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SEASONAL VARIATIONS OF MICROBIOLOGICAL PARAMETERS OF WATER QUALITY OF THE VELIKA MORAVA RIVER SERBIA

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Abstract - In this study, we investigated the level of sanitary pollution and organic contamination of the Velika Morava River, the largest river in central Serbia. Samples of water for microbiological analysis were collected at 5 sites, monthly, from April 2010 to February 2011. Sanitary analysis, i.e. enumeration of total and fecal coliforms and intestinal enterococci, indicated moderate to critical fecal contamination, while organic load assessment (oligotroph to heterotroph ratio, index of phosphatase activity) revealed the category of moderately polluted water. We also investigated seasonal variations of these groups of bacteria and the factors that could contribute to these differences. Our results showed that the microbiological quality of the water in the Velika Morava River during different seasons is affected by numerous factors such as unequal loading of wastewaters, solar irradiation, and relations of flow/dilution and rainfall/runoff.

Key words: Velika Morava River, microbiological analysis, fecal pollution, organotrophs, Serbia

INTRODUCTION

The Velika Morava River is a significant right-bank tributary of the Danube River upstream of the Iron Gate gorge. It is the largest national river in central Serbia, with a catchment area of 37,444 km². The Velika Morava River flows through a region of intense agriculture (over 25,000 ha) and numerous settlements, receiving untreated or incompletely treated wastewater from urban areas and animal farms, which leads to serious degradation of water quality. In order to maintain safe water according to quality targets, and to prevent disease occurrence, the detection of microbial pollution is crucial (Farnleitner et al., 2001).

Sanitary pollution can be caused by point sources such as discharges of sewage from human sources or livestock enterprises and by non-point sources such as pastures, urban and agricultural run-off or water flow (Kirschner et al., 2004). In this study, we investigated the level of sanitary pollution and organic contamination of the Velika Morava River at 5 sampling sites: Varvarin, Ćuprija, Bagrdan, Markovac and Ljubičevo, from April 2010 to February 2011. For sanitary quality assessment, three groups of coliform bacteria were monitored. The first group was the total coliform bacteria that differ considerably in their pathogenic properties, and apart from the intestines of vertebrates and invertebrates, they can also be present in the soil. The use of total coliforms as indicators of fecal pollution in surface waters in international regulations has been abandoned because of their origin in aquatic ecosystems (Caplenas and Kanarek, 1984; Gauthier and Archibald, 2001). The other two studied groups were fecal coliforms (thermotolerant coliforms) and intestinal enterococci (fecal streptococci) whose levels in bathing waters are defined by the directive of the council of the European Economic Community (European Commission, 2006). Excreted by humans and warm-blooded animals, they can pass through sewage treatment plants and survive for a certain time in aquatic environments (Kavka and Poetsch, 2002). The fecal coliform to enterococci ratio was used to indicate the origin of pollution. A ratio lower than 1.5 reveals pollution by water flow, while a ratio higher than 4 is typical for anthropogenic pollution (Geldreich and Kenner, 1969). The presence of *Pseudomonas aeruginosa*, *Bacillus* sp., *Proteus* sp. and sulphite-reducing clostridia in potable water is undesirable, as subsequent growth is often associated with deterioration in water quality.

Organotrophs constitute one of the largest groups of microorganisms that take part in the processes of matter and energy circulation in water ecosystems; they contribute to the biodegradation and transformation of organic matter, and thus take an active part in the self-purification process of water (Azam 1983; Swiatecki, 2003). They are expected to be autochthonous in aquatic ecosystems and they are mainly used for organic load assessment. Assessment of organotrophs is included in the national regulative (Official Gazzete of RS 74/2011, 2011) due to a number of allochthonous pathogenic species that can be identified within this group.

We also investigated seasonal variations in counts of coliforms, fecal enterococci and organotrophs, and the factors that could contribute to these differences. It is to be expected that the composition of recovered species and their quantities will vary depending on many factors, including the physical and chemical characteristics of the water, and therefore, in this work, we monitored temperature, pH, dissolved oxygen, oxygen saturation, NH_4^+, NO_3^- and PO_4^-. To investigate correlations, we performed Canonical Correspondence Analysis (CCA), a multivariate method to elucidate the relationships between the composition of species and their environment. CCA has been used in numerous studies related to aquatic ecosystems (Pantle and Buck, 1955; Descy, 1979; Sladecek, 1986).

