ASSESSMENT OF CONTAMINATION WITH OPPORTUNISTIC PATHOGENIC BACTERIA FROM FAMILY *ENTEROBACTERIACEAE* IN SEDIMENTS OF ISKAR RIVER

Ivaylo Yotinov, Yovana Todorova, Lyubomir Kenderov, Yana Topalova

Abstract: Fecal pollution from municipal wastewater discharges is an extremely serious problem for aquatic ecosystems. This type of pollution is also found in the waters and sediments of the Iskar River in its upper and middle valley. From a microbiological point of view, an indicator of such contamination is the presence of high amounts of opportunistic pathogenic microorganisms of the family *Enterobacteriaceae*. An important feature is that, except in the waters where they are normally found, large amounts of these bacteria can also accumulate in sediments. The purpose of this study is to compare the contamination with opportunistic pathogenic bacteria from the *Enterobacteriaceae* in sediments from the upper and middle part of the Iskar River. In addition, a comparison of the quantity of entero-bacteria with the ecological state indicators was made by Regulation N-4 of 14.09.2012 on characterization of surface water.
From the results of this study, the higher amounts of entero-bacteria were found in the sediments from the middle part of the Iskar River. This is largely related to the strongly negative effect of the urbanized area of Sofia as well as the lack of functioning wastewater treatment plants in the middle of the Iskar River. The results for entero- bacteria from the Upper Iskar subcatchment, also demonstrated a significant contamination with fecal character. As a probable reason for this can be the discharge of Samokov WWTP as well as the unregulated point sources of pollution in this region. On the other hand, there is also evidence of the high self-purification potential of the river ecosystem, which is indirectly proven by the well-represented microbial consortium of aerobic heterotrophic microorganisms in the sediments of the two studied sections of the Iskar River. When comparing fecal pollution indicators with the general environmental assessment, some differences were identified, especially at Lakatnik and Prokopanik stations.

Keywords: *Enterobacteriaceae*, sediments, pollution, river, accumulation, fecal indicators

INTRODUCTION

The Iskar River, and especially the sector – subject of this study, are located in a highly urbanized area, where many of the small agglomerations do not have sewages and built-up wastewater treatment plants. From this point of view, the Iskar River is a natural channel that receives untreated wastewater from the settlements and small industrial enterprises, as well as the produced infiltrate from landfill sites and old septic systems [13]. Pollutants that are entering in surface waters have an extremely diverse background, but the main types can generally be summarized as: 1) organic pollutants (oxygen demanding wastes) mainly generated by households in settlements; 2) organic pollutants of toxic origin (organic synthetic compounds and oils) that are generated by the workshops and plants of various industries located in the area; 3) heavy metals produced by mining and metalworking enterprises in the river basin [18].

River bed sediments are a key component of hydroecosystems where various pollutants, including microorganisms, can deposit and accumulate. The assessment and management of contaminated sediments is more complex than the management of contaminated soils and waters [7]. The main targets for prevention of sediment pollution are limiting the modification and deformation of water basins, reducing the local pollution of hydroecosystems, and increasing the natural self-purification processes in sediments [4]. One of the most serious problems related to pollution of sediments and surface waters is the fecal contamination. This specific type of contamination is due to the unregulated discharge of untreated wastewater and to the presence of multiple point sources for discharge of municipal fecal waters into the natural water bodies. One of the most reliable ways for identification and indication of such type of fecal-household contamination is the application of microbiological indicators for its assessment in surface waters and sediments [1, 8]. The identification and quantification of all pathogenic microorganisms is a complex and costly task. Emphasis is placed on the identification of some key indicator microorganisms with repeatedly proven indicative potential and this monitoring allows effective and efficient control of microbiological contamination [9, 16]. Because the microbiological contamination of water and sediments is mostly the result of fecal wastewater discharge, a small group of non-pathogenic bacteria also contains in human and animal feces - *Enterobacteriaceae* family (i.e.

opportunistic pathogens since under certain conditions some genera can be dangerous to the human body and cause disease) have been identified as the most suitable indicator organisms. To this family belong members of the genera: *Citrobacter, Enterobacter, Escherichia, Hafnia, Klebsiella, Serratia, Yersinia, Shigella, Salmonella* and others. Other indicators for this type of contamination are fecal streptococci and enterococci, coliform bacteria (part of *Enterobacteriaceae*) and sulphite-reducing bacteria [1]. All of these bacteria are relatively easy to isolate and identify, and can therefore serve as a reliable indicator of fecal contamination of surface waters and sediments [3]. Bacteria from *Enterobacteriaceae* are able to survive in the environment for several weeks, their identification is easy and for these reasons they are widely used as the main indicator of the presence of fecal contamination [3, 15].

