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MICROBIOLOGICAL QUALITY OF WATER AND DETECTION OF GENOTOXIC POLLUTION IN DIFFERENT SECTORS OF THE SAVA RIVER WITH PROKARYOTIC AND EUKARYOTIC TEST SYSTEMS

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МИКРОБИОЛОШКИ КВАЛИТЕТ ВОДЕ И ДЕТЕКЦИЈА ГЕНОТОКСИЧНОГ ЗАГАЂЕЊА РАЗЛИЧИТИХ СЕКТОРА РЕКЕ САВЕ ПРОКАРИОТСКИМ И ЕУКАРИОТСКИМ ТЕСТ СИСТЕМИМА

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Microbiological quality of water and detection of genotoxic pollution in different sectors of the Sava River with prokaryotic and eukaryotic test systems

Abstract

The Sava River Basin is under strong impact of stressors related to anthropogenic activity, yet the consequences that the stressors might have on aquatic biota are not properly addressed. This dissertation aims to provide data on genotoxic potential along the Sava River. Research was organized in two campaigns. In the first campaign, preliminary research was conducted to indicate optimal bioindicator, season, sample preservation and bioassays for performing the research on the scale of the whole Sava River. The second campaign consists of comprehensive survey along the 900 rkm of the Sava River. With combined *in vivo* and *in vitro* approach, 12 sites were analyzed in total. The genotoxic potential was assessed using a complex battery of bioassays performed in prokaryotes and aquatic eukaryotes (freshwater fish). Battery comprised evaluation of mutagenicity by SOS/*umuC* test in *Salmonella typhimurium* TA1535/pSK1002. The level of DNA damage as a biomarker of exposure (comet assay) and biomarker of effect (micronucleus assay) and the level of oxidative stress as well (Fpg - modified comet assay) was studied in blood cells of bleak and spirlin (*Alburnus alburnus/Alburnoides bipunctatus* respectively). Results indicated differential sensitivity of applied bioassays in detection of genotoxic pressure. Comet assay showed higher potential in differentiation of the sites based on genotoxic potential in comparison with other assays. Data presented in this dissertation are a snapshot of the current status of the river. Genotoxic potential along the river can be traced to the deterioration of water quality by communal and industrial wastewaters. The major highlight of the study is that the complex set of data was obtained from a single source (homogeneity of analyses for all samples).

Key words: ecogenotoxicology, the Sava River, biomonitoring, bioindicators, biomarkers, wastewaters, DNA damage, SOS/*umuC* test, *Alburnus alburnus, Alburnoides bipunctatus*

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Микробиолошки квалитет воде и детекција генотоксичног загађења различитих сектора реке Саве прокариотским и еукариотским тест системима

Сажетак

Слив реке Саве је под снажним утицајем стресора антропогеног порекла, међутим последице које стресори могу имати на водену биоту нису довољно испитане. Ова дисертација има за циљ да испита генотоксични потенцијал дуж реке Саве. Истраживање је организовано у две целине. У овиру прве целине испитивало се који биоиндикаторски организам је оптималан за истраживање на нивоу читаве реке. Такође испитивао се утицај сезоне на одговор биомаркера као и методологија очување узорка на резултате анализа. У оквиру друге целине рађено је комплексно истраживања дуж 900 ркм реке Саве. Коришћен је комбиновани *in vivo* и *in vitro* приступ, при чему је анализирано укупно 12 локација. Генотоксични потенцијал је праћен употребом сложене батерије биотестова на прокариотским и еукариотским организимима. Батерија је обухватала процену мутагеног потенцијала SOS/*umuC* тестом на бактерији *Salmonella typhimurium* TA1535/pSK1002. Ниво оштећења ДНК, као биомаркер излагања (кометни тест) и биомакер ефекта (микронуклеус тест) као и ниво оксидативног стреса (Fpg - модификовани кометни тест) праћен је у крвним ћелијама риба (*Alburnus alburnus/Alburnoides bipunctatus*). Резултати су показали различиту осетљивост примењених биолошких тестова у детекцији генотоксичног потенцијала. Комет тест је имао већи потенцијал у диференцирању локалитета по генотоксичном потенцијалу у поређењу са другим примењеним тестовима. Подаци представљени у овој дисертацији представљају слику тренутног стања реке. Генотоксични потенцијал забележен дуж реке се може везати за нарушен квалитет воде утицајем комуналних и индустријских отпадних вода. Подаци приказани у овој студији потичу из јединственог извора (хомогеност анализа за све узорке) упркос великој географској површини коју студија покрива што јој додатно даје на значају.

Contents

INTRODUCTION

1. Introduction

1.1 Stressors in environment

Among the stressors in environment, an hierarchy can be established which will separate the stressors in the group of primary and group of secondary stressors. Primary stressors would be the stressors with excesive influence that strongly alter the effects of the ones from the secondary group. The best example of a primary stressors in the aquatic environment would be water discharge while the concentrations of various pollutants in environment can be considered as secondary stressors.

Understanding the interactions of stressors and their effects on the environment are just some of the steps towards solving this serious problem. The impact of stressors is a complex problem which requires multidisciplinary approach for resolve. In aquatic ecosystems, stressors might have a cumulative effect on organisms and therefore it is difficult to determine the impact of single stressor on the chemical and ecological status of water bodies and their ecosystem functionality (Navarro-Ortega et al. 2015).

1.2 The Sava River Basin

The Sava River is the major drainage basin of Southeastern Europe and the largest tributary to the Danube River. The main characteristics of the Sava watershed are summarized according to the report of the International Sava River Basin (SRB) Commission (ISRBC, 2011). The river is 945 km long with a catchment area of 97,713 km^2 extending over Slovenia, Montenegro, Bosnia and Herzegovina, Croatia and Serbia (Kapetanović et al., 2015) (**Fig.1.1**).

Climatic conditions and physical features, such as morphology, geology, pedology and vegetation, of the SRB are highly heterogeneous as the basin is divided into three climatic areas: Alpine, Pannonian and Continental. In the Alpine areas, in Slovenia, the mean annual precipitation is the highest (2000–3000 mm/y) and the mean annual temperature the lowest (6°C). On the other hand, in the Continental area, in Serbia, annual precipitation decreases to around 660 mm/y and the mean annual temperature increases to

about 13°C. The average discharge of the Sava increases downstream from 84 m^3/s at Ljubljana to 255 m^3/s at Zagreb, to 1722 m^3/s at Belgrade.

Throughout the sections of the basins heterogeneity of landscapes is evident including alpine, karstic, deep river valleys and shallow Pannonian flats. The average bottom slope of 15.8 % qualifies the Sava as a typical lowland or middle course river starting in the central flow in Slovenia.

The geology of the SRB is diverse. The southern part of the river basin is characterized by the karst regions while the rest of the river basin, between the External Dinarides and the border of the Sava catchments belongs to the Inner Dinarides zone and Pannonian basin. In this zone the following lithological units are present: sandstone, marls, claystones, intrusive and extrusive igneous rocks (Ogrinc et al., 2010). Within the basin a sharp transition from a gravel-bed river (at the Upper Sava) to a sand-bed river (at the Middle Sava) can be observed (Globevnik et al., 2010).
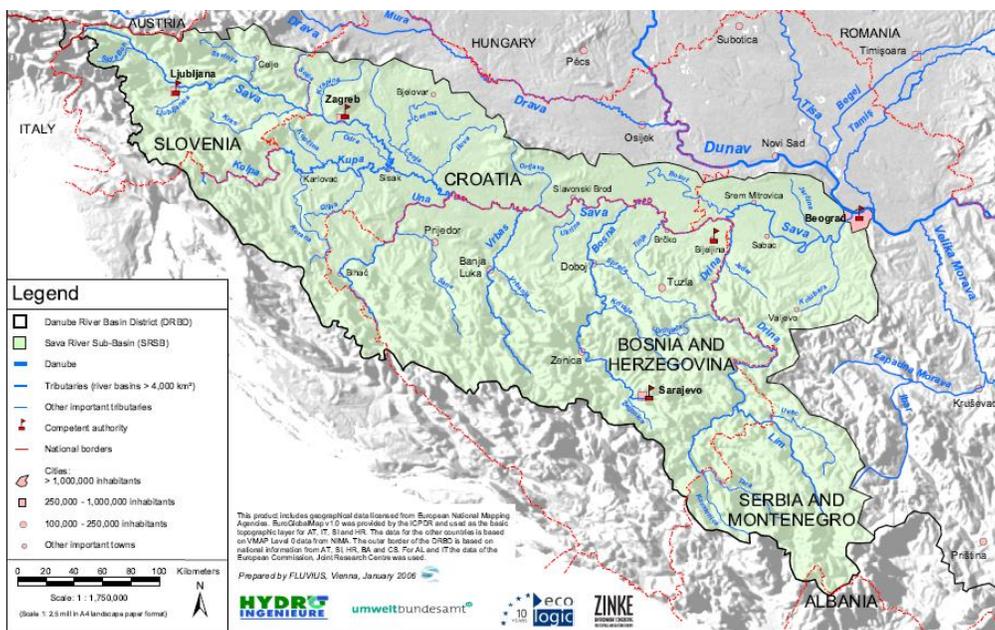
Fig. 1.1 Sava River Basin overview map, image was obtained from the International Comission for the Protection of the Danube River (ICPDR)

1.3 Pressures within the Sava River Basin

The SRB is under the strong impact from diverse anthropogenic activities as indicated in the **Fig. 1.2.** Preliminary data show that only 5.5 % of the water bodies within the basin are characterized by high, 44.8 % by good, 39.9 % by moderate and 9.3 % by poor ecological status (Simić et al., 2015). The upper reaches of the Sava River basin are affected by hydromorphological pressures; the middle reaches by agricultural activities and eutrophication, and the lower reaches by industrial and urban pollution (Navarro-Ortega et al., 2015).

Fig. 1.2 Schematic demonstration of diverse anthropogenic activities within the Sava River Basin

The population in the basin is about 8.2 million (46% of the total population of the four countries that share the basin). On one side the population is concentrated near large cities located at the Sava banks: Ljubljana, Zagreb and Belgrade. On the other side, population is scattered in the large number of smaller settlements and villages. Therefore, the major issue is represented by high quantities of wastewaters which are in some sections processed and in the others completely unprocessed, due to different legislative and economic standards of the countries which share this international river (Kapetanović et al., 2015; Aborgiba et al., 2016).

Land use of the SRB reflects the differences in relief, climate and stream flow (Ogrinc et al., 2010). Agriculture is the dominant activity in the Croatian (40 %) and Serbian (~ 100 %) parts of the watershed, while the upper part, in Slovenia, is mainly covered by forest (more than 50 %). The regions of intense agriculture are the Slavonija in Croatia, the Bosanska Posavina and the Semberija in Bosnia and Herzegovina and the Srem in Serbia, which together cover an area of more than $100,000$ km^2 (Paunović et al., 2012). The Bosnian part of the watershed is dominated by valleys and hills, with about 30 % agricultural area and 30 % forest. For these four countries, the basin catchments area comprises 60-70% of their land and represents the source of more than 80 % of the total available water. The large retention areas of the Sava are one of the most effective flood control systems in Europe. Its management is seen as an international model for sustainable flood management (Vrzel et al., 2016). However, misuse of these retention areas and their conversion in agricultural land creates additional pressures on the quality of water which was especially evident during the extreme flooding event in 2014 (Aborgiba et al., 2016).

The Sava River is also impacted by alteration of hydrological conditions by artificial damming, especially in the upper course. Alterations are also evident in the lower course caused mainly by the backwater effect of the Iron Gate Dam (Djerdap I) which during low flow of Sava is observed almost up to the town of Šabac at 105 km from Belgrade.

Aditional pressure which should be mentioned is the coal processing power plant Nikola Tesla situated in the lower stretch of the river downstream of Obrenovac. This facility also has significant impact on thermal and industrial pollution.