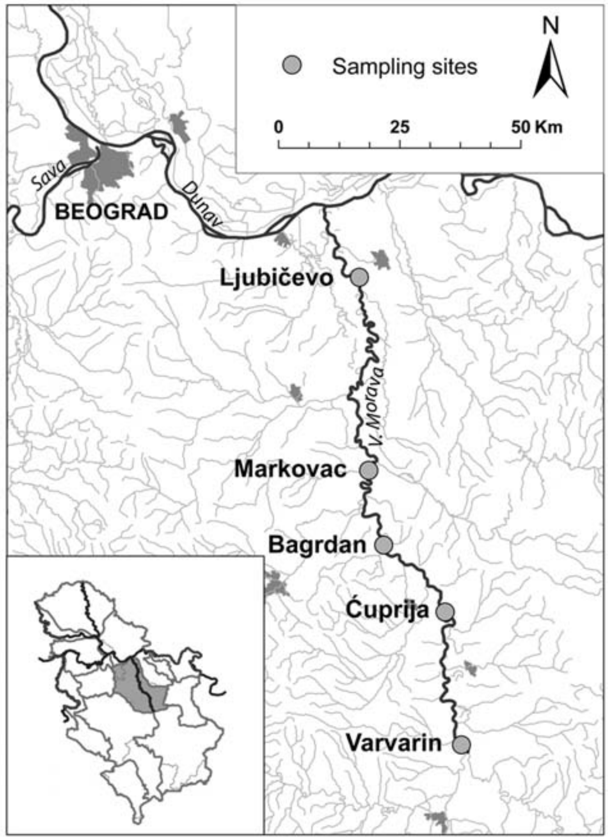

Fig. 1. Sampling sites along the Velika Morava River.

MATERIALS AND METHODS

Study area

During our investigation, samples were collected monthly from April 2010 to February 2011 at five sampling sites (Fig. 1). Varvarin is the furthest upstream sampling site located downstream of the town Kruševac (65,000 inhabitants) and receives a great amount of urban wastewaters. The Ćuprija site is in the small town of Ćuprija; it receives municipal wastewaters (20,000 inhabitants) and is also under the impact of urban wastewaters, and the textile and sugar industry of upstream-located town, Paraćin (25,000 inhabitants). The Bagrdan site is located in the downstream town of Jagodina (35,000 inhabit-

Table 1. Sampling sites along the Velika Morava River

Locality	Latitude	Longitude	Above sea (m)
Varvarin	45° 43.022'	21° 11.348'	173
Ćuprija	43° 56.922'	20° 52.012'	118
Bagrdan	44° 05.099'	21° 11.348'	112.3
Markovac	44° 13.485'	21° 09.174'	109.4
Ljubičevo	44° 36.192'	21° 05.179'	103

Table 2. Classification of ground waters by coliform bacteria and fecal enterococci (Kavka et al., 2006).

	Class	I	II	III	IV	V
	Pollution	slight	moderate	critical	strong	excessive
Total coliforms	100ml	≤ 500	> 500 -10 000	> 10 000 -100 000	> 100 000 -1000 000	> 1 000 000
Fecal coliforms	100ml	≤ 100	> 100 -1000	> 1 000 -10 000	> 10 000 -100 1000	>100 000
Fecal enterococci	100ml	≤ 40	> 40 -400	> 400 -4 000	> 4 000 -40 000	>40 000

Table 3. Physical and chemical parameters of the water of the Velika Morava River. For each season, the most representative month is shown; spring-May, summer-August, autumn-October, winter-December.

