FUNCTIONAL PROFILE OF MICROBIAL COMMUNITY STRUCTURE IN CRITICAL POINTS OF UPPER ISKAR SUBCATCHMENT /BULGARIA/

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Synopsis

The functional profile of microbial community in upper Iskar subcatchment has been studied at two-stage scheme based on the approach of critical control points. The two steps of experimental design have included: 1) analysis of counts of functional microbial groups with a key role for realization of self-purification processes in river ecosystem; 2) taxonomic characteristics of risk water quality factors – coliform bacteria and enterococci. The obtained results are a good base to formulate bioalgorithms for control and management of water quality, microbiological risk and self-purification capacity of Iskar River ecosystem.

INTRODUCTION

The modern strategies for management of water resources point to the role of self-purification processes as a natural highly effective mechanism for maintaining of the ecological state in river ecosystems.

The leading factor for the realization of self-purification processes in rivers is the functioning of microbial segment of biocenosis. The microorganisms occupy the nodal position in the fundamental transformation processes of organics and nutrients, as a result of their flexible metabolism and enormous physiological diversity. The functional profile of microbial community as a presence and proportion, relationships and dominancy of the key groups determine significantly the opportunities of ecosystem for adequate response to pollution with different origin. The state of microbiocenoses is a direct indicator for the effectiveness of the ecosystem functioning and its precise analysis allows a fine regulation of the entire self-purification capacity and water quality by specific controlling approaches and measures (RHEINHEIMER, 1992; SIGEE, 2005).

The discussion of microbial biodiversity should include another important element - a sanitary bacteriological aspect of the problem, which has the most critical manifestation in strategic sources of drinking water. The provision of microbiologically pure water is a priority of any developed country. CHAPMAN, 1992, indicates the presence of pathogenic microorganisms as the most famous risk factor associated with water. The biodiversity and functional stability of autochthonic microbial communities are essential for the incorporation of potentially dangerous alochthonic microorganisms. The connection of microbial communities with water quality has a dual importance: the presence of certain families and strains directly endangering the human health, while the functioning of microbial communities in the entire spectrum of their physiological diversity is the main factor for the realization of self-purification processes and improve water quality.

The aim of this paper is to provide useful information for microbial biodiversity in upper subcatchment of Iskar River and to specify the critical control points (as a time, location and parameter) for formation of microbiological factors of water quality. The functional contribution of microbial community for river metabolism and water quality has recently attracted more attention with respect to conservation, restoration, and management of water sources.

SUBJECT AND METHODS OF RESEARCH

Study area

The study area is situated in north Rila Mountain, Bulgaria, and includes a subcatchment of upper part of Iskar River before the Iskar Dam (an area of 892 km^2 with average latitude of 1314 m). Iskar River and its reservoir are the water sources in Bulgaria with the most important economical and social meaning because of their role for drinking water supply of Sofia (capital of Bulgaria). Iskar is the longest Bulgarian river (368 km) with average stream flow of 8.9 $m^3.s^{-1}$ for 2004. The study part of the river has a clearly determined seasonal character with summer and winter low flow (1–3 $m^3.s^{-1}$), a little increase in flow during the autumn (6–10 $m^3.s^{-1}$) and very expressive spring high water level (15–25 $m^3.s^{-1}$) (KUKURIN, 2005).

Experimental design

The design of the experiments has been based on the principles of the CCPs theory /Critical Control Points/. The sampling sites, target river components and investigated microbial groups have been selected by their key significance for ecosystem functioning and formation of water quality. These CCPs concern the functioning of the important water and ecosystem connections: *(1)* Iskar River – effect of WWTP; *(2)* main river channel – tributary; *(3)* Iskar River – Iskar Dam; *(4)* stream water – hyporheic zone – river bed sediments; *(5)* microbial communities – self-purification – water quality.