1.4 Impact of wastewaters and microorganisms as indicators of pollution

Among the multiple stressors present in the SRB, special attention should be given to wastewaters as the major input for numerous substances in the surface waters (Taebi and Droste, 2004). This issue is especialy evident in transitional countries in which the legislation regarding this topic is not fully established. When disscusing the SRB two distingushed groups can be formed. The first one is formed by the countries which are members of the EU (Slovenia and Croatia) which have established national legislation regarding the emision of wastewaters and subseqently implemented wastewater treatment plants. The other group is formed by Bosnia and Herzegovina and Serbia in which the wastewater treatment is not fully implemented yet. This especially complicates the situation in transboundary sections shared by the countries from different groups as this is the case with Croatia and Bosna and Herzegovina. Recent studies performed at various water bodies in Serbia confirmed that the wastewaters are indeed one of the major impactors on the chemical and ecological status assessment (Kolarević et al., 2013, 2014, 2016; Kračun-Kolarević, 2016; Sunjog et al., 2012, 2015, 2016). One of the most convenient examples is impact of wastewaters of the capital Belgrade on the water quality of the Danube River which is often evident up to the Smederevo, situated 50 km downstream (Kolarević, 2014).

When discussing the impact of wastewaters on surface waters, the focus will be placed on two aspects. The first one is the input of alochtonous microorganisms in surface waters which is of great importance especially due to the health threat these organisms might pose. The contamination of water by faecal pollution leads to exposure to pathogens via drinking water production, recreation or irrigation. However, monitoring of microbiological quality of surface waters is quite neglected despite its importance for human health. In the case of Sava River Basin, there is contradiction as many of the settlements situated on the river banks discharge high quantities of untreated or improperly treated wastewaters directly into surface waters (Kapetanović et al., 2015; Aborgiba et al.,

2016). But, on the other hand, in the middle and lower course, the Sava River flows through the regions of intense agriculture and due to usage of water for irrigation, the evaluation of microbiological quality of the Sava River becomes essential for further river management. One of the pathways of exposure to contaminated water which must not be neglected is flooding. The study of Casteel et al. (2006) indicated that flooding can result in extensive faecal contamination of nearby agricultural soil with pathogens.

As the methods for isolation and cultivation of mentioned pathogens are quite complicated and expensive, as a more convenient solution, assessment of faecal pollution is performed by enteric bacteria which are an indirect indicator of the presence of pathogens in water. Within the enteric bacteria *Escherichia coli* and *Enterococcus faecalis* have been selected as reliable indicators of faecal pollution included in legislation regarding the quality of bathing water at the EU level (EU directive 2006/07/EEC).

1.5 Genotoxic agents as stressors in the environment

The other aspect of the impact of wastewaters is the agents that are released into the environment, which may affect living organisms in different ways. One of them is the interaction with DNA molecules, which can lead to DNA damage and if not corrected by the mechanisms of reparation can cause chromosomal aberrations, mutations, cancers, or may lead to cell apoptosis. Such effects do not have to be immediately apparent; however, the consequences might be very serious considering that mutations that might occur are passed on to posterity affecting fitness and fertility of individuals which will finally be reflected on the level of populations in the ecosystem (**Fig. 1.3**) (Jha, 2008).
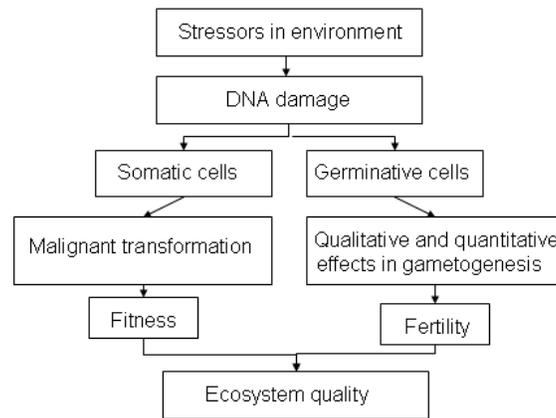
Fig. 1.3 The effects of stressors in environment on the DNA damage and possible consequences on the ecosystem quality

Genetic toxicology or genotoxicology, is a multidisciplinary science that applies knowledge in the field of molecular genetics, DNA repair and mutagenesis, population genetics, mathematical modeling and statistics, to detect the effects and consequences of exposure to genotoxic agents (Kolarević, 2014). Ecogenotoxicology is a subdiscipline of genotoxicology which deals with the genotoxic potential of environmental habitats and the effects in organisms. Since the habitat is a complex relationship of physical and chemical factors, ecogenotoxicology combines biological monitoring studies and the data on physical and chemical agents in environments which might lead to a change in the genetic material directly or might affect dynamics of DNA repair. Such surveys are conducted using specific tests that detect and quantify changes in the DNA of prokaryotic, and eukaryotic organisms.

1.6 Biomonitoring

Biomonitoring represents usage of organisms in monitoring programs. They can be autochthonous organisms, which naturally inhabit ecosystem of interest or allochthonous, organisms that are introduced into the ecosystem and can live in the habitat in which they are adopted. Usage of bioindicators may give information on presence and distribution of contaminants in the environment, as well as the information on sources of pollution (Munn, 1973).

Biomonitoring carried out directly at the site of interest is known as *in situ* biomonitoring (Kolarević, 2014). The studies carried out *in situ* are comonly quite complex for interpretation due to lack of controled experimental conditions and subsequent influence of numerous factors which might interfere with the studied phenomenon.

This kind of survey can be performed passively and actively. Passive biomonitoring involves studying of the effects of environmental stress in organisms inhabiting the particular site while active biomonitoring involves studying the effects of environmental stress in organisms that have been exposed for a certain period of time at the site of interest. Each approach has its advantages and disadvantages and choice of the approach commonly depends on the concept of the research.

1.7 Aquatic organisms as bioindicators

Bioindicators are organism or groups of organisms used in biomonitoring. In the studies related to aquatic ecosystems, mussels and fish are commonly employed. So far reliability of these organisms in assessment of the quality of ecosystems from the ecogenotoxicological aspect has been demonstrated in numerous studies carried out by both active and passive approach (Kolarević et al., 2013; Sunjog et al., 2012, 2014; Vuković-Gačić et al., 2014). Once again, choice of bioindicator depends on the concept of the research, having in mind that response of organisms is related to the trophic level and habitat preference. In either way, there are some criteria which should be followed in the selection of the potential bioindicator:

• Wide distribution in different types of ecosystems;

• The possibility of growing in a laboratory;

• Sensitivity to a large number of pollutants in low doses;

• Low effect at the population level in exploitation of the organisms for experiments.

1.7.1 Mussels

Mussels possess many properties which make them suitable for ussage in monitoring. The response of these sedentary, filter feeding organisms can be easely linked to the certain locality inhabited by these organisms. Concerning the freshwater mussels, usage of autochtonous and alochtonous species in ecogenotoxicology has been demonstrated in the studies of Pavlica et al. (2001), Klobučar et al. (2008) and Kolarević (2014). Choice of the species is strongly related to the studying area, for instance in the study of Kolarević (2014) *Sinanodonta woodiana* was found most apropriate for the monitoring program carried out on monthly basis at the Velika Morava River while the species *Unio pictorum*, *Unio tumidus* and *S. woodiana* were used in the research carried out in the snapshot study along the Danube River.

One of the features which also favourises these organisms in an environmental study is the easy sampling of tissue needed for analysis. The study of Vuković-Gačić et al. (2014) demonstrated aplicability of gills and haemolymph in detection of genotoxic potential. Due to easy collection and minimal manipulation with the cells in genotoxicological analyses, haemolymph can be recomended for complex *in situ* research (Kolarević, 2014).

1.7.2 Fish

Fish are one of the most commonly employed organisms in studying aquatic environments. The interest for these organisms is primarly because of their comercial value. The study of Sunjog (2016) provides a comprenhensive dataset on usage of these animals in studying genotoxicological potential in aquatic environments. As for the mussels, selection of the species for research is dependent on the research area. The study of Sunjog (2016) demonstrated aplicability of barbel (*Barbus barbus*) and European chub (*Squalius cephalus*) in detection of genotoxic potential in various waterbodies. The study of Kračun-Kolarević et al. (2016) showed sensitivity of salmonid species in assessment of genotoxicity in the Adige River basin. The study of Kostić et al. (2016) demonstrated aplicability of the comet assay in freshwater bream (*Abramis brama*) sampled from the Danube. Within the Joint Danube Survey 3, bleak (*Alburnus alburnus*) was chosen as the

most suitable species for the assessment and the genotoxic potential along the whole Danube River (Deutchmann et al., 2016).

In previous studies gills, liver and blood were used for assessment of the effects of genotoxic pollutants (Sunjog, 2014). Among these tissues, blood can be recommended once again due to easy manipulation with the cells in the analyses. Cells are already in suspension which facilitates preparation of the slides used in research.

1.8 DNA damage as biomarker

Biomarkers are biochemical, physiological and histological indicators of the presence of xenobiotics which enable their detection before the effects are reflected at the level of the individuals and at the level of the whole population (Forbes et al., 2006). DNA damage is a complex biomarker which can be studied at the level of molecule, gene and the level of whole chromosomes. A variety of agents in the envirnoment interact with DNA and cause single-strand, or double-stranded breaks, DNA-DNA and DNA-protein cross-linkages, or modification of the bases. Occured damage can have different faiths (**Fig. 1.4**).
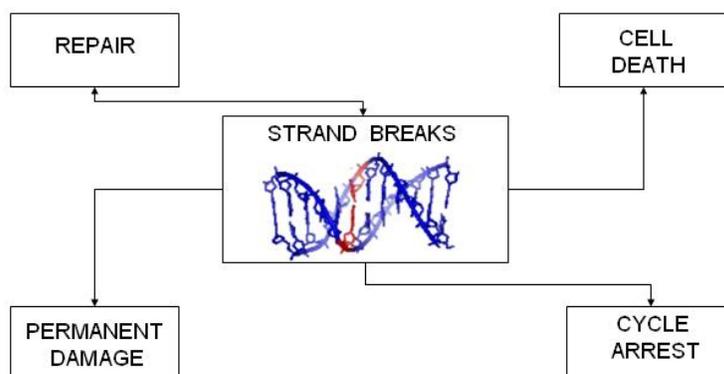
Fig. 1.4 Possible faiths of single and double strand breaks in DNA molecules

Depending on the nature and the level of DNA damage one of the possible paths will be chosen. In the best case, DNA damage can be repaired but in the case of excessive damage the cell cycle can be arrested and DNA damage can be promoted in permanent damage or programmed cell death can be triggered. When discussing the DNA damage as a biomarker of exposure we refer to initial DNA damage which can be detected by the comet

assay. Permanent DNA damage is recognised as a biomarker of effect and can be detected with assays such as the micronucleus test.

Ecogenotoxicology applies assays originally developed in genotoxicology which enables detection of genotoxic potential at the level of molecule, gene and at the level of whole chromosomes. Comet assay detects DNA damage at the level of molecule, SOS/*umuC* at the level of gene while chromosomal damage can be assessed by the micronucleus assay.

1.8.1 Comet assay

Comet assay, also known as the Single Cell Gel Electrophoresis (SCGE) is a fast and reliable method which allows the detection of DNA damage in individual cells (Jha, 2008). Currently, this is one of the most commonly used tests in genotoxicology based on quantification of negatively charged DNA fragments which in electrophoresis move through the gel towards a positively charged cathode. Nuclei with fragmented DNA material form comet like shapes which determined the name of the assay.