The aim of this study is to assess and compared the contamination with opportunistic pathogens from family *Enterobacteriaceae* in sediments of Iskar River (upper and middle part of river).

MATERIALS AND METHODS

The experimental design of this study was constructed so that the used critical control points (CCPs) to provide a maximum reliability of the results, to reflect accurately the ecological status of the Iskar River in the studied areas and to give a clear picture of the pollution, self-purification and accumulation processes in the sediments. Ten CCPs were studied along the river (Fig. 1).

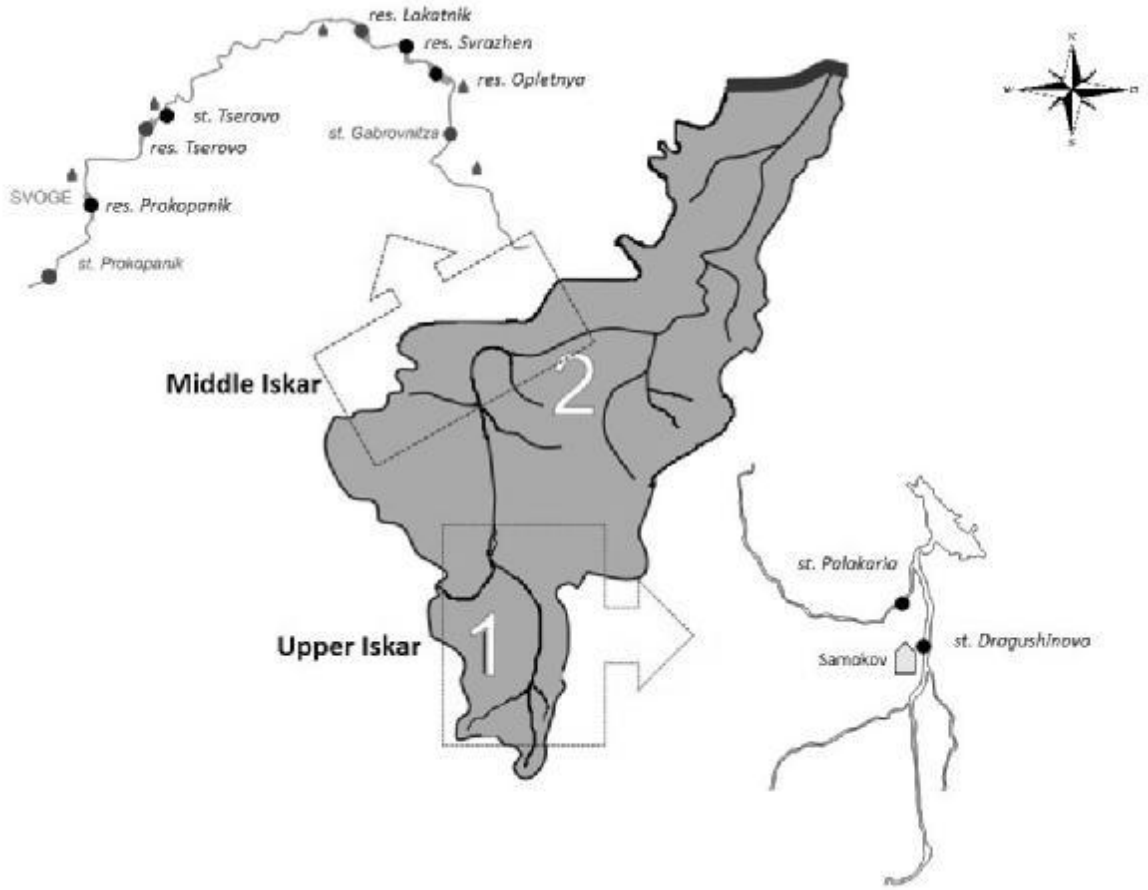


Fig. 1. Map of Iskar River and location of sampling sites

From the upper reaches of the Iskar River, the sampling sites were Dragushinovo and Palakaria – the most risky sites according to the analyses of anthropogenic impact and main sources of pollution. Station Dragushinovo is located immediately after WWTP Samokov and data from it may reflect important risk events. Station Palakaria is located before the confluence of the Palakaria River in the

Iskar River. The Palakaria River is located in an extremely intensive agricultural area, and carries a number of pollutants in its waters. CCPs from the middle part of the Iskar River were located below the significant impact of Sofia city near to the villages of Prokopanik, Tserovo, Lakatnik and Gabrovnitza. These sampling sites cover a sector of the river, located in one of the largest municipalities in Bulgaria (Svoge) with highly presented anthropogenic impact on the river. The study also included 5 CCPs in the impoundment sites of cascade of small hydropower plants Middle Iskar (Prokopanik reservoir, Tserovo reservoir, Lakatnik reservoir, Svrazhen reservoir and Opletnya reservoir) – one of the biggest project for utilization of energy from renewable sources in Bulgaria. The study was carried out in October 2016.