The two selected sampling sites are with the most critical location in upper Iskar subcatchment (Figure 1):

CCP A – located on 903 m altitude, 42°21’963”N, 23°33'357"E, below the town of Samokov and after the influx of town WWTP. The preliminary studies have been registered a certain deterioration in ecological state during the seasons with critical low flows or incidental events and have been determined this site as a major risk factor for water quality in study area (TOPALOVA et al., 2004; TODOROVA & TOPALOVA, 2005).

CCP B – located on 833 m altitude, 42°25’831”N, 23°31'900"E, above Iskar Dam but after the confluence of the Iskar biggest tributary in the study area – Palakarija River. The site is in the close proximity of the Iskar Dam and the self-purification processes in this river section and microbiological risk parameters reflects on the water quality in main drinking source of Sofia city.

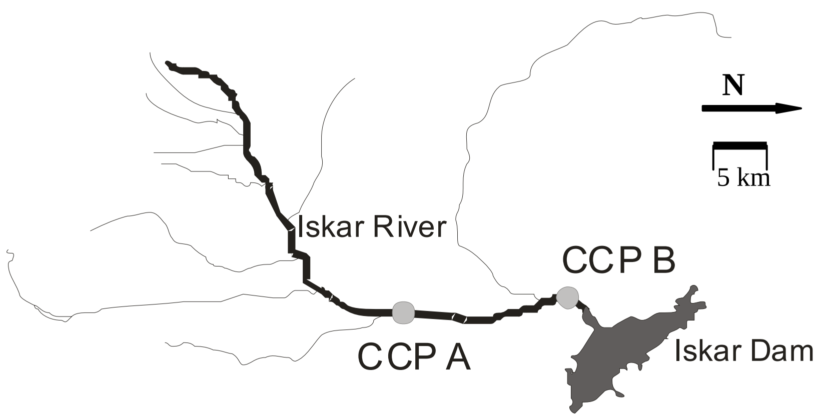


Figure 1: Location of sampling sites in the upper subcatchment of Iskar River
CCP A – Iskar River after town of Samokov and influx of WWTP; CCP B – Iskar River before Iskar Dam

The functional profile of microbial communities in CCP A and CCP B has been investigated at two-stage scheme: (1) analysis of counts of functional microbial groups with a key role for realization of self-purification processes in river ecosystem; (2) taxonomic characteristics of risk water quality factor – coliform bacteria and enterococci. The focus of the study has been placed on interactions of microbial communities in the system “surface water – hyporheic water – sediments”. This is determined by the importance of sediments for the lotic ecosystem metabolism and the insufficient experimental data about the functional biodiversity of microorganisms in this complex system.

Sampling and analytical procedures

The samples were collected during the low flow period of 2008 from the target river components – water, hyporheic water and sediments, transferred into bottles or sterile containers, stored (at 4°C) and processed within 4 hours. Hyporheic water was extracted with Bou-Rouch pump (BOU, 1974) from 15 – 35 cm depth. This shallow hyporheic zone was an active ecotone – linkage of surface and groundwater systems and was preferred because of its important role for the understanding of self-purification processes of their intensity. Sediments were collected with unified particle size (< 1 cm) by sieving.

The physicochemical parameters (temperature, oxygen concentration, and pH) of water and hyporheic water have been analyzed *in situ* immediately after sampling with portable Oxy-meter Handylab Ox1/set and pH-meters Handylab pH11/set (WTW).

After transfer of samples to the laboratory, the water and hyporheic water were analyzed in triplicate for determination of Total Suspended Solids (total SS) by drying to constant mass at 105° C (gravimetric method) and for estimation of organic loading as COD (Chemical Oxygen Demand – dichromatic EPA 410.4/ISO 6060 method), (APHA, 1989). Before measurement of nitrogen and phosphorus concentration the samples were filtered (main pore size 0.45 µm). The nitrogen concentration was calculated as a TCN (total calculated nitrogen) by colorimetric measurement of $N\text{-}NH_4$, $N\text{-}NO_2$ and $N\text{-}NO_3$ according to the BNS-EN-ISO standards and the phosphorus was determined as $P\text{-}PO_4$ (colorimetric method based on phosphomolybdate blue reaction).

