively correlated for the larger bacteria (>1.6 µm) and with the total nitrogen, which was positively correlated for the smaller sizes (0.5-1.6 µm). Once again a weak temporal separation between wet and dry years emerged with high numbers predominantly of large size classes, high turbidity and COD observed in the dry years. The spatial separation of wetlands by size classes (Fig. 5B) was less clear than by morphotypes (Fig. 4A). Obviously because some factors with significant effect on the bacterial size structure like zooplankton grazing were absent among the potential explanatory variables and therefore could not be selected by the applied analysis.

In conclusion, the relation to COD, TN and NH_4-N, proved a statement true that bacterioplankton actively participated in the decomposition of the dead organic matter, self-purification and in remineralisation of nutrients to be used by primary producers (phytoplankton and macrophytes) in the wetlands.

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