THE ANTHROPOGENIC IMPACT ON WATER QUALITY OF THE RIVER DANUBE IN SERBIA: MICROBIOLOGICAL ANALYSIS AND GENOTOXICITY MONITORING

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Abstract - The aim of this work was to examine the impact of urban wastewaters on the water quality of the Danube River in Serbia. Samples of water and sediments for microbiological analysis and genotoxicity monitoring were collected from 6 sites during spring and/or autumn 2010. 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. Mercury-resistant bacteria were detected in all water samples, with high numbers at locations positioned downstream of Belgrade. There was no correlation of the microbiological parameters of the sediment and water samples. Genotoxicity monitoring, performed by the comet assay on hemocytes of mussels *Sinanodonta woodiana*, indicated a significant increase of DNA damage in mussels collected from the studied sites compared with the control group.

Key words: River Danube, microbiological analysis, genotoxicity monitoring, comet assay, *Sinanodonta woodiana*

UDC 579.63(282.243.7)(497.11)

INTRODUCTION

Water ecosystems are nowadays under increased threat from rising human populations accompanied by increased agricultural and industrial growth. Therefore, detection of microbial and genotoxic pollution is crucial for watershed management activities in order to maintain safe waters according to their quality targets (Farnleitner et al., 2001).

The Danube Basin is the second largest river basin in Europe that flows through 17 countries. The total length of the river is 2,857 km. The Serbian reach of the Danube extends a distance of 588 km that covers the middle and part of the lower, 220 km-long stretch. This typical low-land river flows through a region of intense agriculture (over 470,000 ha) whose runoff degrades water quality due to the high concentrations of nitrogen and phosphorus. The Danube flows through numerous industrial and urban centers and receives significant amounts of urban and industrial wastes, leading to serious debasement of the water quality. Industrial and urban wastes are often characterized by numerous toxic and carcinogenic chemicals, such as heavy metals and organic matter, which can contaminate water and enter the food chain, posing considerable danger to public health. Moreover, genotoxic pollution can cause adverse reproductive effects or may even result in an elevated risk of extinction for particular species of the ecosystem (Villeneuve and Garci-Reyeron, 2011).

Mollusks, such as bivalves, are widely distributed sessile filter feeders that can serve as reliable bioindicators of aquatic pollutants (Venier, 1997) and are thus a valuable tool for the screening of pollution and potential environmental harm. They are capable of accumulating pollutants and they exhibit different physiological, histological and molecular responses (Johns and Louma, 1990; Roberts, 1996; Bilos et al., 1998; Narbonne et al., 1999; Dowling and Mothersill, 2001; Pavlica et al. 2001; Binelli et al., 2002; Bolognesi et al., 2004; Klobucar et al. 2008). Woznicki et al. (2004) pointed out the ability of the mussel *Sinanodonta woodiana* (Rea, 1834) to detect the genotoxic potential of certain agents, such as polycyclic aromatic hydrocarbonates (PAHs), with road applications to aquatic toxicology and monitoring.

This study was undertaken to evaluate the impact of wastewaters from large urban settlements, such as Novi Sad, Belgrade, Pančevo and Smederevo, on the water quality of the River Danube in Serbia. In order to follow a more integrated approach and obtain a more reliable picture of the past and present state of this water ecosystem, we analyzed both water and sediment samples and, in addition to standard physical, chemical and sanitary quality parameters (EU-Wastewater directive 91/271/EEC, EU-nitrates 91/676/EEC), we evaluated the organic load and the presence of bacteria resistant to mercury. As the comet assay has become one of the major tools for assessing pollution-related genotoxicity in aquatic organisms (Dixon et al., 2002), we also used the comet assay on hemocytes of *S. woodiana* for genotoxicity monitoring of Danube water.