The abundance of selected physiological bacterial groups was measured by use of count-plate or most probably number techniques in stream waters, hyporheic waters and sediment solids. The sediments were preliminary treated with ultrasonic disintegrator UD – 20 automatic (3 times x 5 sec) for detachment of bacteria from sediment particles. The following key microbial groups were analyzed by use of specific nutrient media according to KUZNETZOV & DUBININA, 1989; APHA, 1989 (Table 1).

Functional group	*Nutrient media and cultivation*
Aerobic heterotrophes (AH)	Nutrient agar, 24-48 h at 35°C
Anaerobic heterotrophic bacteria (AnH)	Nutrient agar, 7-14 days in anaerobic conditions at 35°C
Oligotrophic bacteria (Oligo)	Diluted Nutrient agar (1:10), 48 h at 28°C
Enterobacteriaceae (Colif)	Endo agar, 24-48 h at 35°C
Enterococci (Entero)	Barnes agar, 24-48 h at 35°C
Pseudomonas/Aeromonas	GSP agar, 48 h at 28°C
Nitrificators (Nitrif)	Sarachandra broth, 5 days at 28°C
Denitrificators (Denitr)	Giltay agar, 7-14 days in anaerobic conditions at 28°
Ammonificators (Ammon)	Nutrient broth with Phenolrot, 3-5 days at 28°C
Proteolytic bacteria (Proteo)	Casein agar, 24 h at 35°C

Table 1: Functional microbial groups and methods for their determination

The all data for microbial counts were presented as $CFU.cm^{-3}$ for water phases and $CFU.g^{-1}$ moist weight for sediments.

The taxonomic determination of isolated bacterial genera from *Enterobacteriaceae* family was carried out by express diagnostic tests API (bioMerieux).

RESULTS AND DISCUSSION

Physicochemical and hydrochemical parameters of stream water and hyporheic water during the study period

The main characteristics of stream and hyporheic water are presented in Table 2. The data showed a typical distribution for the mountain river subcatchment during the summer – concentrations of nutrients were relatively low and followed the standing tendencies in this study area (TOPALOVA et al., 2006).

The hyporheic waters from the two sampling sites presented two significant differences: the amount of total SS in CCP B was four-fold higher than this in CCP A, and respectively the COD was 165.59 $mgO_2.dm^{-3}$. The high organic content is a favorable for massive growth of bacteria in nutrient limited streams and creates a local “hot spots” of intensive production. The amount of suspended solids is a risk factor for survival and incorporation of alochthonic bacteria in natural biocenosis due to the effective adsorption of the cells over the substrata surface (McFETERS & STUART, 1972).

	CCP A water	*CCP B water*	*CCP A hyp.w.*	*CCP B hyp.w.*
T, °C	*17.90*	*17.50*	*19.30*	*19.20*
oxygen, $mg.dm^{-3}$	*9.00*	*7.80*	*5.30*	*5.30*
O_2 *saturation, %*	*103*	*90*	*64*	*63*
pH	*7.87*	*6.93*	*7.00*	*6.80*
total SS, $mg.dm^{-3}$	*165*	*140*	*1850*	*7920*
TCN, $mg.dm^{-3}$	*0.23*	*0.38*	*0.50*	*0.53*
$P\text{-}PO_4$*,* $mg.dm^{-3}$	*0.41*	*0.12*	*0.52*	*0.08*
COD, $mgO_2.dm^{-3}$	*4.72*	*7.87*	*48.33*	*165.59*

Table 2: Parameters of water and hyporheic water (hyp.w.)
T – temperature of water; SS – suspended solids; TCN – total calculated nitrogen

Functional profile of microbial communities in CCP A and CCP B

Three segments of microbiocenoses with important indicative role for the realization of main transformation processes and for the formation of water quality parameters have been studied during the low flow period of summer:

- **I segment**: functional groups realizing an organic matter biodegradation – aerobic heterotrophes (AH), anaerobic heterotrophes (AnH) and oligotrophes (Oligo);
- **II segment**: physiological groups with a key role for the nitrogen cycle – proteolytic bacteria (Proteo), ammonificators (Ammon), nitrificators (Nitrif) and denitrificators (Denitr);
- **III segment**: taxonomic groups with specific indicative role – *Pseudomonas*/*Aeromonas* (bacteria with flexible metabolism, able to realize different transformation processes, including a xenobiotic biodegradation); enterococci and coliforms (microbiological indicators for water quality).