Season	Site	t (C°)	Oxygen (mg/l)	Oxygen (%)	pH	NH_4^+ (mg/l)	NO_3^- (mg/l)	PO_4^- (mg/l)
Spring	Varvarin	15.4	8.65	88.4	7.85	0.03	3.0	0.5
	Ćuprija	16.1	8.74	89.9	7.98	0.04	3.4	0.4
	Bagrdan	16.3	8.7	90.3	7.93	0.04	3.0	1.1
	Markovac	16.7	8.75	91.1	7.84	0.08	3.4	1.1
	Ljubičevo	17.4	8.68	91.5	7.81	0.02	3.8	0.9
Summer	Varvarin	24.8	10.4	123	8.20	0.05	2.7	0.8
	Ćuprija	26	/	/	8.60	0.02	2.1	0.6
	Bagrdan	27	13.7	165	8.50	0.03	1.8	1.0
	Markovac	26.5	/	/	8.70	0.05	1.7	0.9
	Ljubičevo	28	12.4	152	8.60	0.34	1.8	2.5
Autumn	Varvarin	13.2	7.48	71.6	8.50	0.22	1.3	42.9
	Ćuprija	14.6	7.58	76.2	8.20	0.11	3.5	38.7
	Bagrdan	12.6	7.69	73.1	8.00	0.12	9.4	/
	Markovac	13.4	7.59	72.8	8.00	0.22	10.3	44.8
	Ljubičevo	13.3	8.27	78.6	8.10	0.19	11.9	16.7
Winter	Varvarin	10.9	8.2	74.5	8.00	0.15	1.2	5.9
	Ćuprija	12	8.13	73.9	7.80	0.74	4.9	3.3
	Bagrdan	12.5	7.77	76.8	7.70	0.70	1.3	3.3
	Markovac	12.6	7.75	76.5	7.80	0.73	3.3	2.8
	Ljubičevo	11.5	8.53	74.5	8.00	0.61	5.6	4.6

/-not measured

Table 4. Correlation matrices of physical, chemical and microbiological parameters of the water of the Velika Morava River.

	Total coliforms	Fecal coliforms	Fecal enterococci	AMB	Heterotrophs	Oligotrophs	PAI
t (C°)	-0,27	-0,48	-0,6	-0,32	-0,35	-0,18	0,08
Oxygen (mg/l)	-0,06	0,03	0,15	-0,12	-0,16	-0,09	-0,12
Oxygen (%)	-0,16	-0,39	-0,51	-0,32	-0,13	-0,26	0,11
pH	-0,22	-0,28	-0,21	0,16	-0,37	-0,35	0,12
NH_4^+ (mg/l)	-0,05	0,16	0,59	-0,04	-0,06	-0,05	-0,01
NO_3^- (mg/l)	-0,4	-0,42	-0,48	-0,15	-0,13	-0,31	0,11
PO_4^- (mg/l)	-0,6	-0,53	-0,36	-0,04	-0,25	-0,13	-0,02

Marked correlation are significant at $p<0.05$.

ants), receiving domestic sewage through the Velika Morava tributaries Belica and Lugomir. The site Markovac is located downstream of the power plant "Velika Morava". The site Ljubičevo is under the impact of wastewaters from the nearby town, Požarevac (35,000 inhabitants). The coordinates of the sampling sites were measured by GPS ("Garmin Etrex") and charted by using ArcView software (map 1:300.000, system WGS_1984) (Table 1).

Physical and chemical analyses

Physical and chemical parameters were measured on site by using the multi-parameter instrument Multi 340i (WTW, Germany).

Microbiological analyses

All samples were processed in the laboratory within 12 h from sampling and a total of 12 parameters were analyzed. Microbiological indicators of sanitary quality and indicators for organic assessment of contamination were analyzed using standard procedures (Official Gazette of SFRJ, number 33/87, Official Gazette of SRJ, number 42/98, Official Gazette of RS, number 74/2011) and the EU Bathing Water Directive 2006/7/EC. For the assessment of recent fecal pollution and the potential presence of pathogenic bacteria, total coliforms (cultivated on eosin methylene blue agar at 37°C for 24 h), fecal coliforms (cultivated on MacConkey agar at 44°C for 24 h) and intestinal enterococci (cultivated on dextrose tellurite agar at 37° for 24 h) were isolated by a membrane filtration method. Classification was performed with class limit values shown in Table 2, proposed by Kavka and Poetsch (2002), and used in the Joint Danube Survey (Kavka et al., 2006).

Identification of isolated coliform bacteria was carried out using an API 20e identification kit (bioMerieux, 1995). The presence of potential pathogen species was tested by cultivation of aerobic mesophilic bacteria (AMB) on meat peptone agar (MPA). To fulfill sanitary analyses, the presence of *Proteus* sp. (cultivated on phenylalanine agar at 37°C for 24 h), sulphite-reducing clostridia (cultivated on sulphite agar at 37°C for 48 h), *Pseudomonas aeruginosa* (cultivated on pseudomonas agar at 42°C for 24 h) and *Bacillus sp.* (cultivated on blood agar at 30°C for 24h) was also determined.

For providing information about the overloading of water with organic compounds, the presence of heterotrophic bacteria, oligotrophic bacteria (pour plate technique with nutrient agar, incubation at 22°C for four days) and phosphatase activity index (PAI) (Matavulj et al., 1990) were monitored.