The determination of microbial number was done by use of count plate technique according to the standards of routine microbiological practice [5]. The studied groups of microorganisms, used nutrient media and cultivation conditions are presented in Table 1.

The sediment samples were pre-treated by ultrasonic disintegrator UD-20 automatic (Techpan), in three repeats of 10 seconds in order to deadsorb the bacterial cells from the surface layer of the sediment particles.

Table 1. Studied groups of microorganisms, used nutrient media and cultivation conditions

Microbial groups	**Nutrient media**	**Duration and temperature for cultivation**
Aerobic heterotrophs (**AeH**)	Nutrient Agar (Scharlau, Brit. Phar.)	24 ±1 h at 28°C
Endo-bacteria (**Endo**)	Endo Agar (Scharlau, Brit. Phar.)	24 ± 1 h at 37°C
сем. *Enterobacteriaceae* (**Entero**)	Endo Agar (Scharlau, Brit. Phar.), Cytochrome-oxidase test	24 ± 1 h at 37°C

Endo-bacteria include all bacteria that grow on Endo Agar. The confirmation of bacteria from *Enterobacteriaceae* was done by use of cytochrome-oxidase test of grown up colonies on Endo Agar (Fig. 2). The test was conducted by wet filter paper method – soaking of filter paper with reagent and scraping fresh colonies using loop. After 10-30 sec the oxidase positive colonies (pseudomonades, aeromonades) colored in blue but the oxidase negative bacteria did not change [2].

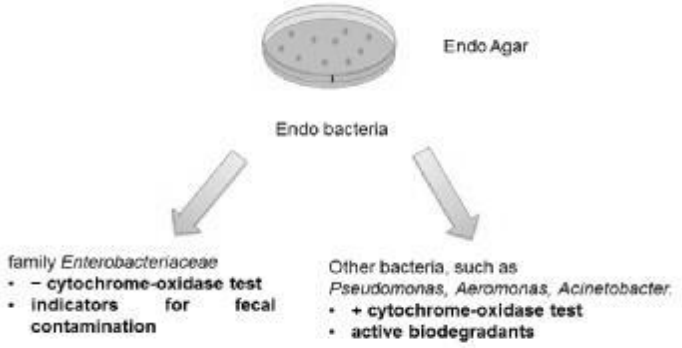


Fig. 2. Scheme for confirmation of bacteria from *Enterobacteriaceae* by cytochrome-oxidase test