I segment

The key physiological groups with an important role in **organic matter transformations** were relatively well presented - about 10^3-10^4 CFU.cm^{-3} for water phases and 10^6 CFU.g^{-1} for sediments (Figure 2).

In the surface waters of CCP A, the influence of WWTP influx was significant – the aerobic and anaerobic heterotrophes were twice more in comparison with the CCP B. Regarding the oligotrophic bacteria, the counts in the two sites were equal – about 12 000 CFU.cm^{-3}. Overall, across all studied river components the oligotrophes prevailed with the exception of surface waters in CCP A and the hyporheic waters of CCP B. The oligotrophes survive better in the cases of nutrient limiting but at organic pollution with incidental or constant character the heterotrophes become dominant. In the hyporheic water of CCP B this dominance was probably a result of the higher values of registered COD. In CCP A such relation with behavior of COD could not be done, even contrary in stream water the lowest organic content was observed. It can be assumed that the local microflora is very well adapted to the permanent nature of the discharge of WWTP and utilizes quickly and effectively the incoming organic matter. This was confirmed by double-high amounts of adsorbed microflora in sediments of CCP A. This river component presented a similar behavior of microbiocenoses as the surface waters. The two microbial communities – stream bacterial plankton and sediment biofilm were in close relationships and registered the anthropogenic effect from WWTP.

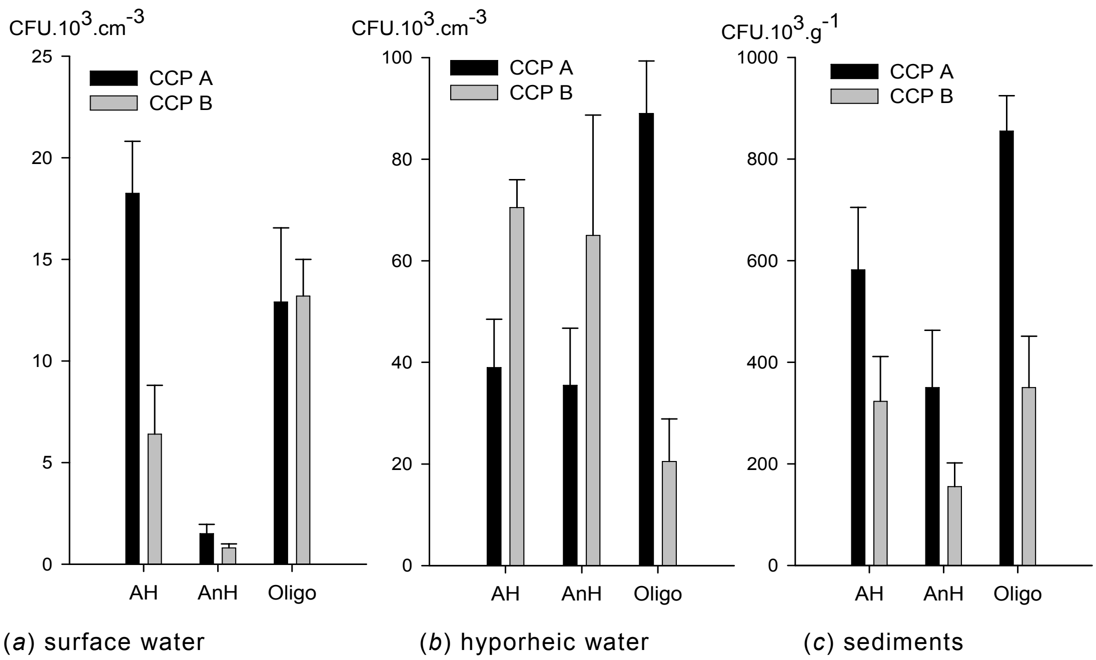


Figure 2: Abundance of physiological microbial groups with key role in organic matter transformations

The anaerobic heterotrophes were weakly presented in the oxygen-rich river waters, while in hyporheic zone (water phase and sediments) they are commensurable with the aerobic heterotrophes counts. The relatively good oxygen saturation of hyporheic zone (64%) rather suggested that the anaerobes were facultative with ability for switching over on various metabolic pathways, depending on the conditions of the corresponding microhabitat.