Statistical analyses

Correlation matrices were calculated using Statistica 6.0 (StatSoft, 2001). Statistical analyses were performed by CCA in the program FLORA ver. 2.0.1.1 (Karadzic et al., 1998). The CCA ordination diagram consists of the following elements: points for bacterial groups (total coliforms, fecal coliforms, fecal enterococci, oligotrophs, heterotrophs and AMB), points for qualitative variables (sampling sites/sampling months) and arrows for quantitative environmental variables (temperature, pH, dissolved oxygen, oxygen saturation, NH_4^+, NO_3^- and PO_4^-). The points for bacterial groups indicate the relative locations of the two-dimensional niches of the bacterial group in the ordination diagram. The coordinates of the points of qualitative variables are related to the coordinates of points of bacterial groups as centroids; means that point to a qualitative variable located closer to a certain point of a bacterial group have a higher abundance of that bacterial group. Quantitative environmental variables are displayed by their correlations with the axes and qualitative environmental variables. The arrow for a quantitative variable runs from the centre of the diagram and ends with arrowhead coordinates that represent the correlations of the quantitative variable with the points of bacterial groups and points of qualitative variables (Braak and Verdonscho, 1995).

RESULTS

Physical and chemical characteristics of the Velika Morava River

Results of physical and chemical analyses are shown in Table 3. During all sampling months, the water reaction was mildly alkaline. Concentrations of nitrates complied with the EU Nitrates Directive 91/676/EC. The highest concentrations of nitrates and phosphates were observed in September and November at the majority of sampling sites. Phosphate concentrations measured in samples from June to February indicated unsatisfactory levels of water quality (European Commission, 1991). Ammonia concentrations reached the highest values from December to February.

Microbiological analysis of the Velika Morava River

The sanitary analyses (Fig. 2) indicated insufficient water quality at all sampling sites during April, May and December 2010 and January and February 2011 (European Commission, 2006). In April and May, fecal coliform numbers ranged from 10^3 to 2.4 x 10^4 CFU/100 ml. A strong fecal pollution (class IV) was detected at the Ćuprija site in May. In these months, fecal enterococci were not isolated. From June to November, a decrease in counts of coliform bacteria was noticed and the majority of sampling sites was moderately polluted (class II). Critical pollution (class III) was detected at the site Bagrdan in August and the Varvarin site in October. September was the least polluted month when fecal coliforms and fecal enterococci indicated slight pollution. In December, January and February the numbers of fecal enterococci ranged from 1.2 x 10^3 to 1.4 x 10^3 CFU/100 ml, indicating critical pollution (III class) at all sampling sites. At the majority of sampling sites from June to November, the number of fecal coliforms was more than 4 times higher than the number of enterococci, showing a human origin of pollution. From December to January, the fecal coliform to enterococci ratio was lower than 1.5, indicating pollution by water flow. Among isolated coliforms, *Escherichia coli*, *Enterobacter asburiae, Enterobacter cloacae, Klebsiella ornithinolytica, Acinetobacter baumannii* and *Citrobacter* sp. were identified. Presence of *Proteus* sp. was noticed in 11%, sulphite-reducing clostridia in 60%, *Bacillus* sp. in 89%, *Pseudomonas* sp. in 62% of samples. Occurrence of this pathogenic species was not related with a specific season or specific sampling site.

The results of organic pollution assessment are shown in Fig. 3. From April to November, only minor oscillations in the counts of organotrophs were noticed. An increased presence of organotrophs was noticed from December to February when AMB counts ranged from 10^3 to 3.1 x 10^3 CFU/ml. The domination of oligotrophs in almost all water sam-