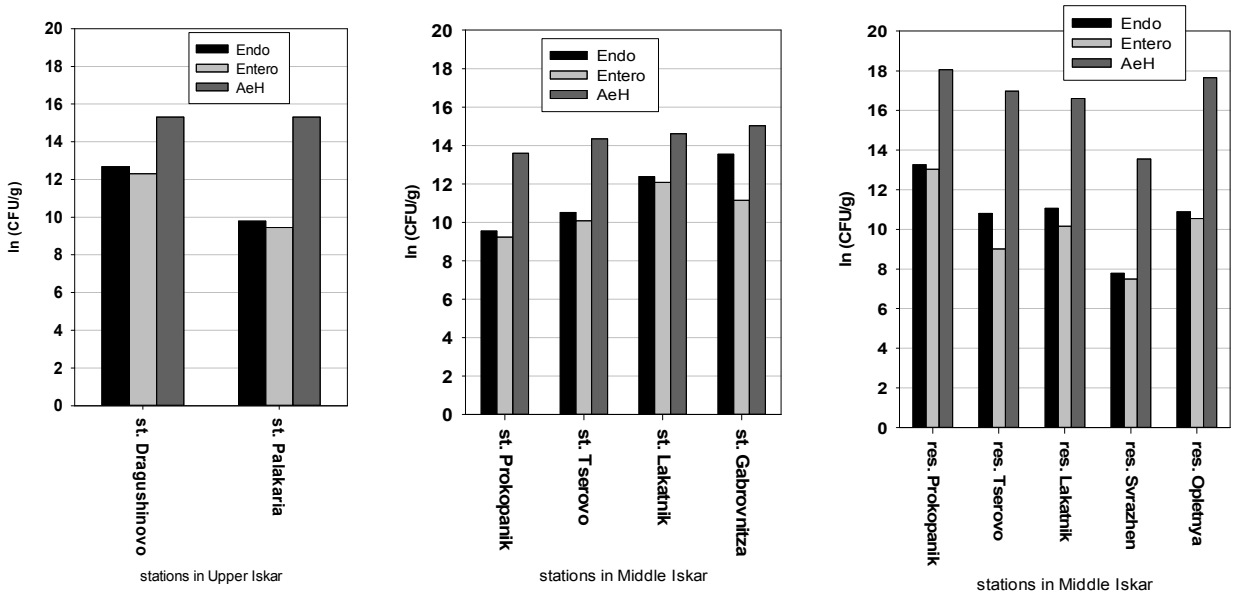
RESULTS AND DISCUSSION

Microbial indicators in sediments of Iskar River

The study of key microbial groups is part of long-term monitoring program in Iskar River. One of the aims is the assessment of Iskar River ecosystem from view of contamination with fecal origin. This type of monitoring requires analyses of specific microbiological indicators in critical control points. The data for the sediments in different parts of Iskar River are presented on Fig. 3.

From the data for microbiological parameters in Upper Iskar sediments, the higher pollution was found near to the village of Dragushinovo (Fig. 3a). The

number of Entero-bacteria in this sampling site exceeded the counts detected in Palakaria River with one order (Table 2). The main reason for this result is the location of sampling site directly below the town of Samokov, especially after the discharge of Wastewater Treatment Plant (WWTP) of Samokov. The high values of Entero-bacteria indicated a fecal contamination – expected effect of this type of impact on river ecosystem (Fig. 3a). The data reveals that the fecal contamination has a potential to accumulate in the river bed sediments. In sediments at Palakaria site, there is a decrease in the number of Entero-bacteria (Fig. 3a). The numbers of Endo-bacteria and in particular the Entero-bacteria at this site are greatly reduced and this determines the low degree of fecal contamination. In the previous studies of this subcatchment it have been shown that this tributary passes through an intensive farming area that heavily loads the surface waters with nitrogen and phosphors but the organic content of waters are very low [10, 11, 12]. The analyses of actual data give an evidence for intensification of self-purification processes in the sediments at this river site - the counts of aerobic heterotrophs exceeding these at Dragushinovo (Table 2, Fig. 3a).



a) b) c)

Fig. 3. Number of microbial indicators presented as ln (CFU/g) in: (a) Upper Iskar; (b) Middle Iskar – river sectors; (c) Middle Iskar - reservoirs