MATERIALS AND METHODS

Sampling area

The samples for analysis were collected during spring and autumn 2010 at 6 sites on the River Danube (Table 1). The Stari Slankamen site which is, located downstream from the city of Novi Sad (300,000 inhabitants), receives a considerable amount of urban wastewaters. The Belegiš site was chosen to measure the effects of the River Tisa (the largest tributary of the Danube River) on water quality. The impact of the largest city in the region, Belgrade (≥1,600,000 inhabitants), was monitored on the downstream located site at Višnjica. The Vinča and the Orešac sites are under great impact from the urban wastewaters, the oil refinery and industry thelocated upstream in and around Pančevo (80,000 inhabitants). The Kostolac site receives effluents from theupstream domestic sewage the located in the town Smederevo (80,000 inhabitants). This site is also under the impact of the polluted water from the Velika Morava River tributary, and the thermal power plant "Kostolac" located upstream. The coordinates of the sampling sites were measured by GPS ("Garmin Etrex") and charted with ArcView software (map 1:300,000, system WGS_1984).

Microbiological analyses

All samples for microbiological analyses were processed in the laboratory within 12 h from sampling, and a total of 16 parameters were analyzed. Microbiological indicators of sanitary quality and indicators for the estimation of organic contamination were analyzed using standard procedures (Official Gazzete of SFRJ, 33/87; Official Gazzete of SRJ, 42/98, Official Gazzetes of RS, 46/91, 53/93, 67/93, 48/94 and 54/96) as used in previous research (Kolarević et al., 2010, Zlatković et al., 2010).

The status was assessed according to the criteria defined in the EU-Bathing Water Directive 2006/7/EEC modified by Kavka and Poetsch (2002). For the assessment of recent fecal pollution and the potential presence of pathogenic bacteria, total coliforms cultivated on eosin-methylene blue agar (HIMEDIA, India) for 24 h at 37°C, fecal coliforms cultivated on MacConkey agar (HIMEDIA, India) for 24 h at 44°C, and intestinal enterococci cultivated on dextrose tellurite agar (Torlak, Serbia) for 24 h at 37°C, were analyzed by membrane filtration method.

Identification of isolated coliform bacteria was performed using the API 20e identification kit (bioMerieux France, 1995). The presence of potential

pathogen species was assessed by cultivation on meat peptone agar (MPA) (Difco, USA). Sanitary analysis was performed by examing the samples for the presence of *Proteus sp.* by cultivation ed on phenylalanine agar (Torlak, Serbia) for 24 h at 37°C, of sulphite-reducting clostridia by cultivationed on sulphite agar (Torlak, Serbia) for 48 h at 37°C, of *Pseudomonas aeruginosa* by cultivation edon pseudomonas agar (Lab M Limited, UK) for 24 h at 42°C and of *Bacillus sp.* by cultivationed on blood agar (Torlak, Serbia) for 24 h at 30°C.

In order to obtain information about the level of organic pollution, the presence of heterotrophic and oligotrophic bacteria (the pour plate technique with MPA agar, incubation at 22°C for four days) and the phosphatase activity index (Matavulj et al., 1990) were assessed.

Microbiological analyses of the sediment included the quantification of coliform bacteria, membrane filtration of diluted sediment samples, incubation on eosin-methylene blue agar (HIMEDIA, India) for 24 h at 37°C, identification of isolated coliforms (API 20e), isolation of bacteria resistant to mercury by the spread plate technique, diluting the sediment samples on MPA (Difco, USA) with mercury for 24 h at 37°C and examination for the presence of potential pathogens by the spread plate method, diluting sediment samples, incubation on MPA (Difco, USA) for 24 h at 37°C.

Genotoxicity monitoring
Biological material

Genotoxicity was evaluated by the comet assay on hemocytes of *S. woodiana* collected from selected polluted sites in the River Danube. Freshwater mussels *S. woodiana* were collected in autumn 2010. Samples were collected with the FBA hand net (Kick and Sweep technique), a benthological dredge and diving. The specimens of *S. woodiana*, shell length range 7-15 cm, were used for the test. To measure the DNA damage response to environmental stress, the samples of mussels were tested within 4 h after sampling.