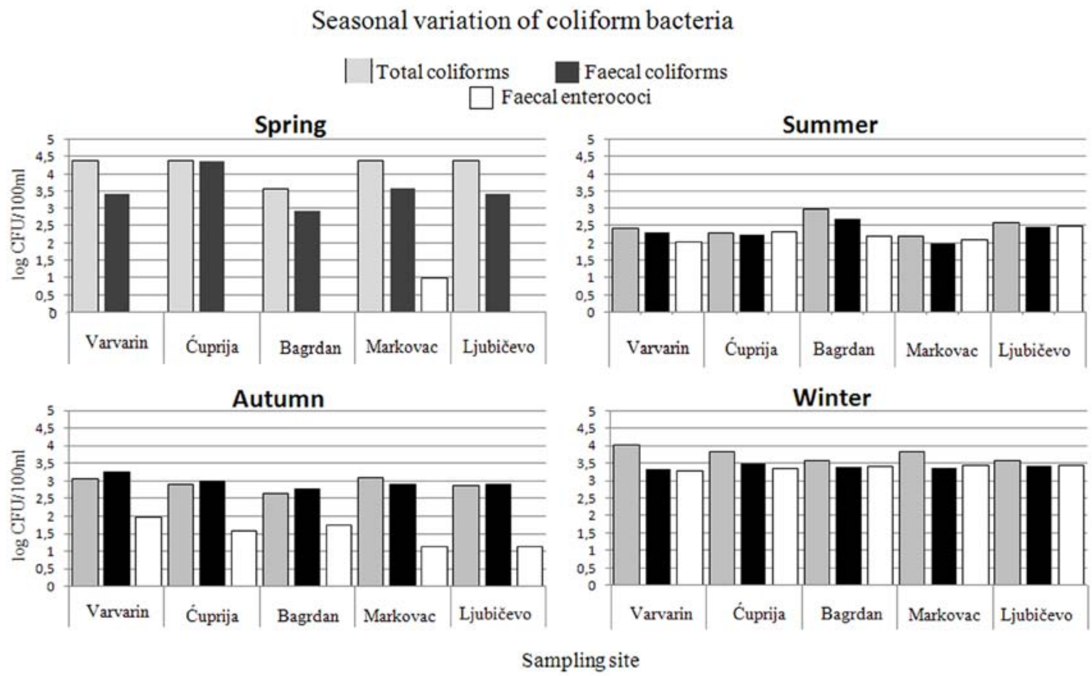

Fig.2. Seasonal variations of total coliforms, fecal coliforms and fecal streptococci at 5 sampling sites along the Velika Morava River. For each season, the most representative month is shown; spring – May: summer – August: autumn – October: winter –December.

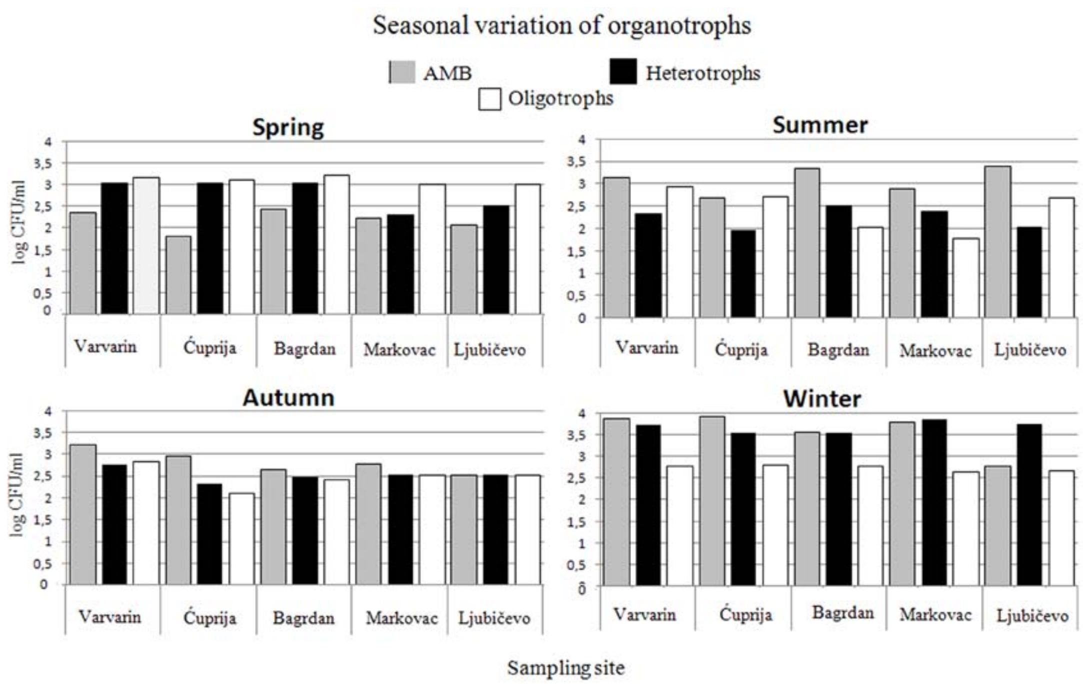

Fig. 3. Seasonal variations of aerobic mesophilic bacteria, heterotrophs and oligotrophs at 5 sampling sites along the Velika Morava River. For each season, the most representative month is shown; spring – May: summer – August: autumn – October: winter –December.