The results obtained for the dynamics of the bacteria in the river sediments show a tendency to increase along the river sector in Middle Iskar (Fig. 3b). The number of bacteria from the Endo-bacteria at the last sampling site (Gabrovnitza) is very high more than 10 times compared to the number in sediments of Prokopanik (Table 3). The high amount of bacteria from the Endo-bacteria and aerobic heterotrophs are both indicative for the predominant origin of organic pollutants in the area (municipal) and for high self-purification potential aimed at its effective elimination. The same trend is also found for the bacteria of the *Enterobacteriaceae* family along the river (Fig. 3b). This dynamics in spatial aspect supposes the permanent retention and survival of the indicator group in the sediments. The origin of the bacteria - indicators of fecal contamination is not directly related to the exploitation working of the Middle Iskar cascade. But probably the change of hydrological regime at sites of impoundment has an auxiliary effect on their accumulation and leads to an increase of risk for the whole ecosystem [14]. For these high values of Entero-bacteria, the contamination with unregulated sources of municipal character in the region of the studied area is contributed to a great extent [14].

In Fig. 3c the data for the quantities of the microbiological parameters in the sediments of the micro-reservoirs in Middle Iskar cascade are presented. The indicator groups show unstable spatial dynamics with decrease in bacterial number along the cascade and a slight increase at the last micro- reservoir. The

lowest number of microorganisms from the three groups was reported in the sediments of the micro-reservoir at Svrazhen (Table 4). The high number of microorganisms indicates the retention of organic pollutants in the sediments of the micro reservoirs, but also the realization of more intensive biotransformation processes – in impoundment sites the processes of utilization and elimination of pollutants in the system are concentrated functionally. An evidence for this is the high level of aerobic heterotrophic microorganisms. The highest number of bacteria from the *Enterobacteriaceae* is found in Prokopanik reservoir and Opletnya reservoir – the first and the last reservoir of the cascade. While the high values of Entero-bacteria at Prokopanik reservoir have logical explanations in its location at the beginning of the cascade and accumulation of all organic pollutants coming from the heavily urbanized area of Sofia, the high values of these bacteria at the Opletnya reservoir can be found in the sediment leakage in cascade sequence. These results are likely to be directly related to the similar dynamics in river bed sediments and confirm connections throughout the hydroeconomic system. The role of sediments as a permanent habitat for survival, retention and accumulation of microorganisms is also confirmed [14].

Table 2. Number of key microbial indicators in sediments of Upper Iskar

Upper Iskar	Endo, CFU/g	Entero, CFU/g	AeH, CFU/g
st. Dragushinovo	0,324 x 10^6	0,222 x 10^6	4,474 x 10^6
st. Palakaria	0,018 x 10^6	0,013 x 10^6	4,491 x 10^6

Table 3. Number of key microbial indicators in river sediments of Middle Iskar

Middle Iskar	Endo, CFU/g	Entero, CFU/g	AeH, CFU/g
st. Prokopanik	0,014 x 10^6	0,010 x 10^6	0,801 x 10^6
st. Tserovo	0,037 x 10^6	0,024 x 10^6	1,700 x 10^6
st. Lakatnik	0,237 x 10^6	0,178 x 10^6	2,210 x 10^6
st.Gabrovnitza	0,756 x 10^6	0,069 x 10^6	3,380 x 10^6

Table 4. Number of key microbial indicators in reservoir sediments of Middle Iskar

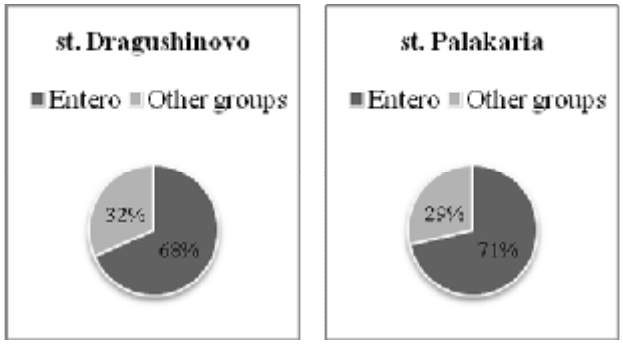
Middle Iskar	Endo, CFU/g	Entero, CFU/g	AeH, CFU/g
reservoir Prokopanik	0,574 x 10^6	0,454 x 10^6	69,852 x 10^6
reservoir Tserovo	0,049 x 10^6	0,0081 x 10^6	23,587 x 10^6
reservoir Lakatnik	0,064 x 10^6	0,026 x 10^6	16,100 x 10^6
reservoir Svrazhen	0,0024 x 10^6	0,0018 x 10^6	0,766 x 10^6
reservoir Opletnya	0,054 x 10^6	0,038 x 10^6	46,160 x 10^6