Within the development of the assay, many versions have emerged which may provide additional information on the nature of the detected genotoxic potential (Fig. 1.5). Most commonly employed are neutral version which enables detection of double strand breaks, alkaline version which enables detection of single and double strand breaks and alkali labile sites, and assay coupled with glycosylases which in addition to alkaline version enables detection of oxidative damage of DNA molecule (Collins, 2004). Oxidative stress has been recognized as one of the major pathways in induction of DNA damage in environments and for majority of priority hazardous substances the ability of formation of reactive oxygen species has been well documented (Abdollahi et al., 2004; Liu et al., 2009). This makes glycosylase coupled comet assay version especially interesting for studying of the environmental stress.
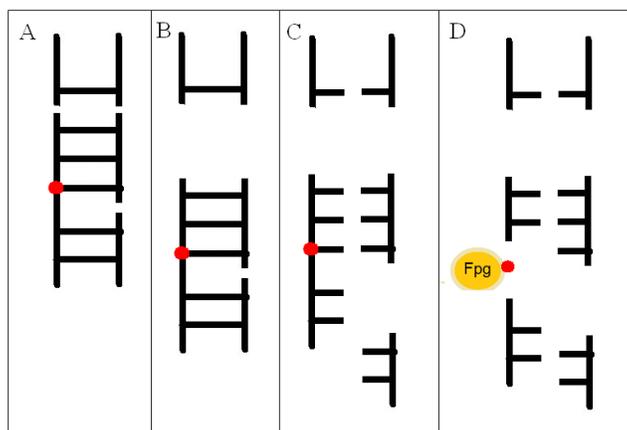
Fig.1.5 Different scenarios in interpretation of damage in DNA molecule with single and double strand break and oxidative damage marked with red dot (A) within the electrophoresis in the neutral (B), alkali (C) and Fpg-modified (D) version of the comet assay

Mentioned versions of the assay differ in protocol but few steps as lysis and electrophoresis are common for all procedures. In the first step of the protocol cells embeded in agarosis are subjected to lysis to get rid of the cell membrane and enable work with isolated nuclei. After lysis, in the neutral version of the assay, nuclei are subjected to electrophoresis while in the alkaline version slides are first placed in alkaline conditions to unwind the helix of DNA. In the version of the assay coupled with glycosylases, samples are digested with the enzyme prior to denaturation and elecrophoresis. Alkaline version demands neutralisation of the slides after the electrophorsis due to high pH which might affect further analysis of the slides. Afterwards, slides are fixed in methanol which facilitates analyses of the slides as the gels are dehydrates which places all the cells on slides in the same plane. Fluorescent stains such as acridine orange (AO), ethidium bromide (EB), cyber grin, cyber gold, etc. are used for visualisation of the slides and comets are scored under the flurescence microscope using image analysing software, most commonly, Comet IV Computer Software (Perceptive Instruments, UK) as shown on the Fig. 1.6. This software automatically detects the level of damage by few different parameters: the intensity of the tail (Tail Intensity - TI), tail moment (Olive Tail Moment - OTM) and tail

length (TL). TI is the percentage of fluorescence in the tail of the comet compared to the fluorescence of the entire comet. OTM parameter is derived by combining the parameter TI and distance centers of mass of the head and tail (Olive and Judit, 2006; Cok et al. 2011; Rosenberger et al., 2011). TL is the length of the tail of the comet (Kolarević, 2014).
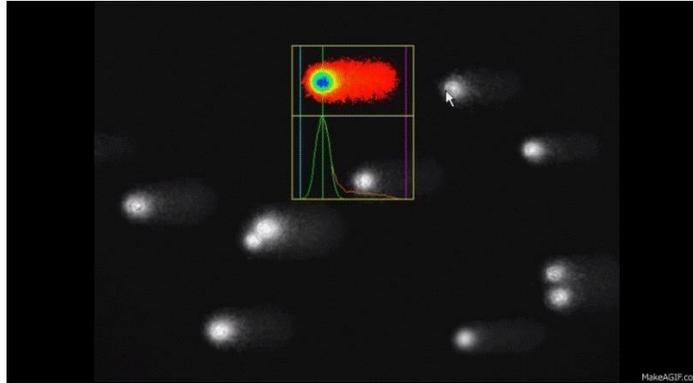
Fig. 1.6 Screenshot from the Comet IV Computer Software for DNA damage analyses

1.8.2 SOS/*umuC* test

SOS/*umuC* test detects the genotoxic effects at the gene level. Prokaryotic cells have specific complex molecular responses which enable them to survive the various types of stress caused by external or internal factors. One of the mechanisms of protection is SOS response, which is initiated in the case of DNA damage. Single or double strand breaks activate repair mechanisms where the expression of involved genes is in well-established order as indicated in **Fig. 1.7**. The first step is the increase of expression of genes involved in the nucleotide excision repair, and if the products of these genes cannot repair the damage, the second step is the expression of the genes responsible for the translesion synthesis. This regulation is very important as in this way DNA damage is primarily

repaired by the error free mechanisms, and if these mechanisms cannot remove the damage, error-prone mechanisms come into play.

For this assay a specific strain of *Salmonella typhimurium* TA1535 is constructed. This strain is auxotrophic for histidine, it caries *rfa* mutation which means deficiency in lipopolysaccharide layer resulting in greater permeability of the cell wall. It is also carries *uvrB* mutation which makes this strain deficient in excision repair which further increases the SOS response. Further on, the strain is *lacZ* mutant which makes lactose operon inactive. Modification also includes multicopy of plasmid with gene for ampicillin resistance gene and fused *umuC-lacZ* gene.
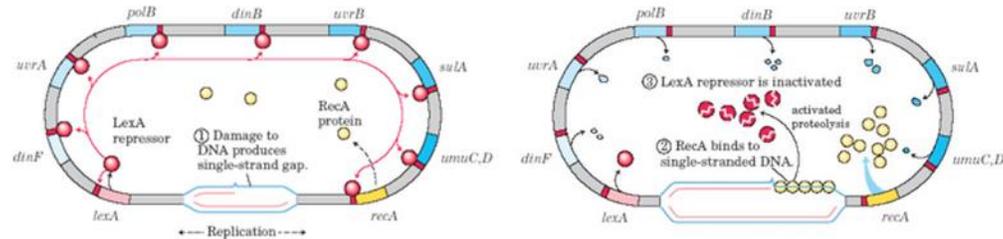
Fig. 1.7 Scheme of the cascade activation and repression of the genes involved in the repair mechanisms in *S. typhimurium*
(http://2012hs.igem.org/Team:Heidelberg_LSL/Project_SOS)

In the assay, fusion of *umuC* and *lacZ* genes enables quantification of the response. Product of the lacZ gene is enzyme β-galactosidase which breaks down the substrate o-nitrophenyl β-D-galactoside (ONPG) in ONP, yellow colored product (McDaniels et al., 1990). The concentration of the product can be quantified spectrophotometrically. In addition to genotoxic, using of this test also enables detection of cytotoxic effect.

To enable the extrapolation of the results obtained in the assay from prokaryotic to eukaryotic models, modification which considers metabolic activation with S9 fraction from the liver rat is included. If potential promutagen is present in the water sample, enzymatic treatment is required in order to activate the substance.

1.8.3 Micronucleus test

Micronuclei are structures built by the condensing of fragments, or entire chromosomes, which are not included in the main nucleus during anaphase, chromatids during anaphase-centric and lagging chromosome fragments and unlike centric chromosomes are not affected by the division spindle, and therefore do not move towards the poles of the cell (Heddle, 1973; Al-Sabt, 1995). As such, they are not included in the nucleus of the daughter cells during mitosis, as they lack the centromere (Fig. 1.8). It can sometimes happen that these structures join the nucleus of the daughter cell, and then can be observed as the so-called buds.
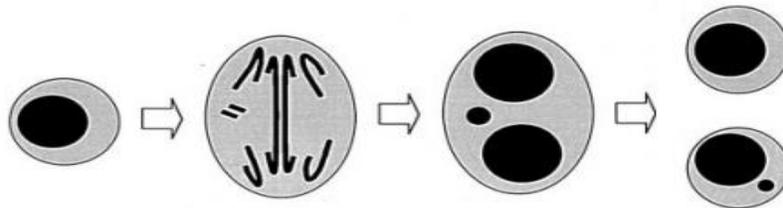
Fig. 1.8 Scheme showing process of the formation of the micronucleus (modified image from the internet)

If the micronucleus has a diameter larger than 1/3 of the diameter of the main nucleus, such cells are referred as binuclear. In this case, each has its own nucleus membrane and show the same intensity of coloration. Cells stricken with apoptosis can be seen within the micronucleus assay; in these cells large numbers of micronuclei can be spotted in the cytoplasm.

When discussing aquatic organisms, fish blood and hemolymph of invertebrates are tissues most commonly used in research due to fast, easy and inexpensive reparation of the slides for analyses. Slides are usually fixed in methanol which enables prolongation of the time needed for analyses. Slides can be analyzed by light and fluorescent analysis which makes this method applicable in the majority of moderately equipped laboratories.

1.9 Assessment of genotoxic potential along the Sava River

The Sava River Basin is under high impact of pollutants originating from quite diverse sources of pollution. So far it is known that hotspots of faecal and industrial

polution exist throughout the basin. However, as available literature provides only partial data on the level of genotoxic pollution along the Sava River it is evident that comprehensive research of genotoxic potential along the Sava River is needed.

This thesis is focused on the identification of the hotspots of faecal and genotoxic pollution at multiple sites along the Sava River. Detection of faecal pollution was performed by the microbiological indicators. Identification of the hotspots of genotoxic pollution is performed by a complex battery of bioassays which comprised of prokaryotic and eukariotic test systems.

When discussing the usage of the organisms the most apropriate in the survey of the whole Sava River, the major disadvantage of usage of moluscs would be the lack of distribution in the upper course of the river in which Sava has characteristics of a alpine and subalpine watercourse (Lucić et al., 2015). In the middle and lower stretch, *Corbicula fluminea* and *Unio pictorum* could be taken into consideration as the most frequent representatives of the moluscs along the Sava River (Lucić et al., 2015).

On the other hand, the most apropriate fish species for carying the research along the Sava River would be European chub or minnow species (*A. alburnus* and *Alburnoides bipunctatus*) considering the data on ichtiofauna in the Sava River basin (Simonović et al., 2015). As the dataset is already available for the bleak in the Danube River, this species could be esspecially interesting due to comparison of datasets of these two large rivers. In the Sava River, two minnow species can be chosen, which are ecological equivalents. Spirlin (*A. bipunctatus*) is characteristic for the upper section and bleak is present in the middle and lower sections of the river. Both spirlin and bleak are active and fast swimmers, with a short life-span, with consequently high metabolic rates which can lead to high accumulation of metals and metalloids. Also, they are both epipelagic fish not exposed to the contaminants in the benthic zone. Based on these features, bleak were successfully used for estimation of metals and metalloids in aquatic environments (Uysal et al., 2009; Petkovšek et al., 2012; Mercai et al., 2014).

This thesis is part of the research of the project GLOBAQUA (FP7, EU) which is focused on discovery of the potential consequences of the scarcity of water on aquatic ecosystems, detection of potential stressors and determination of their impact on the ecosystem, as well as exploitation this knowledge for legislation that will help to minimize the impacts of global change. Due to the multidisciplinary nature of the problem the project involved scientists from different fields: chemistry, geology, geomorphology, biology, as well as experts from the social economy and political science, political rights (Navarro-Ortega et al., 2015).

OBJECTIVES

2. Objectives

The main goal of the study was identifying the hotpsots of faecal and genotoxic pollution along the whole Sava River. As the study was carried out along 900 rkm of the Sava River in a short period of time and specific field conditions, preliminary research was needed which would facilitate work on-site and provide success of the research. Therefore research was organized in two major parts. The first one was preliminary study focused on only one site at the Sava River which was exercise for the second part, which comprised the whole river survey.

Within the preliminary research following goals were set:

- Selection of the site on the Sava River which is known to be under the impact of industrial pollution and urban wastewaters
- Identification of physico-chemical parameters and faecal pollution as microbiological indicators on the selected site on the Sava River
- Detection of the level of DNA damage by using the comet assay in freshwater organisms which belong to different trophic levels (mussels and fish) on the selected site on the Sava River during different seasons
- Evaluation of season related response of selected organisms

The outcomes of the preliminary study were used for planning the whole river survey which had the folowing objectives:

- Selection of multiple sites along the Sava River in compliance with national routine monitoring program of the countries within the basin
- Identification of physico-chemical parameters and faecal pollution as microbiological indicators
- Detection of genotoxic (mutagenic) potential of water samples with prokaryotic SOS/*umuC in vitro* test
- Detection of genotoxic potential of selected sites at the Sava River by using the comet assay and micronucleus tests in aquatic organisms
- Identification of hotspots of faecal and genotoxicological pollution along the Sava River

MATERIAL AND METHODS

3. Material and methodes

3.1. Material

3.1.1 Bacterial strain
Salmonella typhimurium TA1535 pSK1002 – kindly provided by National Institute of Biology, Ljubljana Slovenia

3.1.2 Solutions

3.1.2.1 Comet assay related
<u>Solution I - lysis (pH 10)</u>

For 1 L of solution:

NaCl "Carlo Erba Reagents" (Milano, Italy)............................146,6 g

EDTA "Sigma" (St.Louis, MO, USA)...37,2 g

Tris   "Sigma" (St.Louis, MO, USA)...1,21 g

dH$_2$O...1.000 mL

Triton X-100 "Sigma" (St. Louis, MO, USA)

Before usage add 1% Triton X-100 and stear.