II segment

The comparison of the microbial groups from the two sampling sites involved in the **nitrogen cycle** showed that the surface waters and sediments of CCP A had the greater abundance of microorganisms in contrast to the hyporheic waters, where the higher counts were reported for the CCP B (Figure 3).

The quantitative parameters of the groups participated in the first stages of non-specific nitrogen transformations /degradation of N-contained organic/ - ammonificators and proteolytic bacteria were relatively high in the range of 10^3-10^5 CFU.cm^{-3} (or g^{-1}).The proteolytic bacteria was a dominant group and indirectly indicated the prevailing protein nature of organic matter in upper Iskar subcatchment.

The more specific groups of denitrifying and nitrifying bacteria were poorly represented, as the nitrificators were not exceeding 500 CFU.cm^{-3} (or g^{-1}). A significant part as a proportion this group occupied in surface waters, while the denitrificators had a key role in sediment microflora. These results determine the processes of nitrification and denitrification as critical for functioning of nitrogen cycle still more the denitrification is the only way to eliminate completely from river ecosystem the surplus amount of nitrogen. In the past periods the local nutrient pollution had been registered in CCP A and the balance in nitrogen transformation was a natural highly effective mechanism for realization of successful self-purification.

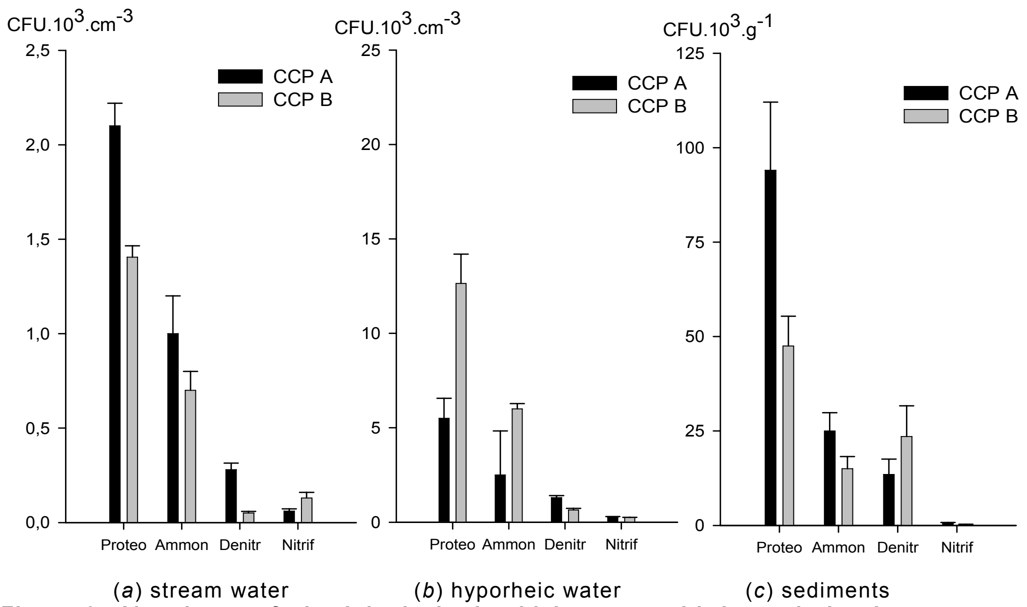


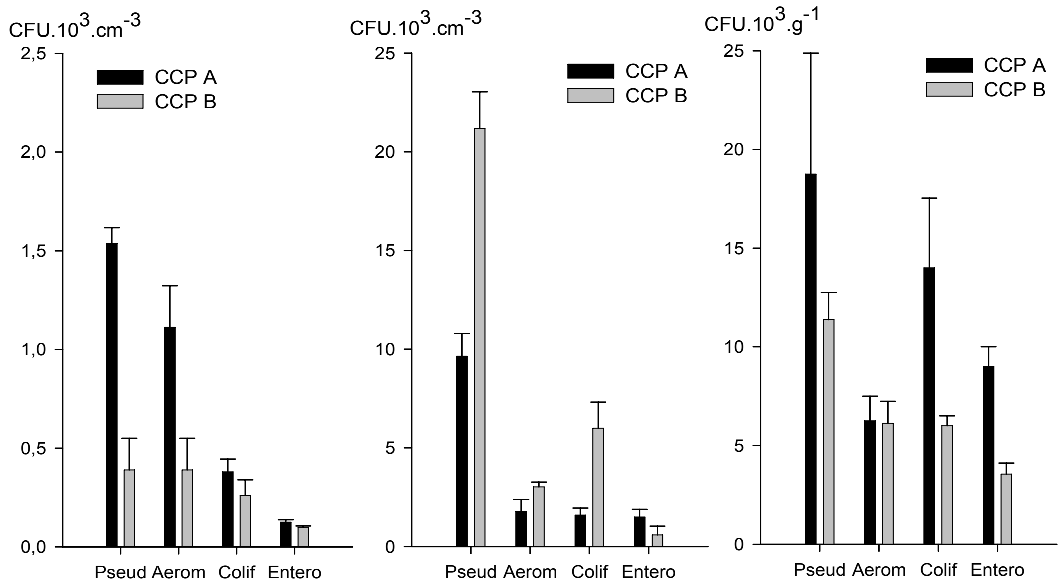
Figure 3: Abundance of physiological microbial groups with key role in nitrogen transformation processes

III segment

The group *Pseudomonas/Aeromonas* have been developed as an indicator of the effectiveness of biodegradation processes in artificial conditions of WWTP, where the microbial counts directly correlate with the potential of the biological system to respond to xenobiotic shock pollution (TOPALOVA & DIMKOV, 2003). In nature waters, *Pseudomonas/Aeromonas* are subject to assessment