Proportion of family *Enterobacteriaceae* in the structure of Endo-bacteria

As already noted in the Materials and Methods section, the Endo-bacteria includes except the *Enterobacteriaceae* and other gram-negative but cytochrome oxidase positive bacteria - e.g. the genera *Pseudomonas, Aeromonas, Acinetobacter* and others. In this part of the results the percentage of cytochrome oxidase (-) Entero-bacteria and cytochrome oxidase (+) bacteria in the strucuture of the Endo-bacteria is compared and discussed.

The results for percentage distribution of the Endo-bacteria in Upper Iskar show high but equal proportion of *Enterobacteriaceae* in the sediments of two sampling sites (Fig. 4). In Middle Iskar river sites, the similar proportion is determined for the sediments of three sampling sites (Fig. 5). The proportion of family *Enterobacteriaceae* in the structure of the bacteria from Endo-complex is about 65-75%.

The different result is obtained for river bed sediments in Gabrovnitza site - very low percentage of Entero-bacteria, which means that the remaining groups of microorganisms in the Endo-bacteria make up 91% and are the basis for intensive self-purification processes in the river sediments (Fig. 5d).



a) b)

Fig. 4. Percentage distribution of Endo-bacteria in sediments of Upper Iskar

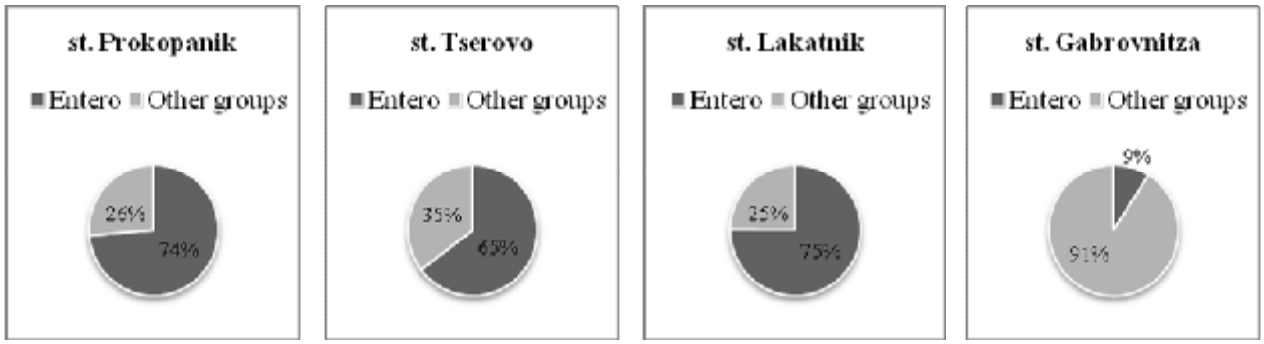


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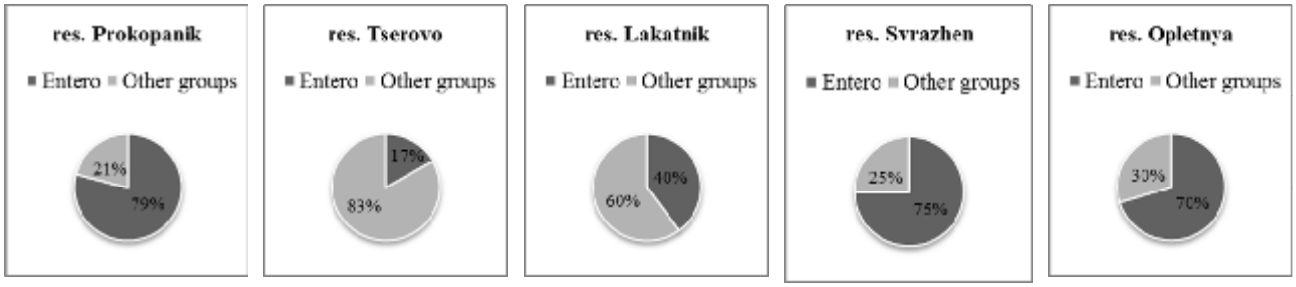
Fig. 5. Percentage distribution of Endo-bacteria in river sediments of Middle Iskar