A group of mussels used for negative control were kept for 15 days under controlled aquarium conditions (six glass aquaria 100 L each) in dechlorinated, well-aerated water (the concentration of $O_2 > 8$ mg/L, O_2 saturation > 90%, pH 7.2-8.1) at 18-24°C, with a bottom environment composed of fine sand that had been washed with clean water to eliminate debris and treated with heat (4 h, 250°C) before the set-up of the aquaria system, in order to eliminate potential disease vectors. The animals were fed every third day with dry leaves of string nettle (*Urtica dioica*) macerated and minced with a pestle and mortar (Gačić et al., 2010, Tomović et al., 2010).

Hemolymph collection

Hemolymph was collected in the dark from the posterior adductor muscle sinus of each freshwater mussel by a hypodermic syringe, and transferred to 1.5 ml microtubes, centrifuged for 10 min at 1,000 rpm and subjected to the standard alkaline comet procedure. The hemocytes' viability of hemocytes was determined with 0.4% Trypan blue dye. Cells that stained blue were considered non-viable.

Comet assay

The alkaline comet assay procedure was performed under yellow light essentially as described by Singh et al. (1988). Microscopic slides were precoated with 0.5% NMP agarose and air dried for 24 h. To form a second, supportive layer, 80μl of 1% NMP agarose was gently placed on the top of the 0.5% NMP layer and spread over the slide using a coverslip. The slide was placed on ice for 5 min to allow complete polymerization of the agarose. After the coverslips were removed, 30μl of hemolymph pellet suspension, gently mixed with 70μl of 1% LMP (37°C) agarose, was pipetted onto the supportive layer of 1% NMP agarose and covered with a coverslip. After 5 min on ice, the coverslips were removed and the slides were lowered into freshly made cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1.5% Triton X-100, pH 10) for 1 h. To allow DNA unwinding the slides were placed in an electrophoresis chamber containing cold alkaline electrophoresis buffer (300 mM NaOH,

1 mM EDTA, pH 13) for 20 min. Electrophoresis was performed at 25 V and 300 mA for 20 min. After electrophoresis, the slides were placed into freshly made neutralizing buffer (0.4 M Tris, pH 7.5) for 15 min. Staining was performed with 20 μl per slide of EtBr (2 μg/ml). The slides were examined with a fluorescence microscope (Leica, DMLS, Austria) at 400 x magnification, with a 510-560 nm excitation filter and a 590 nm barrier filter. The microscopic images of comets were scored using Comet IV Computer Software (Perceptive Instruments, UK). Images of 25 cells were collected from each of two replicate slides per sample and of the parameters available, the tail moment was chosen as the most relevant measure of DNA damage.

Statistical analyses

Statistical analyses were performed by the Mann-Whitney U-test using Statistica 6.0 Software (StatSoft, Inc.).

RESULTS AND DISCUSSION

Environmental parameters, such as temperature, salinity, pH and dissolved oxygen play a major role in the distribution of bacteria in all aquatic systems. Therefore, the basic physical and chemical parameters of water were determined at all the sampling sites (Table 2). An increased concentration of NH_4 was detected at the Višnjica site while increased concentrations of PO_4 were detected at sites Belegiš, Orešec and Kostolac.

Microbiological analyses

Microbiological analysis of the river water is obligatory for use-related purposes such as drinking water production, irrigation and recreation. Standard sanitary analysis includes the enumeration of fecal indicator bacteria and potentially pathogenic bacteria, the presence of sulphite-reducing clostridia, *Pseudomonas aeruginosa*, *Proteus* sp. and *Bacillus* sp. Fecal indicator bacteria, i.e. total coliforms, fecal coliforms (thermotolerant coliforms), and intestinal enterococci (fecal streptococci) are ex-
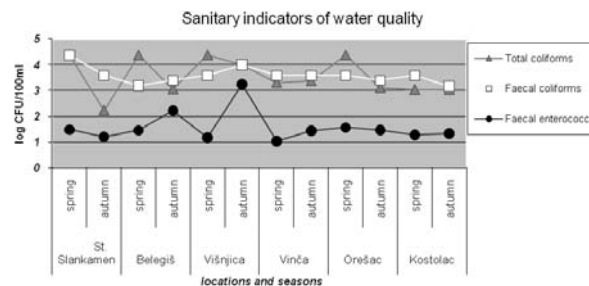