primarily because of the pathogenic representatives in both genera. The above discussed indicative abilities are still subject to further research, but it can be assumed that the good quantitative presence of the group is an indicator of the possibilities of natural microbiocenoses to realize the full set of metabolic capabilities to eliminate pollutants with a different nature, and to develop the self-purification full capacity on ecosystem level.

The distribution of *Pseudomonas/Aeromonas* in the spatial aspect on vertical plain and along the river section is shown on Figure 4. In surface waters the pseudomonades and aeromonades were approximately with equal proportions, while in hyporheic water and river sediments the *Pseudomonas* dominated because of its flexibility and tolerance to environmental conditions. In hyporheic waters of CCP B, the registered microbial counts were higher than in CCP A on the background of clearly shown opposite trend for the other components. In stream waters and sediments next to the influx of WWTP, the pseudomonades were twice/thrice as much than in CCP B.

The two indicators with a critical importance for the formation of microbiological parameters of water quality (coliforms and eneterococci) represent a considerable interest in study subcatchment. The both groups were at higher counts in surface water and especially in sediments of CCP A. This result confirms by another angle the hypothesis that the sediment microflora more sustainable over time and space records by structural changes the previous disturbances in the quality of surface water as a result of constant influence with variable intensity - the discharge of treated waters from WWTP. The registered total counts of coliforms were beyond the requirements of first and second category for drinking water quality after the Bulgarian and European legislations (see Regulation no. 12/18.06.2002, Official Bulletin of Bulgarian Government 63/28.06.2002) and determined this parameters as a risk factor with highly critical significance.