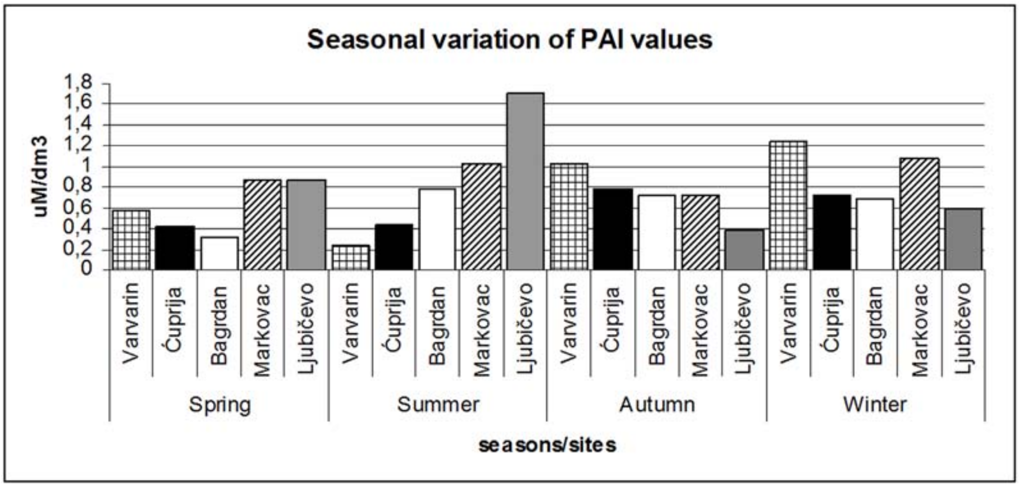

Fig.4. Seasonal variations of the phosphatase activity index at 5 sampling sites along the Velika Morava River. For each season, the most representative month is shown; spring – May: summer – August: autumn – October: winter –December.

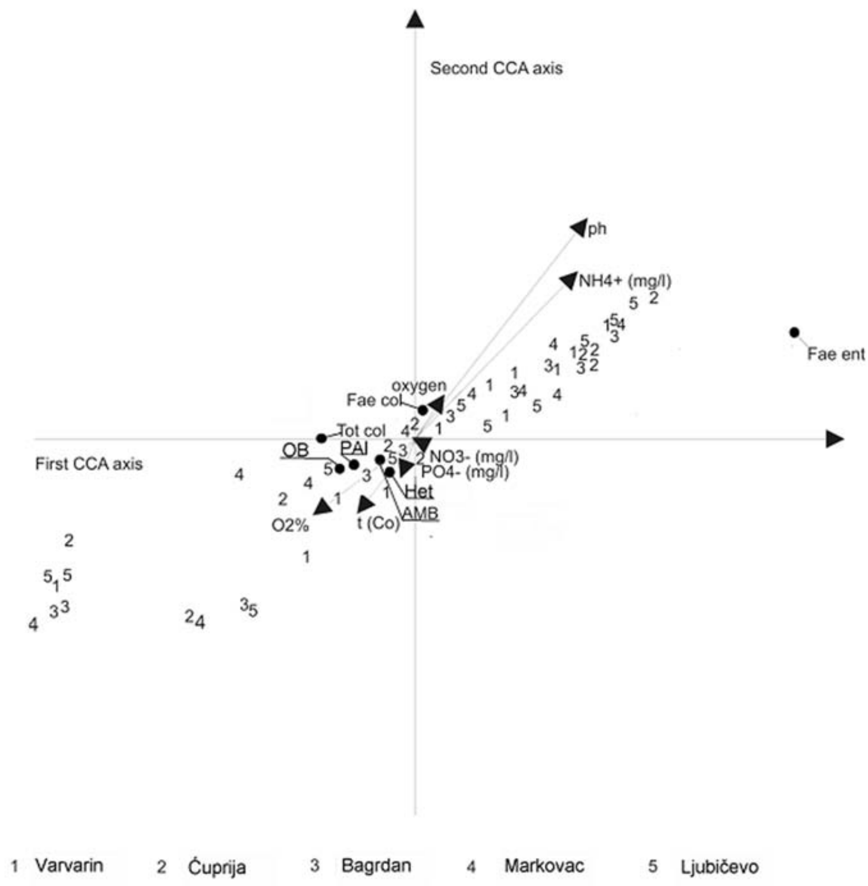

Fig.5. Analyses of samples by CCA according to the sampling site. Fae col-fecal coliforms; Tot col – total coliforms; Fae ent – fecal enterococci; OB – oligotrophs, Het –heterotrophs.