Fig. 1. Sanitary indicators of water quality in of samples collectyed from the River Danube during spring and autumn 2010. The results are shown as log CFU/100ml.
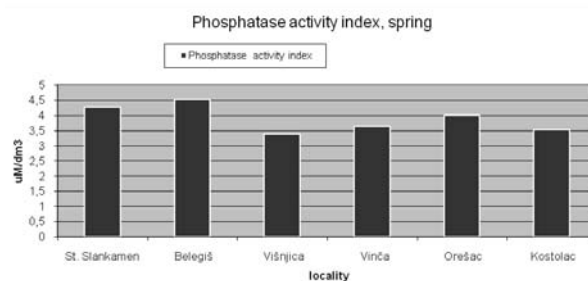

Fig. 2. Phosphatase activity index in water samples collected from the River Danube during spring 2010. The results are shown as μmol/dm³.
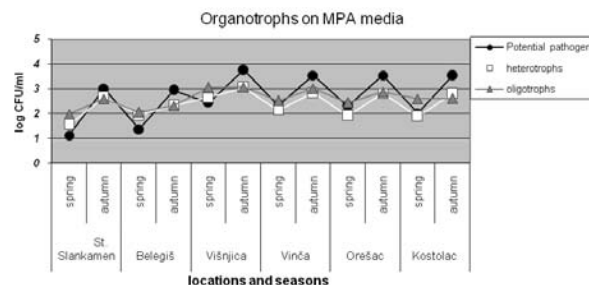

Fig. 3. Number of organotrophic bacteria isolated from water samples from the River Danube during spring and autumn 2010. The results are shown as log CFU/ml.

creted by humans and warm-blooded animals, pass through the sewage treatment plants, and survive for a certain time in the aquatic environment (Kavka et al., 2002). The coliform bacteria differ considerably in their pathogenic properties. Aside from the intestines of vertebrates and invertebrates, they can also be present in the soil. Total coliforms indicate water pollution, but this does not have to directly correlate with an anthropogenic source, while fecal coliforms are used to

Table 1. Sampling sites along the River Danube.

Locality	Latitude	Longitude	Above sea (m)
Stari Slankamen	45° 9.056'	20° 14.837'	91.4
Belegiš	45° 1.812'	20° 21.370'	88.3
Višnjica	44° 50.381'	20° 33.520'	79
Vinča	44° 46.146'	20° 37.159'	77.2
Orešac	44° 39.305'	20° 49.449'	72.1
Kostolac	44° 44.138'	21° 9.425'	69.6

Table 2. Physical and chemical analysis of water.

Site	Slankamen		Belegiš		Višnjica		Vinča		Orešac		Kostolac	
Season	S	A	S	A	S	A	S	A	S	A	S	A
t (C°)	12.1	15.9	12.5	17.0	12.0	17.8	12.2	18.0	12.4	17.9	12.5	18.3
Conductivity (μS/cm)	438	nd	437	nd	356	nd	368	nd	378	nd	400	nd
Oxygen (mg/l)	11.9	7.54	12.4	7.91	9.86	6.4	10.4	7.2	9.9	6.99	10.3	6.8
Oxygen (%)	110	76.2	111	81.6	88.4	67.3	96.4	75.6	92.5	73.4	95.7	71.4
pH	8.7	8.0	8.8	7.8	8.19	7.8	8.1	7.8	8.0	8.0	8.17	7.8
NH_4^+ (mg/l)	0.06	0.06	0.05	0.04	0.21	0.19	0.02	0.11	0.02	0.06	0.03	0.12
NO_3^- (mg/l)	1.8	2.7	3.8	1.3	1.6	3.1	7.6	8.7	5.5	2.2	3.2	1.0
PO_4^- (mg/l)	1.4	1.0	0.7	4.5	0.8	1.8	1.1	32.2	1.3	3.2	7.6	9.5