(*a*) surface water (*b*) hyporheic water (*c*) sediments

Figure 4: Abundance of microbial groups with a specific indicatory role

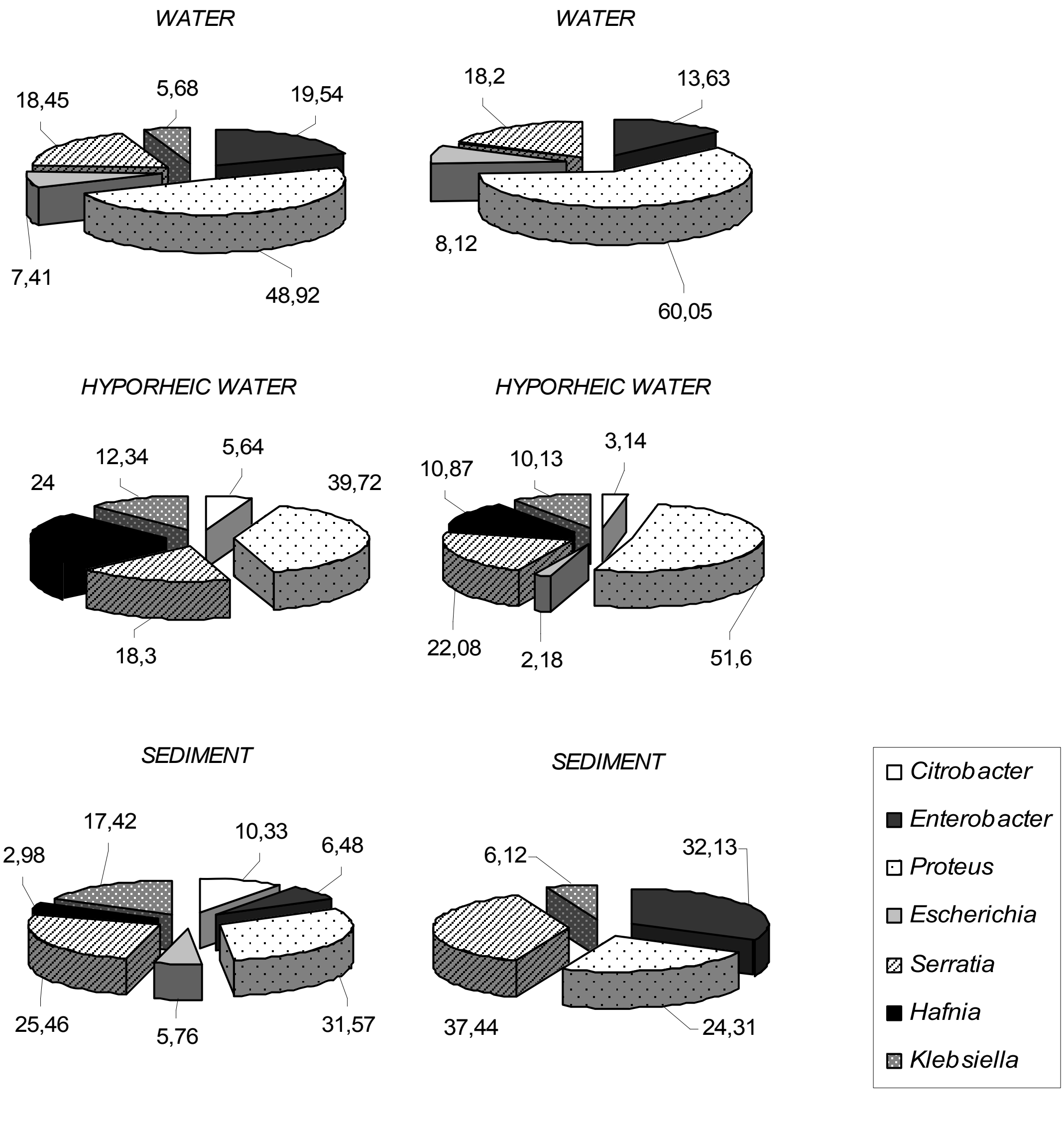
Taxonomic characteristic of coliform bacteria

The importance of *Enterobacteriaceae* family (coliform bacteria) as indicator of contamination with fecal origin and as a potential risk factor for human health requires carrying out a detailed study of their taxonomic characteristic. The percentage quotas of the main taxonomic determined genera in the three river components of CCP A and B are presented on Figure 5.