<u>Solution II – denaturation and electrophoresis (pH 13)</u>

For 1 L of solution:

10 M NaOH "Superlab" (Belgrade, Serbia)..................................30mL

0.2 M EDTA "Sigma" (St.Louis, MO, USA)................................5 mL

dH$_2$O...965 mL

<u>10 M NaOH</u>

For 500 mL of solution:

NaOH   "Superlab" (Belgrade, Serbia)...200 g

dH$_2$O..do 500 mL

<u>0.2 M EDTA</u>

For 500 mL of solution:

EDTA ”Sigma“ (St.Louis, MO, USA)...37,2 g

dH_2O...500 mL

Solution III - neutralisation (pH 7.5)

For 1 L of solution:

Tris ”Sigma“ (St.Louis, MO, USA)...48,44 g

dH_2O..1.000 mL

Keep at cold.

1% NMP first layer

For100 mL:

NMP ”Eurobio“ (France)..1 g

dH_2O...99 mL

1% NMP second layer

For 100 mL:

NMP ”Eurobio“ (France)..1 g

1xPBS ”The Cell Culture Company“ (Austria).............................99 mL

1% LMP

For 100 mL:

LMP ”Bio-Rad Labaratories“ (CA, USA)..1 g

1xPBS ”The Cell Culture Company“ (Austria).............................99 mL

1xPBS

For1 L solution:

10x PBS...100 mL

Sterile dH_2O..900 mL

HBSS+EDTA (solution with anticoagulant)

For100 mL solution:

HBSS "Sigma" (St. Louis, MO, USA).......................................100 mL

EDTA "Sigma" (St.Louis, MO, USA)...186 mg

Adjust pH at 7.5

Ethidium bromide SI

10 mg/mL, "Sigma" (St.Louis, MO, USA)

Acridine orange SI

10 mg/mL, "Sigma" (St.Louis, MO, USA)

Acridine orange/ethidium bromide

For 1 mL solution:

Acridine orange SI...10 μL

Ethidium bromide...10 μL

dH$_2$O...980 μL

Acridine orange for comet

2 μg/mL, "Sigma" (St.Louis, MO, USA)

Fpg-enzyme (Trevigen, Germany)

Primary stock dissolved in Fpg buffer (Trevigen, Germany), 500 U/mL

For the assay, enzyme should be diluted 300x in Fpg buffer (Trevigen). For preservation, primary stock should be aliquoted in smaller volume, diluted 100x in Fpg buffer with addition of 10% glycerol.

Fpg buffer (Trevigen, Germany)

HEPES „Sigma" (St.Louis, MO, USA)...2.383 g

KCl (Zdravlje, Serbia)...7.455 g

EDTA „Sigma" (St.Louis, MO, USA)...3.722 g

Bovine Serum Albumine HEPES „Sigma" (St.Louis, MO, USA).............0.1 g

dH$_2$O...1000 mL

pH adjusted at 7.2

Fpg buffer by Akcha et al. (2003)

HEPES „Sigma" (St.Louis, MO, USA)...2.383 g

KCl (Zdravlje, Serbia)...7.455 g

EDTA „Sigma" (St.Louis, MO, USA)...3.722 g

dH$_2$O...1000 mL

pH adjusted to 7.2

3.1.2.2 SOS/*umuC* related

S9 liver microsomal fraction (Moltox, USA)

S9 induced with arochlor isolated from liver of male rat, concentration of proteins 36.4 mg/mL.

4-Nitroquinoline 1-oxide (4NQO) „Sigma" (St.Louis, MO, USA)

Primary stock 10 mg/mL in water

Benzo(a)pyren „Sigma" (St.Louis, MO, USA)

Primary stock 10 mg/mL in DMSO

B-Buffer

Na$_2$HPO$_4$ 2H$_2$O „Sigma" (St.Louis, MO, USA)..20.18 g

NaH$_2$PO$_4$x H$_2$O „Sigma" (St.Louis, MO, USA)..5.5 g

KCl (Zdravlje, Serbia)..0.75 g

MgSO$_4$x 7H$_2$O,„Sigma" (St.Louis, MO, USA)..0.25 g

dH$_2$O..1000 mL

Autoclave, adjust pH na 7.0±0.2. Upon usage add 1 g SDS and 2.7 mL 2-mercaptoethanol. Store at 4 °C.

1M Na$_2$CO$_3$

Na$_2$CO$_3$ (Alkaloid Skoplje, Macedonia)..20.18 g

dH$_2$O ....1000 mL

ONPG (2-nitrophenyl-ß-d-galactopyranoside)

ONPG (Boehringer wannhein GMBH, Germany)..........................45 mg

Phosphate buffer..10 mL

Phosphate buffer

Na$_2$HPO$_4$ 2H$_2$O „Sigma" (St.Louis, MO, USA)..1.086 g

NaH$_2$PO$_4$x H$_2$O „Sigma" (St.Louis, MO, USA)..0.538 g

dH$_2$O ………..100 mL

Autoclave, adjust pH to 7.0±0.2.

3.1.3. Media
Medium for cryopreservation of fish blood

RPMI 1640 medium (PAA, Austria)....................................600 μL

Fetal bovine serum, FBS „Sigma" (St.Louis, MO, USA ......200 μL

DMSO „Sigma" (St.Louis, MO, USA)..................................200 μL

Medium for cryopreservation of haemocytes

Leibovitz´s L-15 medium (PAA, Austria)....................................120 μL

dH$_2$O ……...680 μL

Glycerol (Zorka, Srbia)...200 μL

Adjust pH to 7.5, then add glycerol

1x TGA medium

Tryptone (Lyophilichem, Italy)................................10 g

NaCl "Carlo Erba Reagents" (Milano, Italy)...........5 g

HEPES „Sigma" (St.Louis, MO, USA)..................11.9 g

dH$_2$O ………...980 mL

Adjuce to pH 7.0±0.2, autoclave, add 2 g (D(+)-glucose (anhydrous)) in 20 mL dH$_2$O (sterile) and 50 mg ampicillin.

10x TGA medium

Tryptone (Lyophilichem, Italy)................................10 g

NaCl "Carlo Erba Reagents" (Milano, Italy)...........5 g

HEPES "Sigma" (St.Louis, MO, USA)..................11.9 g

dH$_2$O ………...80 mL

Adjuce to pH 7.0±0.2, autoclave, add 2 g (D(+)-glucose (anhydrous)) in 20 mL dH$_2$O (sterile) and 50 mg ampicillin.

10x TGA medium + cofactors for S9

Tryptone (Lyophilichem, Italy)................................10 g

NaCl "Carlo Erba Reagents" (Milano, Italy)...........5 g

HEPES "Sigma" (St.Louis, MO, USA)..................11.9 g

KCl (Zdravlje, Serbia)...2.46 g

MgCl$_2$ x 6H$_2$O "Sigma" (St.Louis, MO, USA)......1.63 g

dH$_2$O ………...80 mL

Adjuce to pH 7.0±0.2, autoclave, add 2 g (D(+)-glucose (anhydrous)) in 20 mL dH$_2$O (sterile) and 50 mg ampicillin.

Before use add 148 mg NADP (sodium salt) (Carl Roth) and 76 mg glucose-6-P (disodium salt) „Sigma" (St.Louis, MO, USA) in 5 mL 10x TGA + cofators.

Tryptose Sulfite Cycloserine Agar(TSC) Scharlau (Spain)

Add 45 g of dry media in 1 L of disstiled water. Autoclave, cool to 60 ℃ and add 4 vials of selective supplement.

3.2 Research area and collection of the specimens

3.2.1 Sampling in preliminary research – the site Duboko

The study area is presented in **Fig. 3.1.** The study was carried out in winter (January/February) and summer (June) 2014 on the site (4) situated on the Sava River downstream of the town Obrenovac (2) (circa 50,000 inhabitants). Untreated wastewaters from this town are discharged in the Kolubara River (3), which confluences the Sava River downstream Obrenovac. The largest thermal power plant in Serbia "Nikola Tesla" and fly ash disposal field (1) are situated few km upstream of the sampling site.
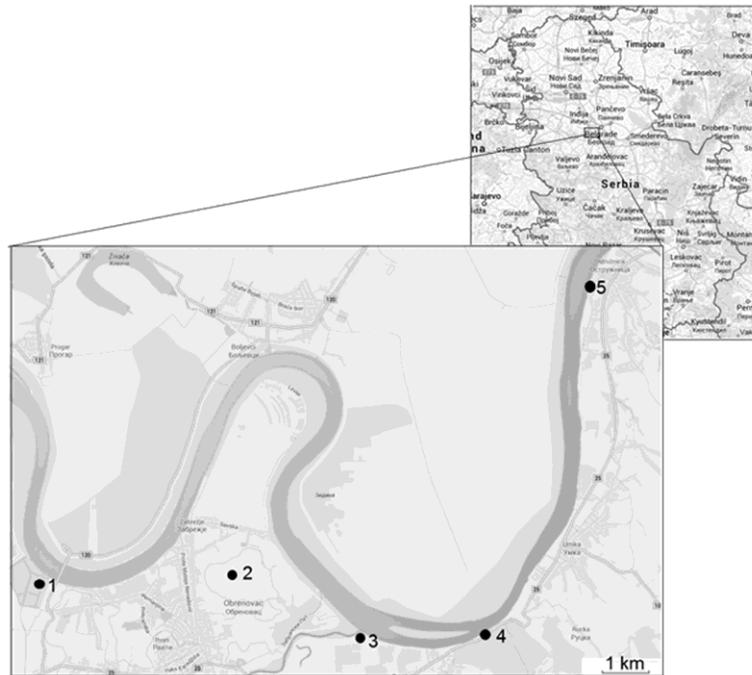
Fig. 3.1 Description of the investigated stretch (1) Thermal power plant "Nikola Tesla", (2) town Obrenovac, (3) Kolubara River, (4) sampling site, (5) site used for routine monitoring of the water quality by the Agency for Environmental Protection, Serbia

Research comprised analyses of microbiological indicators of pollution, evaluation of cell viability and DNA damage level by comet assay in hemocytes/blood cells in freshwater mussels/freshwater fish available at the sites (**Table 3.1**).

Mussels – The species *Unio tumidus* (Fig 3.2) is autochthonous, common for the lower stretch of the Sava River (Paunović et al., 2012) and widely distributed in the study area. The abundance of this species in other potamon-type rivers in the Danube Basin is approximately 30% (Tomović et al., 2013, 2014). Adult specimens of *U. tumidus* (5 − 7 cm shell lengths) were collected from 2 - 5 m water depth by diving and transported to the laboratory in dark cool box.

Fig 3.2 *Unio tumidus* (image source internet)

Fish – bream species, ***Blicca bjoerkna*** and ***Ballerus sapa*** (**Fig 3.3**) are highly similar in body size, nutrition and daily cycles. In the lower stretch of the Sava River they are widely distributed and available throughout the year with abundance of 33% (for bream species based on the study of Jovičić et al. 2014). Selected fish species are not economically important, but represent a significant part of the food chain. They are bottom feeders, with diet consisting of smaller representatives of the benthic fauna and plants. Since they live in bottom habitat, it can be assumed that they could be exposed to the simultaneous action of genotoxic pollution from sediment and food, but also from water - through the respiration process. Fish were collected with the help of local fishermen by angling with a rod. Condition factor of collected specimens was calculated according to formula: $CF = W/L^3 \times 100$, where W is weight (g) and L is total length of fish (mm) Bervoets et al. (2003).
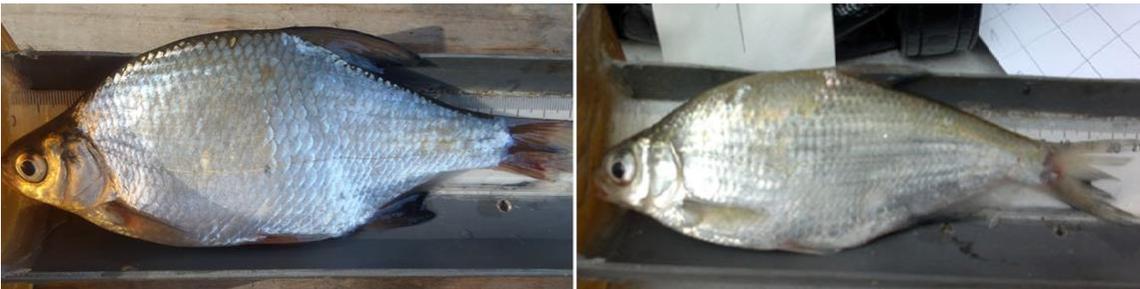
Fig. 3.3 Bream species analyzed in preliminary research *Blicca bjoerkna* (left) *Ballerus sapa* (right)

Table 3.1 Number of specimens used for each season

Species	Season	winter	Summer
U. tumidus	No. of individuals	5	5
B. bjoerkna/B. sapa	No. of individuals	5	3
	Condition Index (CI)[a]	1.0	1.2

[a] significant difference among the CI values was not observed

3.2.1.1 Cryopreservation -preliminary research

Before the Sava River Survey (in August), preliminary cryopreservation experiments were performed on specimens of *U. tumidus* and *A. alburnus* collected at the Duboko site **(Table 3.2).**

Table 3.2 Number of specimens used for cryopreservation experiments

Species	No. of individuals	
U. tumidus	4	
A. alburnus	No. of individuals	Condition Index (CI)
	8	0.68

3.2.2 Sampling during the Sava River Survey

The study area is presented in the **Fig. 3.4.** Sampling was performed at 12 sites along the Sava River in August and September 2015. Overview of the analyses and specimens used in research is provided in the **Table 3.3.** Description of the sites and pollution pressures is given in the **Table 3.4**.