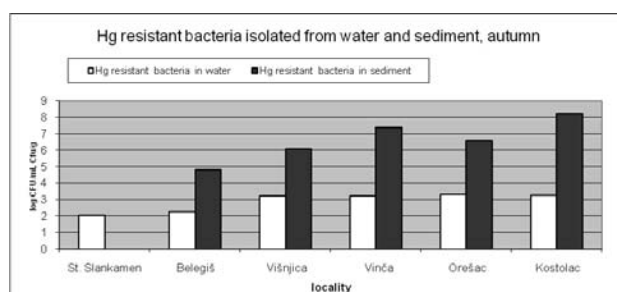S – spring 2010; A – autumn 2010; nd – not determined

Fig. 4. Numbers of mercury-resistant bacteria isolated from water and sediment samples collected during autumn from the River Danube. The results are shown as log CFU/ml and log CFU/g respectively.
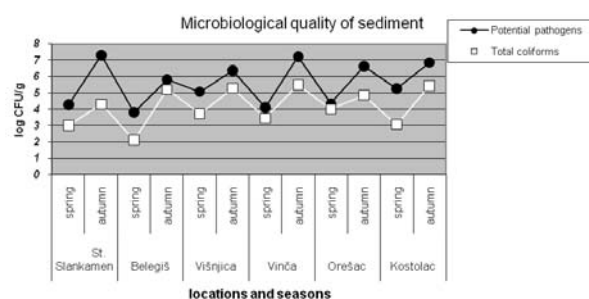

Fig. 5. Microbiological parameters from sediment samples collected from the River Danube during spring and autumn 2010. The results are shown as log CFU/g.

indicate sanitary pollution. The fecal coliforms to enterococci ratio points to the origin of pollution. A ratio lower than 1.5 indicates pollution by the runoff from agricultural surfaces, while a ratio higher than 4 is typical for anthropogenic pollution (Geldreich et al., 1969).

The sanitary analysis revealed a moderate to critical fecal contamination of the water at the ma-