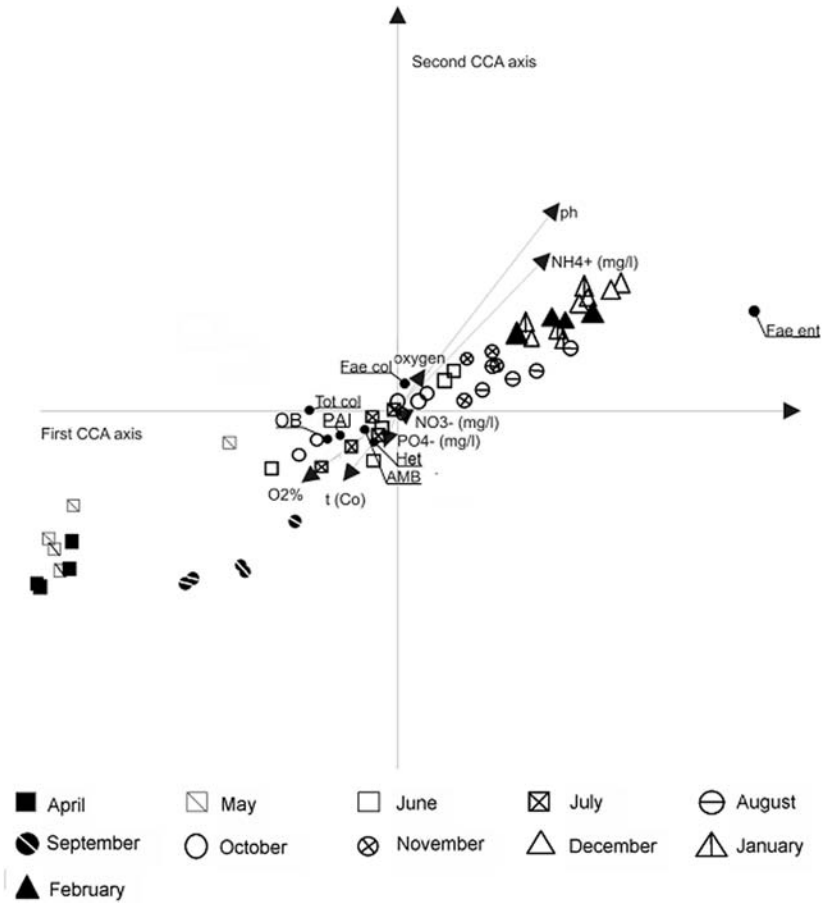

Fig.6. Analyses of samples by CCA according to the sampling month. Fae col – fecal coliforms; Tot col – total coliforms; Fae ent – fecal enterococci; OB – oligotrophs, Het –heterotrophs.

ples from April to November indicated a satisfactory level of self-purification; on the other hand, a domination of heterotrophs was noticed at all sampling sites from December to February, indicating an unsatisfactory level of self purification.

Organic load was also assessed by the phosphatase activity index (Fig. 4). Analysis of the results indicated slight pollution (IIB) in samples collected in April and May, with the most polluted site being Ljubičevo. From June to August, the majority of samples were moderately polluted (II-III class); once again, the most polluted site was Ljubičevo. Slight (class IIB) to moderate (II-III) pollution was detected from September to February, when the highest pollution was detected at the Varvarin site.

Seasonal variation of investigated parameters

A CCA was performed on the physical, chemical and microbiological parameters to get an overview of the biotic and abiotic conditions and to relate the observed patterns to the changes in the composition of the bacterial communities.

The samples were separated along CCA axes according to the sampling site (Fig. 5). The resulting graph shows overlapping clusters along the first CCA axis without grouping of samples according to the sampling site.