Label	Site name
1	Litija
2	Vrhovo
3	Čatež
4	Zagreb
5	Jasenovac
6	Sl. Brod
7	Županja
8	Sr. Mitrovica 1
9	Sr. Mitrovica 2
10	Šabac 1
11	Šabac 2
12	Beograd
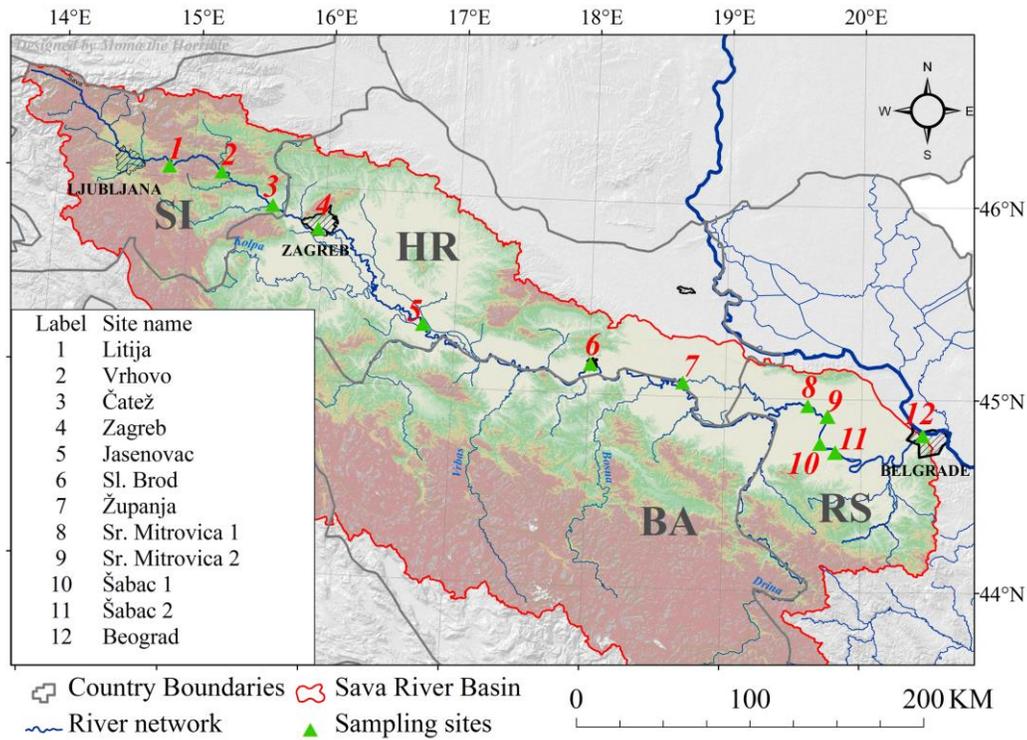
Fig. 3.4 Sampling sites along the Sava River

Research comprised analyzes of microbiological indicators of faecal pollution, mutagenic potency of water samples by SOS/*umuC* test and evaluation of DNA damage by comet test, Fpg-modified comet test and micronucleus test in blood of two minnow species *Alburnus alburnus* and *Alburnoides bipunctatus* (**Fig 3.5**) selected based on the dataset obtained in previous survey of the Sava River (Simonović et al., 2015). Spirlin (*A. bipunctatus*) is characteristic for the upper section and bleak (*A. alburnus*) is present in middle and lower sections of the river. Both spirlin and bleak are active and fast swimmers, of the short life-span, with consequently high metabolic rates which can lead to high accumulation of metals and metalloids. Also, they are both epipelagic fish not exposed to the contaminants in the benthic zone.

Specimens of fish were collected by hand net (mesh size 1 cm^2). Condition factor of collected specimens was calculated according to formula Bervoets et al. (2003) indicated previously.

Fig. 3.5 Minnow species analyzed within the Sava River Survey *Alburnus alburnus* (left) *Alburnoides bipunctatus* (right) (image source, internet)

Table 3.3 Overview of the parameters analyzed within the Sava survey 2015

Parameter	Microbiological indicators	SOS/*umuC*	Genotoxicity (standard and Fpg - comet assay and micronucleus)	N of specimens	Condition Factor (mean ± SE)
Media	water	water	fish		fish
Litija	+	+	*A. bipunctatus*	8	0.80±0.07
Vrhovo	+	+	*A. bipunctatus+A. alburnus*	6+2	0.66±0.03
Čatež	+	+	*A. bipunctatus*	4	1.00±0.17
Zagreb	+	+	*A. alburnus*	7	0.66±0.02
WWZ*	+	+	x	x	x
Jasenovac	+	+	*A. alburnus*	9	0.63±0.02
S. Brod	+	+	*A. alburnus*	8	0.64±0.01
Županja	+	+	*A. alburnus*	7	0.68±0.02
S. Mitrovica1	+	+	*A. alburnus*	7	0.67±0.02
WWSM*	+	+	x	x	x
S. Mitrovica2	+	+	*A. alburnus*	8	0.68±0.02
Šabac 1	+	+	*A. alburnus*	5	0.89±0.09
WWŠ*	+	+	x	x	x
Šabac 2	+	+	*A. alburnus*	8	0.70±0.02
Belgrade	+	+	*A. alburnus*	8	0.66±0.02

x-not assessed, *WW-wastewater outlet at the site listed above

Table 3.4 Brief overview of the major pressures at the selected sites

Site	Code	Site description
		Slovenia
Litija	1	Small size town, about 6.000 inhabitants
Vrhovo	2	Small settlement, less than 1.000 inhabitants; sampled downstream of the reservoir made by damming
Čatež	3	Small settlement, less than 500 inhabitants; sampled downstream of the reservoir made by damming
		Croatia
Zagreb	4	Capital of Croatia, about 800.000 inhabitants, sampled downstream of the city, wastewater outlet detected at the site (WWZ)
Jasenovac	5	Small settlement, less than 500 inhabitants; sampled upstream of the Una River confluence
Slavonski Brod	6	Mid-size settlement, about 60.000 inhabitants, sampled upstream of the town
Županja	7	Mid-size settlement, about 13.000 inhabitants, sampled downstream of the town
		Serbia
Sremska Mitrovica	8/9	Mid-size settlement, about 40.000 inhabitants, sampled in the town and downstream of the town, wastewater outlet detected at the site situated downstream of the town (WWSM)
Šabac	10/11	Mid-size settlement, about 50.000 inhabitants, center of chemical industries, sampled upstream and downstream of the town, few wastewater outlets detected at the site situated between the sites (WWŠ)
Belgrade	12	Site situated upstream of the Belgrade, impact of minor villages situated nearby and the town Obrenovac (about 50.000 inhabitants)

3.3 Methods

3.3.1 Detection of total coliforms, *Escherichia coli* and enterococci

Defined Substrate Technology (DST) was used to detect total coliforms (TC), *E. coli* (EC) and enterococci (FE). This enables isolation and identification of certain groups of bacteria by enzymatic hydrolysis of specific substrates (Buckalew et al., 2006). Quantification was performed with Colilert Quanti-Tray 2000 system, which provides a Most Probable Number (MPN) result, based on color/fluorescence change in 97 wells (**Fig 3.6**). For coliform bacteria two dilutions were analyzed (1:10 and 1:1000) while for enterococci only one dilution was analyzed (1:10). Powdered reagents Colilert-18 and Enterolert-E were used for cultivation of coliforms and enterococci respectively. A diluted water sample was mixed with powdered reagents and poured into Quanty-Tray, a sterile plastic disposable 97-well tray. For coliforms, trays were incubated at 37 ºC for at least 18 h. In the preliminary study on the Sava River, BIORAD MPN plates (**Fig 3.7**) were used for enterococci (two dilutions 1/2 and 1/20). Enterococci were incubated for at least 24 h at 44 ºC.

After incubation, an appearance of yellow color in wells was indicative of the presence of total coliforms. Fluorescence in wells, under UV illumination (365 nm) was used as an indication of the presence of *E. coli*, in Colilert, and enterococci in Enterolert-E. The number of positive wells was scored and converted to MPN using tables provided by the manufacturer. Assessment of the water quality was based on the total coliforms, *E. coli* and enterococci classification scheme suggested by Kirschner et al. (2009) and expressed as MPN/100 ml.

Fig 3.6 Colilert/Enterolert-E Quanti-Tray 2000 system

Fig 3.7 BIORD MPN system for detection of enterococi

3.3.2 Detection of presumptive *Clostridium perfringens* by the membrane filtration method

Before the filtration, water samples were pasteurized for 15 min at 60 ºC. *C. perfringens* (CP) were enumerated by membrane filtration (nitro-celulose filters 0.2 µm pore size) and incubation on Tryptose Sulphite Cycloserine (TSC) media according to ISO 14189:2013 and the number of cells expressed as CFU/100 ml of sample. Incubation was carried out at 24 h at 44 ºC. Dark colored colonies were considered as presumptive positive results (**Fig 3.8**).
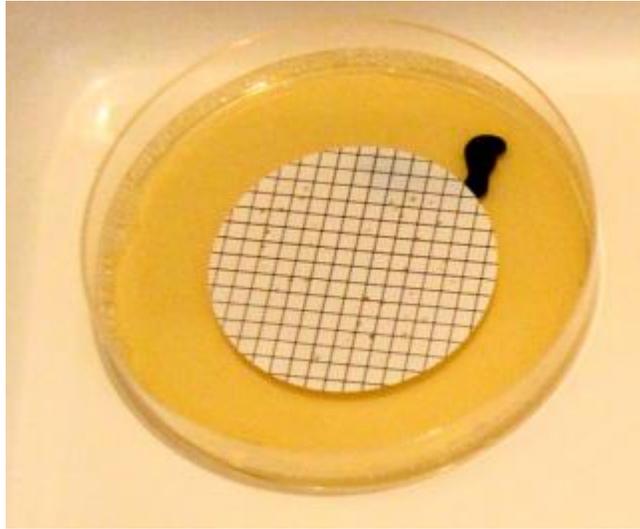
Fig 3.8 Colonies of presumptive *C. perfrigens* on TSC media

3.3.3 Mutagenic potency of water samples - SOS/*umuC*

From each site a sample of 50 mL of water was taken and stored at -20 °C. The SOS/*umuC* assay was applied on stored water samples filtrated through 0.2 μm pore size filters using the protocol described by Žegura et al. (2009). The overnight culture of *S. typhimurium* TA1535/pSK1002 was diluted 10 times with fresh TGA medium and incubated at 37 °C for 1.5 h with aeration until the bacteria reached the exponential growth phase. Treatment was performed in microtiter plates by adding 180 μL of water sample, 20 μL of 10 x TGA and 70 μL of bacterial culture or in the case of metabolic activation 180 μL water sample, 20 μL 10 x TGA with cofactors and 70 μL of S9 bacterial culture mixture prepared as described in the ISO standards (2000). 4-Nitroquinoline (4-NQO, final concentration 0.5 μg/mL) was used as a positive control in experiments without metabolic activation while benzo(a)pyren (final concentration 10 μg/mL) was used in experiments with metabolic activation. Sterile bidistilled water was used as the negative control. The microtiter plate was incubated at 37 °C for 2 h with aeration. After the treatment, the incubation mixture was then diluted 10 times with fresh TGA medium in new microtiter plates and incubated for a further 2 h. The bacterial growth rate was determined by measuring absorbance at 600 nm at microtitar plate reader. ß-Galactosidase activity was determined after using ONPG as a substrate for 20 min at 25 °C (**Fig 3.9**). Absorption was

measured at 405 nm using a reference solution without bacteria. The bacterial growth rate was calculated using the following formula: G = sample OD600/control OD600. A growth ratio less than 0.75, that represents 25% inhibition of biomass was considered to be an indication of cytotoxicity.