jority of the sampling sites (Fig. 1). Fecal coliform counts ranged from 2.5×10^3 to 5.4×10^4 CFU/100ml. Strong fecal pollution (> 10,000 CFU/100ml) was detected at Slankamen locality in spring and at Višnjica locality in autumn (EU Bathing Water Directive 2006/7/EEC). At all sampling locations, the number of fecal coliforms was more than 4 times higher than the number of enterococci, indicating a great impact of human urban pollution (Geldre-
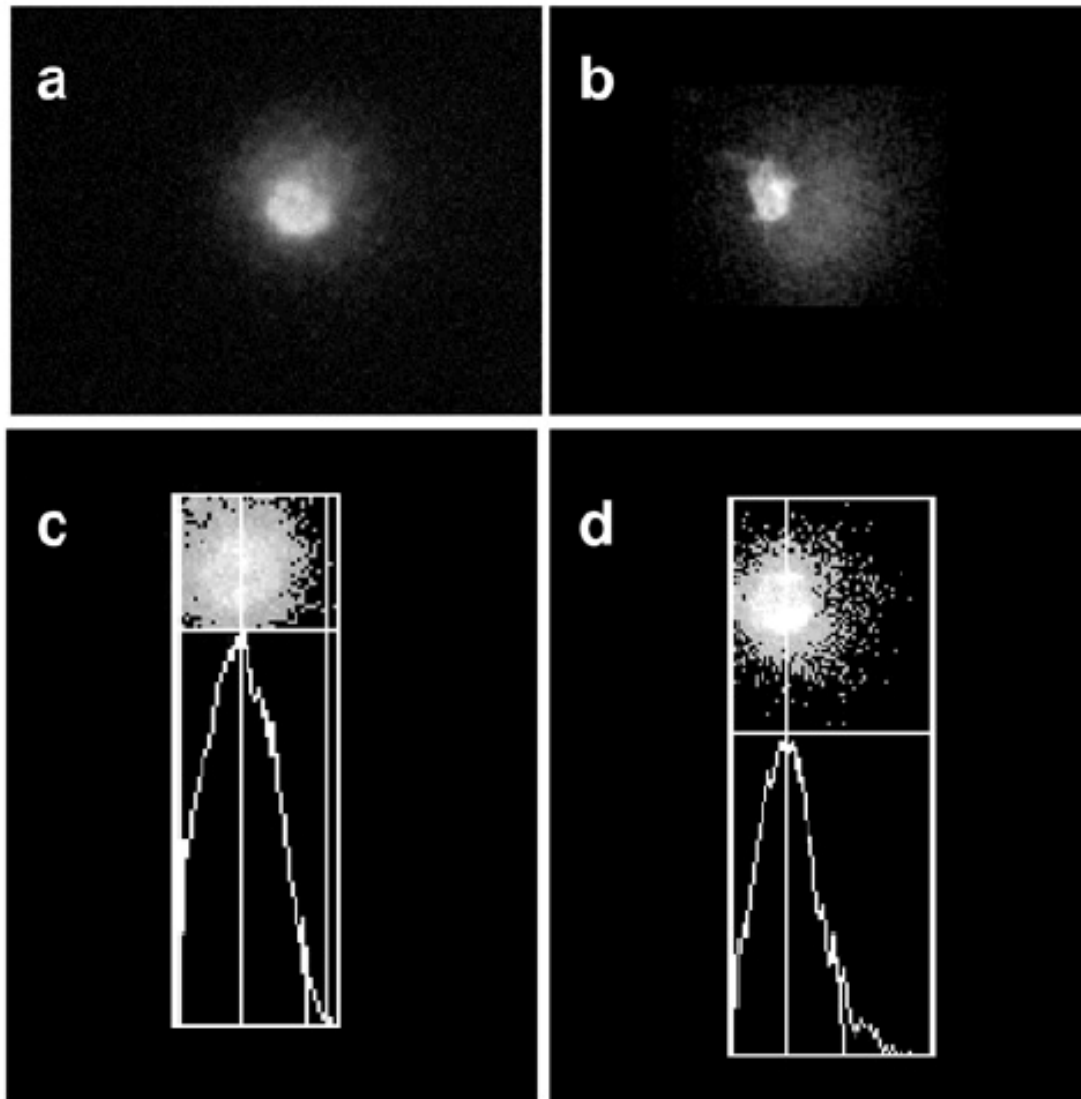
Fig. 6. (a) The nucleoid core of hemocytes from the control group of mussels with no DNA damage, as demonstrated by the absence of DNA fragment migration away from the nucleoid core. (b) The nucleoid of a hemocyte from a mussel collected at a polluted location showing a high degree of DNA damage, with a greatly reduced nucleoid core and a large cloud of DNA fragments migrating away from the core, resulting in the characteristic comet tail. (c, d) The nucleoids described above analyzed using Comet IV Computer Software (Perceptive Instruments, UK).

ich et al., 1969). The isolated coliform bacteria were identified as *Klebsiella* sp.*, Citrobacter* sp.*, Entero-bacter* sp. and *Escherichia coli*. The presence of sulphite-reducing clostridia, as well as *Bacillus* sp. was noticed in all water samples, while *Pseudomonas aeruginosa* was isolated only from the samples collected in autumn.

The origin of organic pollution in an ecosystem can be attributed to organic manure, fertilizers, high stocking density, feed waste, fecal matter, algal bloom and human interference (Moriarty et al., 1997; Lloberra et al., 1991). We used the index of phosphatase activity to evaluate the organic pollution of water of the River Danube. Analyses of the results
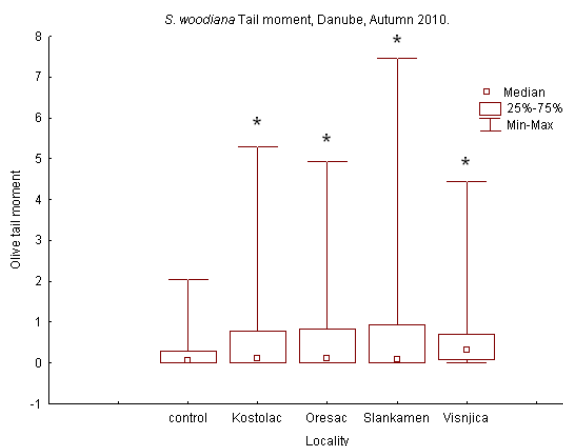
Fig. 7. Nuclear DNA damage in hemocytes of *S. woodiana* after sampling from the River Danube. The results of 100 comets per group are shown. * indicates significant difference between the control group and mussels tested directly upon sampling (p<0.05)