The percentage distribution of the bacteria from Endo-complex in the sediments of the micro-reservoirs from the Middle Iskar cascade does not follow a clear trend (Fig. 6). The highest percentage of Entero-bacteria was registered in sediments at Prokopanik, Svrazhen and Opletnya reservoirs. The percentage of cytochrome oxidase (+) bacteria is higher in the sediments of Tserovo reservoir and Lakatnik reservoir, which means that there is a well-developed microbial community with a clear biodegradation potential not only in terms of trivial but also of toxic organics.



a) b) c) d) e)

Fig. 6. Percentage distribution of Endo-bacteria in reservoir sediments of Middle Iskar

Comparison of the data for *Enterobacteriaceae* number and ecological status of Iskar River (middle part)

In order to better assess the status of river sediments, a comparison between *Enterobacteriaceae* number and ecological situation in the river was made. Ecological status was calculated using Biotic Index (BI) for macroozoobenthic community [17]. Score results identified ecological status after Regulation N-4 using range for R4 river type (semi-mountains rivers in

Ecoregion 12, Pontic Province). Saprobic situation was estimated using Pantle-Buck saprobic index (S_{PB}) in order to show more precisely the differences between sites [6].

The data for number of *Enterobacteriaceae* and ecological status of the river showed that, the microbiological parameters immediately indicate the fecal contaminations, but the influence of organic pollution on the saprobic state of the river is realized downstream - after the transformation of the pollutant and its inclusion in the ecosystem energy flow. These interrelated parameters show the following dynamics along the river length:

1. In Prokopanik the ecological state is deteriorated, corresponding to α-mesosaprobity (Table 5). This is mainly due to the organic pollution and eutrophication of the river from Sofia and the region. At the same time, the number of *Enterobacteriaceae* is low (Table 3). Probably the distance about 20 km along the river (Sofia - Prokopanik) is sufficient for realization of initial main transformations of organics and reduction of fecal bacteria number but the residual organic compounds still affect the saprobity.

2. In Tserovo the ecological status is the highest for all the examined sites. Here we find a stable β-mesosprobity (Table 5), which corresponds to the climax status of the water communities (not only macrozoobenthos but all communities). This is ensured by the powerful self-purification processes in the river and the two impoundments of cascade (at Prokopanik and Tserovo). In contrast, the *Enterobacteriaceae* number increases. This specific dynamics is a reliable indicator of local sewage pollution due to the unpredicted treatment facilities in the villages and presence of unregulated point discharges from households near to the river.

3. In Lakatnik, the two parameters - ecological status (according to BI and S_{PB}) and the presence of fecal indicators (*Enterobacteriaceae* number) show continuing local pollution. Probably, this pollution is not particularly high and the ecosystem utilizes the organic matter without high stress, and the saprobic state is deteriorating insignificantly (Table 5). On the other hand, the highest values of the Entero-bacteria number is detected here, which clearly demonstrates the specific type of the pollution – fecal contamination.

4. In Gabrovnitza the ecological state again turns into a-mesosaprobity (Table 5) as it is at the first sampling site. The data for microbiological indicators (Table 3) also detect a high organic pollution in the end of the studied river sector. Although the value of the number of *Enterobacteriaceae* decreases compared to this at Lakatnik, the high abundance of aerobic heterotrophs and Endo-bacteria is indicator for intensified processes of organic utilization. This fact shows that despite the positive impact of the impoundment sites for self-purification processes, the absence of wastewater treatment plants for the local agglomerations in Svoge municipality determines the pollution from households as a major problem in the region.