Induction ratio (IR) was calculated by the formula: sample OD405/control OD405 x G. An induction ratio 1.5 was taken as the threshold at which the sample was considered as genotoxic (ISO, 2000). All treatments were performed in triplicates in three individual experiments.
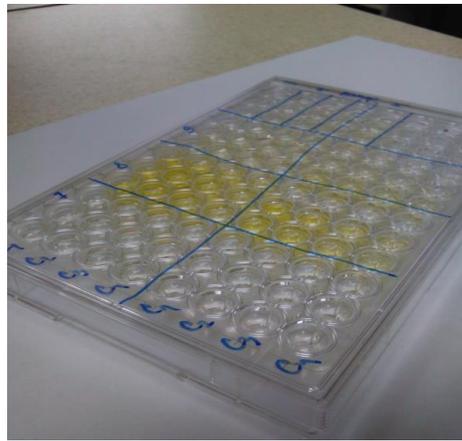
Fig 3.9 SOS/*umuC* plate with positive wells with intense yellow color (Milovanović, 2016)

3.3.4 Hamolymph collection and preservation

Haemolymph was collected by protocol described in Kolarević (2013). Approximately 1 mL of haemolymph was taken from adductor muscle using three mL syringes (23 G needle) and mixed with equal volume of HBSS with addition of 5 mM EDTA. Samples were centrifuged at 2,000 rpm and pelleted cells were resuspended in 100 µL of residual supernatant. Suspensions prepared as indicated were used for cell viability assay and comet test.

To study the effects of cryopreservation, haemolymph was preserved based on instructions of Kwok et al. (2013). Briefly, 1 mL of haemolymph was mixed with equal volume of cryoprotective medium (L15 medium supplemented with 20 % glycerol) and

immediately frozen liquid nitrogen. In analyzes, cryotubes were taken from liquid nitrogen and immediately thawed at 21 ºC in water bath. Afterwards, suspensions were centrifuged (2,000 rpm, 10 min, 4 °C), the supernatants were discharged and pellets were suspended in 100 µL of residual supernatant and prepared suspensions were further used for cell viability and comet assay.

3.3.5 Fish blood sample collection and preservation

In preliminary study performed at the site Duboko on bream species fresh blood samples were analyzed directly upon sampling while within the Sava River Survey analyzes were performed on cryopreserved blood samples of minnow species.

Immediately after sampling, fish specimens were anesthetized with clove oil prior to dissection and blood was collected directly from the heart with 3 mL syringes (21 G needle rinsed with sodium heparin). For fresh samples of bream species (*B. bjoerkna* and *B. sapa*) one drop of blood of each specimen was diluted 20x in 4 ºC cooled 1xPBS. Samples were additionally diluted upon arrival in laboratory in 1xPBS and subjected to comet assay as indicated in chapter 3.3.8.

Blood samples of minnow species *A. alburnus* and *A. bipunctatus* collected within the Sava River Survey were cryopreserved based on methodology described in Akcha et al. (2003) with slight modifications. Briefly, one drop of blood of each specimen was diluted 20x in 4 ºC cooled medium (RPMI 1640 supplemented with 25% FBS). Due to storage limitations, blood samples from two specimens were pooled together. To all samples, cryoprotective agent was added (DMSO final concentration 20%) and immediately frozen in liquid nitrogen until the analysis (up to 3 weeks). Cryotubes were taken from liquid nitrogen and immediately thawed at 21 ºC in water bath. Afterwards, suspensions were diluted in 1xPBS to obtain approximately 50,000 cells/mL, the suspensions were centrifuged (2,000 rpm, 10 min, 4 °C), the supernatants were discharged and pellets were suspended in 100 µL of residual supernatant and prepared suspension were further used for comet assay.

The effects of cryopreservation on cell viability and the level of DNA damage were assessed in preliminary experiments prior to Sava River Survey, in 8 specimens of *A. alburnus* collected at the site Duboko situated in the Sava River.

3.3.6 Assessment of cell viability based on differential fluorescence staining
Viability was assessed in haemolymh and blood suspensions. For each sample, 20 µL of suspension was mixed with 2 µL of acridine orange/ethidium bromide solution and observed at 400x magnification under fluorescence (Leica DMLS, Austria, excitation 510-560 nm, emission 590 nm). Viability was assessed based on 100 counted cells in each sample (**Fig 3.10 a** and **b**).
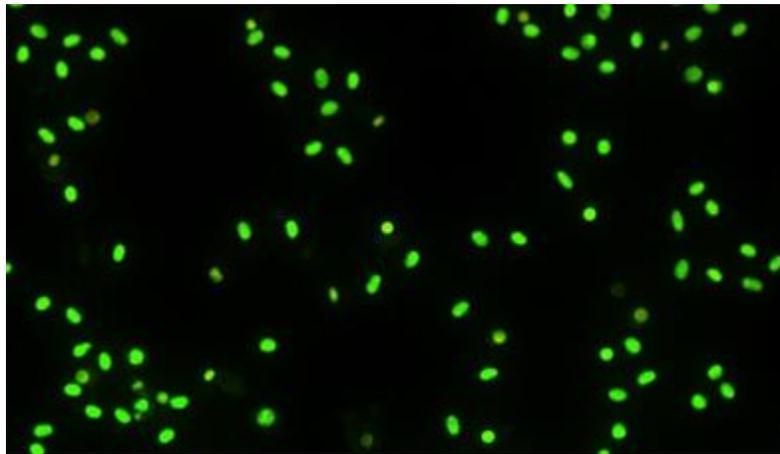
Fig 3.10a Smear of fish blood cells stained with AO/EB differential staining, green cells are recognized as viable
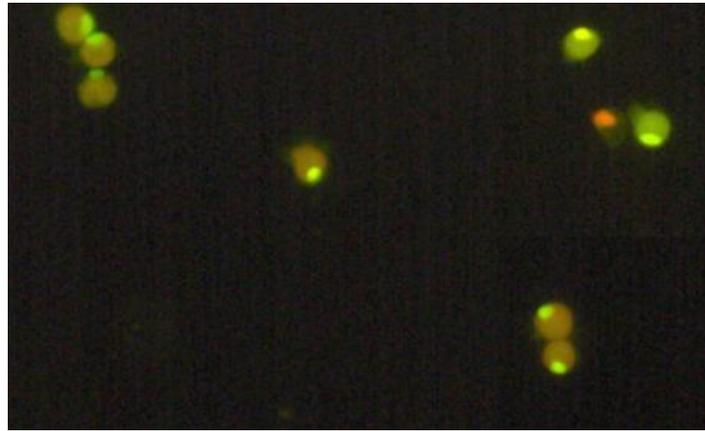
Fig 3.10b Smear of haemolymph cells of *U. tumidus* stained with AO/EB differential staining, cells with green nuclei are considered as viable while cells with orange/red nuclei are considered as nonviable

3.3.7 Comet assay

The comet procedure was performed under yellow light as described in Kolarević et al. (2016) with slight modifications. Scheme of procedure is shown in **Fig 3.11.**
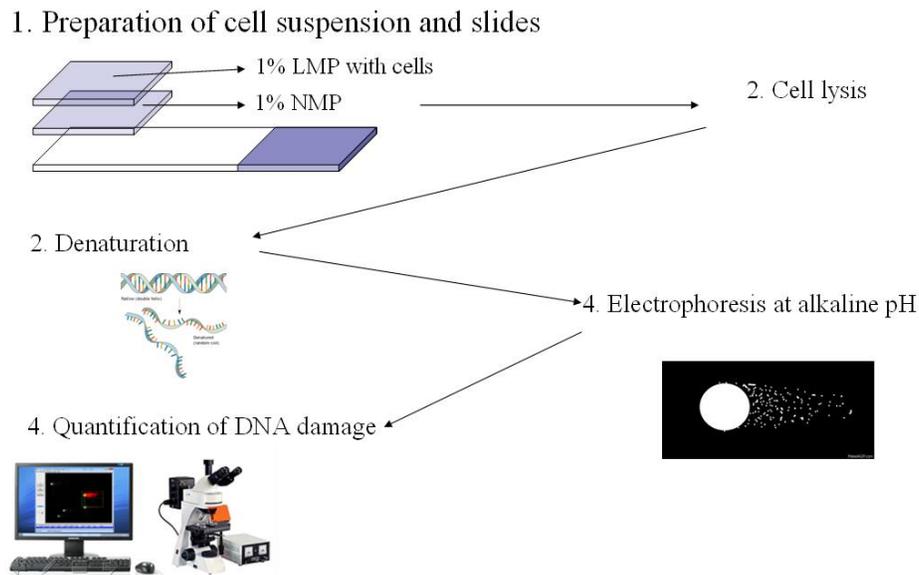
Fig 3.11 Scheme of experimental procedure in alkaline comet assay

Briefly, microscope slides were pre-coated with 1% normal melting point (NMP) agarose and air dried for 24 h. The second, supportive layer was formed of 80 μL of 1% NMP agarose. The final layer was formed of 30 μL of cells suspension (prepared as described earlier) gently mixed with 70 μL of 1% low melting point agarose (37 ℃). The slides were held in freshly made cold (4 °C) lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10 % DMSO, 1.5% Triton X-100, pH 10) for 2 h. To allow DNA unwinding, slides were placed in an electrophoresis chamber containing cold (4 °C) alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min. Electrophoresis was performed with a voltage gradient 0.75 V/cm and amperage 300 mA for 20 min at (4 °C). Afterwards, neutralisation was carried out in freshly made cold (4 °C) neutralizing buffer (0.4 M Tris, pH 7.5) for 15 min. Slides were preserved by fixation in cold methanol at 4 °C for 15 min. Staining was performed with 20 μL per slide of acridine orange (2 μg/mL). The slides were examined with a fluorescence microscope (Leica, DMLS, Austria, under magnification 400 X, excitation filter 510-560 nm, barrier filter 590 nm). Microscopic images of comets were scored using Comet IV Computer Software (Perceptive Instruments, UK). Tail intensity (TI % - percentage of DNA in the tail of the comet) was chosen as a measure of DNA damage. For each sample 100 nucleoids were scored. As the possible indication of apoptosis, excessively damaged nuclei or so called hedgehogs (HH) were counted for each slide using a hedgehog tool available in the Comet IV Software (**Fig 3.12**).
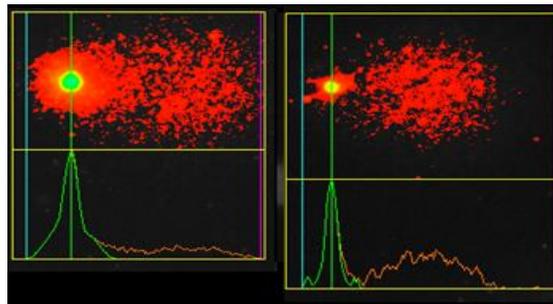
Fig 3.12 Image of damaged nuclei (left) and hedgehog (right) scored in Comet assay IV Software

3.3.8 Fpg – modified comet assay

   For each sample of blood of specimen collected within the Sava Rover Survey, two slides were prepared for Fpg - modified assay as described in previous section, one for buffer and one for the enzyme (**Fig 3.13**). After one hour of lysis slides were washed 3 times in cold (4 °C) washing buffer (100 mM KCl 100 mM, 10 mM Na_2EDTA and 10 mM HEPES, adjusted pH 7.2). At slides prepared for buffer, 45 µL of buffer (100 mM KCl, 10 mM EDTA, 10 mM HEPES, 0.1 mg/mL BSA, adjusted pH 7.2) was added while on slides prepared for enzyme 45 µL of 300 x diluted Fpg enzyme (Trevigen, Maryland) was added and covered with coverslips. Slides were incubated for 30 min at 37 °C in humidity chamber. Afterwards slides were held for 5 min at 4 °C, coverslips were removed and slides were subjected to denaturation step as for the standard comet assay protocol described in the previous section. The net contribution of the 8-hydroxy-2′-deoxyguanosine (8-oxoG) in final DNA damage evaluated by Fpg-modified comet assay was calculated by subtraction of the mean TI% values obtained from slides exposed to buffer only from the mean TI% values obtained from the slides exposed to Fpg–enzyme (Collins, 2004).
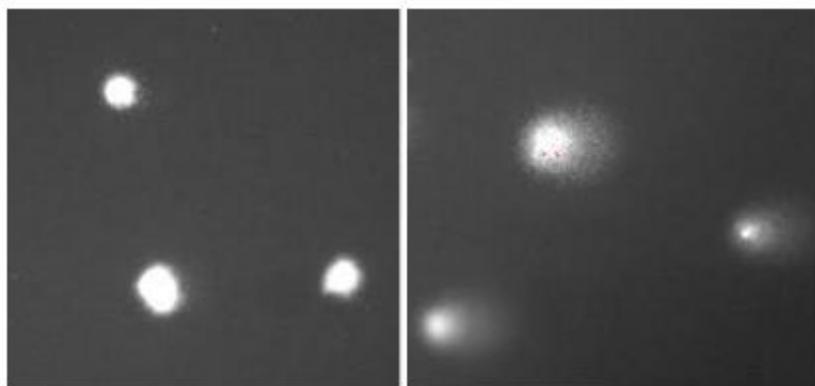
Fig 3.13 Image of common nuclei from slide subjected buffer (left) and Fpg enzyme (right) scored in Comet assay IV Software