The biodiversity on level “morphological types of colonies” in the two experimental sites with the crucial meaning did not show large differences. The total amount of 18 morphological type colonies were differentiated (single colonies in the ratio of less than 1% have not been studied) and were referred to the 7 taxonomic bacterial genera. The greatest biodiversity is reported to sediments of CCP A – the all 7 genera were detected. The registered higher bacterial variety in hyporheic waters and sediments were expected. In both sites the dominant genera were the same: *Proteus* and *Serratia*.

The genus *Proteus* (predominantly *P. vulgaris*) was presented with the highest percentage part (between 25 and 60 %) in the different river components. The pathogenic strain *P. mirabilis* was not identified. The interesting trend was observed – in surface waters the dominance of *Proteus* was full, while in hyporheic zone and especially in the sediments this genus was co-dominant with *Serratia*. The *Proteus* is widespread bacteria in natural waters because of its great adaptability; it participates in the ammonification processes and is an indicator of protein nature of organic matter in upper subcatchment of Iskar River.

Escherichia was permanently presented in the microflora of both sites. The high percentage of the genus in surface waters indicated a possible alochthonic origin in the river channel with subsequent incorporation on the sediment particles. This result shows the potential role of sediments as a source of pathogenic microorganisms ("natural depot") for surface waters and refers to the recommendation the sediments to be incorporated into the system for monitoring of water quality in the upper subcatchment of Iskar River (TOPALOVA et al., 2004).



(*a*) CCP A (*b*) CCP B

Figure 5: Taxonomic biodiversity of coliform bacteria in CCP A (*a*) and CCP B (*b*)

CONCLUSION

The detailed profile of functional biodiversity in microbial communities from two critical sites in upper Iskar subcatchment shows several tendencies that can be summarized as follows:

1. The studied functional microbial groups are represented with higher quantitative indicators in surface water and sediments from CCP A (immediately below the WWTP influx). The microbiocenoses in this site endured the negative effect of many factors, but at the first place must be put the anthropogenic impact of Samokov WWTP and near located villages. The increased levels of microbial counts were observed mainly in groups assimilating the organic loading and participating in the first stages of transformation of pollutants – aerobic heterotrophes, ammonificators, proteolytic bacteria, *Pseudomonas/Aeromonas* and indicators of faecal contamination. The hydrochemical data did not register the disturbance of water quality characteristics (even contrary - the nutrients were with the lowest concentrations in surface waters of CCP A), but the microbial communities were with permanent structural changes. In hyporheic waters, the results followed the opposite trend. It is probably because of the higher nutrient concentrations, COD and suspended solids in hyporheal of CCP B.

2. The aerobic heterotrophic and oligotrophic bacteria were well presented in the studied river components. The anaerobic heterotrophes in surface waters were with low quantitative parameters, but their count in hyporheic zone was balanced with number of aerobes. The result was an indicator for existence of various metabolic pathways for utilization of different substrates in this microhabitat. This was confirmed by the relatively high number of bacteria from *Pseudomonas/Aeromonas* group – $10^3 \div 10^5$ CFU.cm^{-3}, which is an indirect indicator of the potential of ecosystem for elimination of xenobiotic compounds.

3. The key processes in the nitrogen transformations are the denitrification and the nitrification because of the lower number of microbial groups that realized them. But the physiological characteristics of these microorganisms (lower speed growth, requirements for specific conditions, low affinity constants, etc.) reinforce this critical meaning, too.

4. The coliform bacteria in surface water categorized the studied subcatchment between the A2 and A3 category. The increased microbial counts of this indicative group in the sediments of CCP A require the sediment component to be included in the monitoring scheme of water quality due to the long-term and successful incorporation of alochthonic pathogenic bacteria with potential for future de-/adsorption in the whole system of hydrological connections.

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