The samples were also separated along the CCA axes according to the sampling month (Fig. 6). Results were analyzed, considering April and May as spring, June, July and August as summer, September, October and November as autumn and December, January and February as winter. A difference was observed along the first CCA axis between the values of samples collected during spring and those collected in winter. Points for winter samples were grouped around points for fecal coliforms and fecal enterococci. While the clusters of spring and winter were clearly separated, those of summer and autumn overlapped along the first CCA axis. The variables representing pH and NH_4^+ concentration pointed in the direction where the winter samples were found; oxygen saturation and temperature pointed to the cluster of spring samples, while the PO_4^- concentration pointed to overlapped clusters of autumn and summer.

According to correlation matrices (Table 4), all investigated groups of bacteria correlated negatively to water temperature and oxygen saturation with a statistical significance in the case of fecal coliforms and fecal enterococci. On the other hand, a positive correlation with statistical significance was observed between fecal coliforms, fecal enterococci and NH_4^+ level. All groups of organotrophs and coliforms correlated negatively to inorganic nutrients (NO_3^- and PO_4^-). Correlation was statistically significant in the case of total coliforms, fecal coliforms and fecal enterococci.

DISCUSSION

In this study, we performed complex microbiological analyses to evaluate the impact of wastewaters originating from urban settlements such as Požarevac, Jagodina, Paraćin and Kruševac on the level of sanitary pollution and organic contamination of the Velika Morava River. We also investigated seasonal variations of water quality. At all sampling sites deterioration of the water quality by fecal and organic pollution was present. The origin of fecal pollution can be mainly attributed to the great amount of raw or not properly treated wastewaters, since wastewaters in Serbia are still not processed before being released into watercourses. On the other hand, the origin of organic pollution can be attributed to organic manure, fertilizers and feed waste originating from agriculture. An unequal loading from point sources (fecal pollution) and disperse emissions (organic pollution) led to variations in the pollution level; therefore none of the selected sites could be set aside by the studied parameters as the most polluted during all seasons of sampling.

We observed seasonal differences in the fecal pollution level assessed by the quantification of coliform bacteria. Fecal enterococci and fecal coliforms showed a positive correlation with ammonia concentration and a negative correlation with water temperature and oxygen saturation. Variation in ammonia concentrations during the seasons indicated an unequal loading with wastewaters, which could contribute to the observed variation in microbiological pollution. In winter, ammonia concentrations reached the highest level, while the level of dissolved oxygen was the lowest, which could be caused by the decomposition of fecal matter. Moreover, when comparing summer and autumn, the increase of fecal coliforms and fecal enterococci was observed. As emphasized in earlier studies (Sinton et al., 1994; McCambrige and McMeekin, 1981; Nemi, 1976), solar irradiation reduces the number of fecal coliforms. It is expected that months with a higher level of solar irradiation are also the months with a higher water temperature, which could explain the observed negative correlation of fecal indicators with temperature.

Total coliforms are the group that commonly inhabits aquatic ecosystems and therefore they are expected to show higher robustness to variation of physical and chemical parameters compared to fecal coliforms and fecal enterococci. A higher tolerance to solar irradiation was also observed in organotrophs, through a statistically insignificant correlation with temperature and only minor oscillation in their counts during the different periods of the year.

The highest values of nitrates and phosphates were recorded in autumn, due to increased agricultural activity during this season and the large amount of rainfall. Both natural and artificial fertilizers contain high concentrations of nitrates and phosphates, which can enter aquatic ecosystems by runoff, rainfall rinsing, etc. Increased rainfall also means an increased flow and dilution, which probably led to the negative correlation in the numbers of organotrophs and the level of nutrients.

In conclusion, the dynamics of bacterial functional groups in the Velika Morava River are affected by numerous factors such as unequal loading, solar irradiation, dilution, rainfall and the origin of investigated groups of bacteria. The present study shows that continuous monitoring during the year is obligatory for a proper assessment of microbial pollution. Moreover, microbiological investigations of water could provide us with information about the relation between abiotic and biotic factors of water quality through an understanding of the role of each bacterial functional group in the ecosystem, which could provide the data necessary to explain the functioning of the particular investigated system. Microbiological control of water quality of the Velika Morava River should be obligatory to ensure the adequate quality of water for domestic, agricultural and industrial uses. Moreover, it was shown that pollution originating from tributaries significantly undermines the water quality of the Danube River (Kirchner et al., 2009; Kolarević et al., 2011a; Kolarević et al., 2011b). The results of this study provide much needed data from Serbia for the International Joint Danube Survey and contribute to the attainment and maintenance of proper microbiological quality of water bodies in Serbia.

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