3.3.9 Micronucleus assay

Slides for micronucleus assay were prepared by making a smear of 100 µL of blood on clean microscope slides for each sample. Slides were air dried for 30 min and then fixed in cold methanol for 30 min at 4 ℃ and air dried for 24 h. Upon scoring, slides were stained using acridine orange (25 µg/mL) and examined at 1000x dry magnification. For each sample at least 3,000 cells were examined. Nuclear aberrations were scored by criteria of Fenech et al. (2003). Diameter for micronuclei was between 1/3 and 1/16 of the main nuclei (**Fig 3.14**).
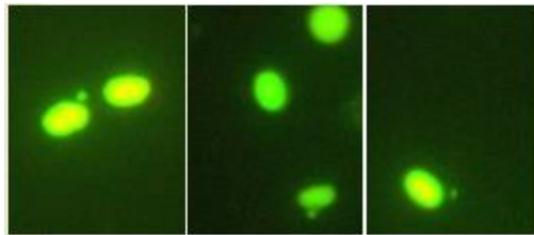
Fig 3.14 Image of erythrocytes in blood smear of minnow containing micronuclei

3.3.10 Statistical analyses of data in gentoxicological bioassays

Statistical analysis of the results obtained in the experiments was carried out using Statistica 6.0 Software (StatSoft, Inc.) and SPSS 20.0 (Inc., Chicago, IL, USA). Kolmogorov-Smirnov test was used to determine if data were normally distributed. Data on MN frequency were analyzed by one-way ANOVA followed by Tukey's post-hoc test. Comet assay data were analyzed by Kruskal-Wallis one-way ANOVA followed by Dunn's Multiple Comparison Test since they were not normally distributed. The level of significance for all comparisons was set at $p < 0.05$. Correlation analyses were carried out using Pearson's correlation test with significance level $p < 0.05$.

3.3.11 Ranking of the sites in the Sava River Survey by integrated biomarker response (IBR)

The IBR ranking of the sites was performed based on parameters – *E. coli* numbers (EC), metal pollution index (MPI), condition factor (CF), mutagenicity (SOS with metabolic activation), comet assay (CA), oxidative stress approximated with net contribution of 8-oxoG sites (OS) and micronucleus assay (MN). IBR was assessed as described by Beliaeff and Burgeot (2002). Briefly, the value of each parameter (X_i) was standardized by the formula $Y_i = (X_i - mean)/SD$, where Y_i is the standardized parameter response, mean and SD are calculated based on values for the selected parameter for all sites. Z_i was then calculated as $Z_i = Y_i$ if the studied parameter respond to contamination by induction or $Z_i = -Y_i$ if the parameter respond to contamination by inhibition. The minimum value Z_i for each parameter was marked (min) and the scores for the studied parameters were computed as $S_i = Z_i + |min|$. Scores for each parameter (S_i) for particular site were used as radius coordinates of the studied parameters of the star plots. Individual areas Ai of the star plot were calculated according to the formula: $Ai = S_i \times S_{i+1} \times \sin(51.43°) / 2$, where S_i and S_{i+1} represent the individual parameter scores and their successive star plot radius coordinates. The IBR value is calculated as following: IBR= sum of all A_i, where A_i is the area represented by two consecutive indicators on the star plot, and n is the number of indicators used in the IBR calculation. Scores for each parameter (S_i) were used for ranking of the parameters while the IBR values were used for final ranking of the sites. The site with the lowest rank was considered as the site with the lowest level of stressors.

3.4 Metadata used for the studies

3.4.1 Preliminary research at the sampling site Duboko

Data on physico-chemical and chemical parameters (from the study Kračun-Kolarević (2017) and Serbian Environmental Protection Agency SEPA, Belgrade, Serbia) were summarized in **Supplementary material 1** in the **Tables 1** and **2**. This data was used in conjunction with our genotoxicological data for making final conclusions.

3.4.2 Research performed within the Sava River Survey

    Data on physico-chemical and chemical parameters in water of the studied sites were kindly provided from the Institute for Biological Research "Siniša Stanković", Belgrade, Serbia and summarized in **Supplementary material 2** in **Table 1**. Data on the concentration of metals in water samples at the selected sites were kindly provided by Jožef Štefan Institute, Ljubljana, Slovenia and summarized in **Table 2**. The data on concentration of metals in fish tissue was kindly provided by the Faculty of Natural Sciences, Kragujevac, Serbia and summarized in Table 3. This data was used in conjunction with our genotoxicological data for making final conclusions.

RESULTS

4. Results

4.1 Preliminary research – the site Duboko

4.1.1 Physico-chemical and chemical parameters

The data provided by the Institute for Biological Research "Siniša Stanković" on physico-chemical parameters, is given in **the Supplementary material 1 Table 1**. Studied parameters, except temperature, were in a similar range during winter and summer. In both seasons pH values were mildly alkaline. According to the national legislation (Official Gazette, 2011) based on NO_3^-, NH_4^+ and PO_4^{3-} concentrations belong to classes III and IV of water quality. The higher pollution based on NO_3^- and NH_4^+, was observed in the winter season.

The concentrations of total and dissolved metals in water (**Supplementary material 1 Table 2**) were also in a similar range in both studied seasons. The most important difference was the concentration of dissolved Zn (four times higher in winter).

4.1.2 Microbiological indicators of faecal pollution

Presence of faecal pollution was evident in both seasons at the site Duboko (**Table 4.1**). The number of *E. coli* and enterococi showed critical pollution with a similar log MPN range during the seasons. Total coliforms were in a similar range and concentration of *C. perfrnigens* was lower in summer.

Table 4.1 Microbiological indicators of faecal pollution

Month	Total coliforms log MPN in 100 mL	*E. coli* log MPN in 100 mL	Enterococci log MPN in 100 mL	*C. perfringens* log CFU in 100 mL
Winter	3.7	3.5	3.2	2.4
Summer	3.5	3.1	2.3	1.7

4.1.3 Assessment of the level of DNA damage in freshwater mussel haemocytes and fish blood cells

Prior to the comet assay, viability of haemocytes and blood cells was estimated by using diferential fluorescent staining. Viability of haemocytes was 81% and 90% in winter and summer, respectively. Viability of fish blood cells was 99% and 94% in winter and summer, respectively.

The results of the level of DNA damage in mussel haemocytes and fish blood cells are summarized in the **Fig 4.1** For both organisms, significant increase of DNA damage was detected in the summer.
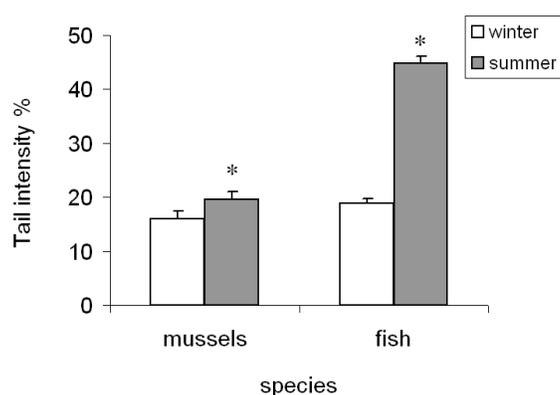
Fig 4.1 The level of DNA damage in mussels and fish specimens collected in the winter and summer at the Duboko site,*significant increase in comparison with the winter season

4.1.4 Effect of cryopreservation

During the preparation for the Sava River Survey we have decided to use cryopreservation of the samples. In preliminary experiments, the effects of cryopreservation on cell viability and the level of DNA damage were assessed in 8 specimens of bleak collected at the site situated in the Sava River near Belgrade prior to the Sava River survey. The effects of cryopreservation were tested in freshwater mussels *U. tumidus* and freshwater fish, bleak.

As indicated in the **Fig. 4.2** the effects of cryopreservation on cell viability and DNA damage induction in fish *A. alburnus* were assessed. Cell viability was reduced 18% while cryopreservation did not additionally increase the DNA damage level. Although the values were within the same range, the level of DNA damage after cryopreservation was significantly lower when compared to the fresh sample. However, significant increase of HH frequency was noticed.
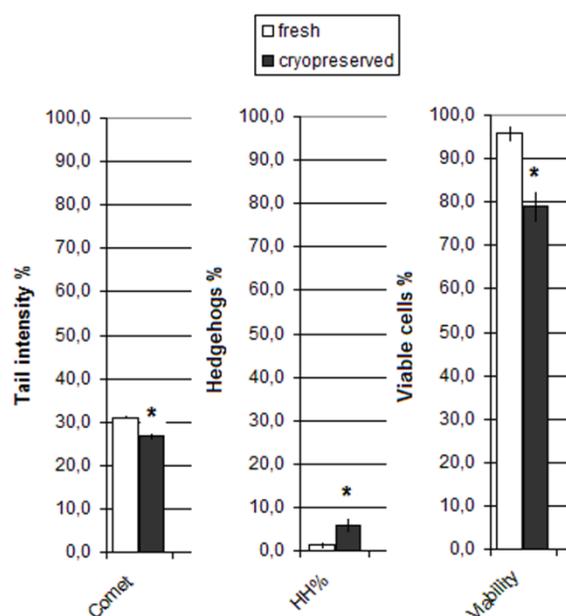
Fig 4.2 Cryopreservation effects on the cell viability and DNA damage level in *A. alburnus*
*statistical significance in comparison to the fresh sample (p < 0.05)

The effects of cryopreservation on cell viability and DNA damage observed in freshwater mussels *U. tumidus* are summarized in **Fig. 4.3.** Cell viability was reduced from 80% to 60% and cryopreservation significantly increased the DNA damage level. Significant increase of HH frequency was also noticed.
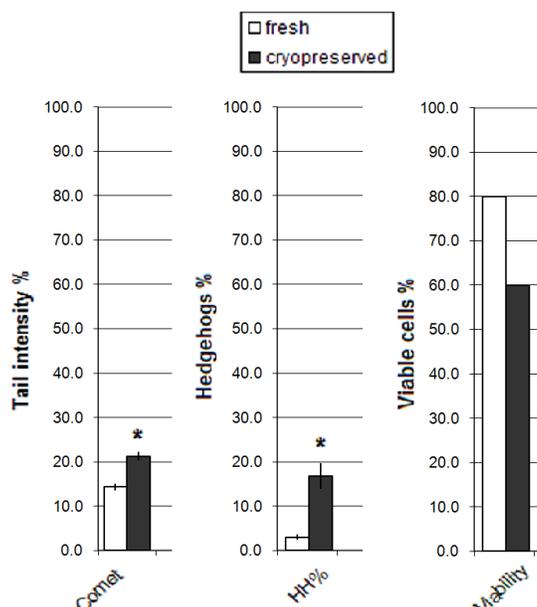
Fig 4.3 Cryopreservation effects on the cell viability and DNA damage level in *U. tumidus*

*statistical significance in comparison to the fresh sample ($p < 0.05$)

4.1.4 Outcomes of the preliminary research at the site Duboko

- Physico-chemical, chemical and microbiological indicators showed a similar level of pollution during both sampling seasons.

- Freshwater mussels and fish showed a higher response to pollution in summer season.

- Negative effects of cryopreservation on cell viability and DNA damage were more evident in mussels.

- By taking into consideration the results obtained in the preliminary study and the distribution of used organisms throughout the Sava River Basin, it was concluded that fish species should be chosen for the Sava River survey.

- The outcomes of the preliminary study were used for planning the Sava River Survey.