Table 5. Data for ecological status of Iskar River after Biotic index (BI) and Saprobic index (S_{PB})

Sampling site	BI	S_{PB}
Prokopanik	2-3 (moderate)	2,70 (α-meso)
Tserovo	3 (moderate)	1,95 (stable β-meso)
Lakatnik	3 (moderate)	2,53 (β/α- meso)
Gabrovnitza	2-3 (moderate)	2,75 (α-meso)

Despite the ability of microbocenoses to overcome the negative anthropogenic impacts through functional restructuring and adaptation and the obtained score for "moderate" ecological state, it can not be denied that the high values of Entero-bacteria at this river stretch reveal a significant contamination by fecal origin [3].

CONCLUSIONS

The data from this study show a presence of fecal indicator bacteria in ecosystem of Iskar River with potential retention and survival of these contaminants in sediments. In upper part of Iskar River the main source for this specific contamination is the discharge of WWTP Samokov and no point sources with municipal and agricultural nature. The main reasons in the area of Middle Iskar are the absence of village sewages and wastewater treatment plants as well as the existence of unregulated point sources of domestic pollution from small settlements. In Middle Iskar, the highest values for the number of *Enterobacteriaceae* and the most severe fecal contamination respectively are detected in sediments of Prokopanik reservoir – the first impoundment site in the cascade of small hydropower plants that accumulates in its sediments much of the incoming organic pollutants. But the sediments of both parts of river have well-presented microbial consortium in structural and functional aspect that contributes to

high self-purification capacity of ecosystem and to elimination of different negative impacts.

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СРАВНЕНИЕ НА ЗАМЪРСЯВАНЕТО С УСЛОВНО ПАТОГЕННИ МИКРООРГАНИЗМИ ОТ СЕМ. *ENTEROBACTERIACEAE* В СЕДИМЕНТИ ОТ ГОРНОТО И СРЕДНОТО ПОРЕЧИЕ НА Р. ИСКЪР

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Резюме: Замърсяването с отпадъчни води от фекално-битов характер представлява изключително сериозен проблем за водните екосистеми. Такъв проблем се установява и във водите и седиментите на р. Искър в нейното горно и средно поречие. От микробиологична гледна точка индикатор за такова замърсяване е наличието на високи количества условно патогенни микроорганизми от сем. *Enterobacteriaceae*. Важна особеност е, че освен във води, където нормално те се установяват, големи количества от тези бактерии могат да се акумулират и в седименти. Целта на това изследване е да се направи сравнение на замърсяването с условно патогенни микроорганизми от сем. *Enterobacteriaceae* в седименти от горното и средното поречие на р. Искър. Като допълнение към това е направено и сравнение на количеството на ентеро-бактериите с показателите за качество на водите от Наредба Н-4 от 14.09.2012 г. за характеризиране на повърхностните води.

От резултатите беше установено по-високо количество на ентеро-бактерии в седиментите от средното поречие на р. Искър. Това до голяма степен може да се обвърже със силно отрицателния ефект, който оказва урбанизираният район на гр. София върху реката. В района на средното поречие на р. Искър липсват функциониращи пречиствателни станции за отпадъчни води, което поражда значителен риск от битово-фекални замърсявания. Резултатите за ентеро-бактериите от Горен Искър също доказват съществено замърсяване от фекално-битов характер. Като вероятна причина може да се посочи недостатъчно ефективното функциониране на ПСОВ „Самоков", както и нерегламентираните точкови източници на замърсяване в този район. От друга страна, са налице доказателства и за високия самопречиствателен потенциал на речната екосистема по отношение на органичното замърсяване, което косвено се доказва от добре представения микробен консорциум на аеробните хетеротрофни микроорганизми в седиментите на двата изследвани участъка на р. Искър. При сравнението на индикаторите за фекално замърсяване с общата екологична оценка, бяха установени известни различия, особено при станциите Лакатник и Прокопаник.

Ключови думи: *Enterobacteriaceae*, седименти, замърсяване, река, акумулация, фекални индикатори

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