indicated a light (class IIB) to moderate (class III) pollution at the majority of the sampling sites (Fig. 2) (Khol 1975, Matavulj et al., 1990). While the sanitary pollution detected at studied sites can be mainly attributed to the great amount of raw or not properly treated urban wastewaters, increased agricultural activity in this area during the sampling period probably contributed to the detected organic pollution. Organic load assessment was also performed by using an oligotroph to heterotroph ratio. The domination of oligotrophs in almost all the water samples indicated a satisfactory level of self purification (Fig. 3).

Mercury-resistant bacteria were detected in all the water samples with a high number of CFU/ml on the locations positioned downstream of Belgrade (Fig. 4). The presence of mercury-resistant bacteria points tothe potential pollution of these localities with mercury. The origin of this pollution can mainly be attributed to the oil refinery of Pančevo and the activity of the thermal power plant "Kostolac".

The quality of the sediments, as dynamic and integral components of aquatic ecosystems, is acquiring increasing importance to river water quality evaluation. In our study, the microbiological parameters of the sanitary analyses of the sediment and wa-

ter samples were not in correlation. Total coliform numbers ranged from 10^2 to 5×10^5 CFU per gram of sediment. The highest number of bacteria was detected at the Vinča location during the autumn. Mercury-resistant bacteria were isolated from all sediment samples with the exception of those from the Slankamen locality (Fig. 5.). The obtained results of the microbial analyses of the sediment could be caused by the accumulation of previous pollutions because sediment serves as a kind of memory of pollution events for the evaluation of river water quality (Avnimelech, 1998).

Genotoxicity monitoring

The assessment of DNA damage in the hemocytes of *S. woodiana* mussels, sampled from the studied locations in autumn 2010, revealed a significant difference of the tail moment values (p<0.05) compared to the hemocytes isolated from the control group (Fig. 6). Moreover, there was a difference in the DNA damage in the mussels sampled at the different locations. In the group of mussels taken from the Višnjica site, a significantly higher migration of DNA was detected in comparison with the Kostolac (p=0.029175) and the Slankamen sites (p=0.04731) (Fig. 7).

The significant level of DNA damage that was observed in the mussels collected from the Slankamen site can be linked to pollution caused by the increased agricultural activities (artificial fertilization of the soil) at this time of the year. DNA damage detected in the mussels sampled at the Kostolac site can be attributed to the power plant "Kostolac". The Orešac site can be linked to pollution caused by the oil refinery in the town Pančevo through the Danube's tributary, the river Tamiš. The genotoxic potential detected at the Višnjica site could originate from the wastewaters and industry of the upstream-located city of Belgrade. Due to high volumetric emission rates, and therefore high loading values, municipal wastewaters can have a noteworthy genotoxic potential and a strong relationship exists between surface water genotoxicity and pollutions (White and Rasmussen, 1998). Well-known genotoxic agents which can be found in wastewaters, such as N-nitroso

compounds, PAHs (Hoffman et al. 1984; White and Rasmussen, 1998) may lead to the genotoxic results detected at polluted sites. The DNA damage evaluated using tail moment values indicated the presence of genotoxic agents at the sampling locations, although natural inter-individual genetic variation should be born in mind when working with live organisms (Nacci et al., 1996; Mitchelmore and Chipman, 1998).

In conclusion, the obtained results show that the pollution detected at the studied sites can be mainly attributed to the high amount of raw or not properly treated urban wastewaters, while increased agricultural activity in this area during the sampling periods probably contributed to the organic pollution. Moreover, our study demonstrates that the comet assay using *Sinanodonta woodiana* is a sensitive and reliable method that is useful forthe detecting genotoxic pollutants in aquatic environments.

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