4.2 The Sava River Survey

4.2.1 Physico-chemical and chemical parameters

Data on physico-chemical parameters (provided by IBISS, Belgrade, Serbia) are given in the **Supplementary material 2 Table 1**. Generaly, pH at the all sites was neutral. Considering the typology of the Sava River, a lower temperature of water was expected and detected in the upper stretch of the river. Similarly, higher oxygen saturation was present at the sites of the upper stretch. Concentration of NH_4 was at the limit of detection. According to the national legislation (Official Gazette, 2011), based on the concentration of NO_3, water quality could be placed in II and III class.

4.2.2 Concentration of metals and metalloids in water

Data on concentration of metals and metalloids in water (provided by Jožef Štefan Institute, Ljubljana, Slovenia) are given in the **Supplementary material 2 Table 2**. The data revealed that the concentrations of elements in water were low in comparison to values characteristic for the Sava River, based on available literature (Milačič et al., 2017). Based on the metal pollution index, the least polluted site was Čatež while the highest concentrations of metals were detected in Belgrade. Only As and Ni had an increasing trend in concentration values in the lower stretch of the river when compared to the upper stretch.

4.2.3 Concentration of metals and metalloids in fish tissue

Data on concentration of metals and metalloids in fish tissue (provided by the Faculty of Natural Sciences, Kragujevac, Serbia) are given in the **Supplementary material 2 Table 3**. The average concentrations of metals and metalloids in spirlin and bleak samples (whole body content) and metal pollution index (MPI) are presented in the **Supplementary Table 3**. A pattern was observed at all sampling sites with the highest Zn concentrations (23.09 ± 6.65 to 47.67 ± 9.73) and at almost all of the sampling sites with the lowest Co concentrations (0.0001 ± 0.0004 to 0.009 ± 0.016) (Table 2). The highest concentrations and number of metals (Al, Co, Fe, Mn, Ni and Sn) were recorded at Županja sampling site. On the other hand, the lowest concentrations of metals and metalloids (Al, Cu, Fe, Hg, Se, Zn) were determined at the Belgrade sampling station. The highest MPI

was calculated for Županja sampling station (0.33) and the lowest for the Belgrade sampling station (0.14). Kruskal-Wallis test revealed that mean concentrations of metals and metalloids were significantly different ($p < 0.05$) between all sampling sites, except between the sites Sremska Mitrovica and Županja, Sremska Mitrovica and Šabac 2.

4.2.4 Indication of presence of wastewaters

Microbiological indicators (**Fig 4.4**) showed that the majority of the river samples were slightly and moderately polluted (class I and II). The numbers of TC ranged from 613 (Vrhovo) to 307,600 (Županja) MPN/100 mL. The numbers of EC ranged from 10 (Jasenovac) to 32,300 MPN/100 mL (Županja). At the 8 out of 9 sites the numbers of FE were below the limit of detection (<10 MPN/100 mL), while the highest numbers were recorded at the site Županja (1,317.2). The numbers of CP ranged from 0 (S. Mitrovica 2) to 963 (Županja) CFU/100 mL. Wastewaters (WW) discharge points were found at the sites Zagreb (ZWW), S. Mitrovica (SMWW) and Šabac (ŠWW). Samples collected from the WW discharge points at the sites S. Mitrovica and Šabac indicated excessive faecal pollution affecting water quality at downstream situated sites.

Wastewaters discharge points were found at the sites Zagreb (WWZ), S. Mitrovica (WWSM) and Šabac (WWS). Samples collected from the WW discharge points at the sites S. Mitrovica and Šabac indicated excessive faecal pollution affecting water quality at downstream situated sites (S. Mitrovica 2 and Šabac 2 respectively). Therefore, the sites Županja, S. Mitrovica 2 and Šabac 2 are recognized as hotspots of faecal pollution.
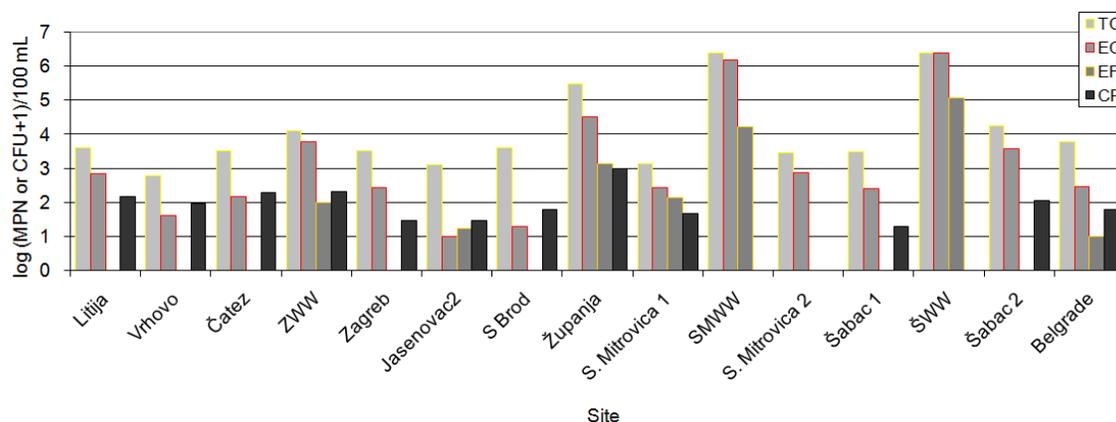
Fig 4.4 Numbers of indicator bacteria (log MPN-CFU/100 mL) measured in water samples of the Sava River and in wastewater (WW) samples collected within the survey; TC- total coliforms, EC – *E. coli*, EF – *E. facealis*, CP – *C. perfringens*

In **table 4.2,** the correlations between the numbers of microbiological indicators of pollution are shown. Significant correlations have been observed between the number of total coliforms and *E. coli* and also with number of *C. perfringens*.

Table 4.2 Correlations between the numbers of microbiological indicators of pollution; TC- total coliforms, EC – *E. coli*, EF – *E. facealis*, CP – *C. perfringens;* significant correlations are marked in red

Correlation	EC		EF		CP	
	r	p	r	p	r	p
TC	0.63	0.012	0.28	0.318	0.64	0.010
EC			0.26	0.349	0.29	0.301
EF					0.30	0.278

When comparing the data of faecal pollution and data on concentration of metals and metalloids in fish tissue, significant correlation ($r = 0.78$, $p = 0.008$) was observed between the number of *E. coli* and MPI (**Fig 4.5**).

log MPN/100 ml *E. coli* = -0,3823+13,4233*x; 0,95 Conf.Int.

MPI:log MPN/100 ml *E. coli*: r² = 0,6047; r = 0,7776; p = 0,0081
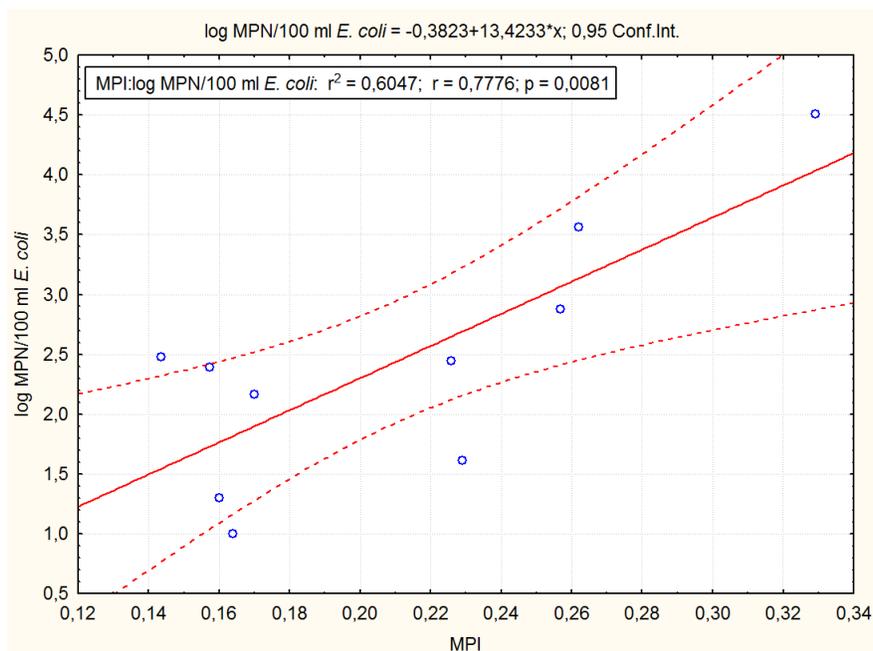
Fig 4.5 Correlation between the numbers of *E. coli* in water and metal pollution index in tissue of fish

4.2.5 SOS/*umuC*

The results of mutagenic potency of water samples measured as SOS induction rate in SOS/*umuC* test, with or without S9, are summarized in the **Fig 4.6**. In the tested water samples cytotoxic effects were not recorded and the threshold value of 1.5 induction was not breached in any case. The highest induction was detected at the sites Litija and Vrhovo. Positive controls for experiment (4NQO without metabolic activation and benzo(a)pyren with metabolic activation) affirmed the validity of the experimental system.
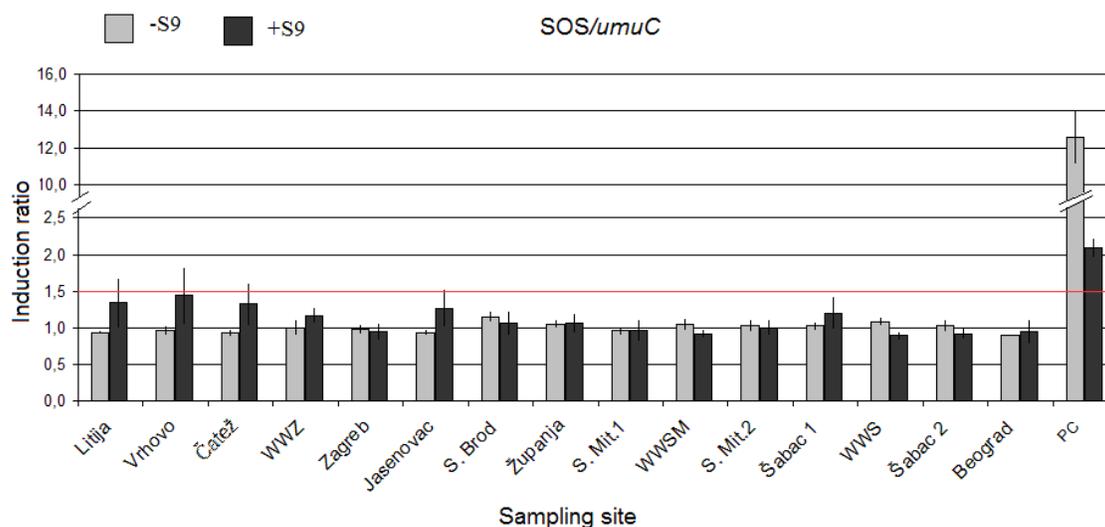
Fig 4.6 SOS induction rate (mean ± SE) in SOS/*umuC*; red line represents threshold induction value (1.5); PC - positive controls: 4NQO (0.5 µg/mL) in experiments without metabolic activation and benzo(a)pyren (10 µg/mL) in experiments with metabolic activation

4.2.6 Assessment of cell viability in cryopreserved samples

Average cell viability in cryopreserved samples was about 80% (**Table 4.3**). The lowest viability was observed in a sample collected at the site Vrhovo (65 ± 6 %) indicating a possible cytotoxic effect. Frequency of the HH was lower than 13% in all samples. Significant negative correlation was observed between the cell viability and HH frequency (r = -0.61, p = 0.0361) (**Fig 4.7**).

Table 4.3 Cell viability, frequency of hedgehogs and frequency in fish blood samples

(mean ± SE)

Site	Viability %	HH%
Litija	77 ± 2	5.5 ± 3.1
Vrhovo	65 ± 6	10.8 ± 0.5
Čatež	79 ± 3	2 ± 1.4
Zagreb	84 ± 2	3.8 ± 1.1
Jasenovac	71 ± 2	5.5 ± 2.7
S. Brod	82 ± 3	0
Županja	79 ± 3	3.3 ± 3.3
S. Mitrovica 1	77 ± 7	12.8 ± 4.3
S.Mitrovica 2	74 ± 3	4.3 ± 1.3
Šabac 1	78 ± 4	10.5 ± 5.5
Šabac 2	85 ± 1	0.3 ± 0.3
Belgrade	87 ± 1	3.3 ± 1.2
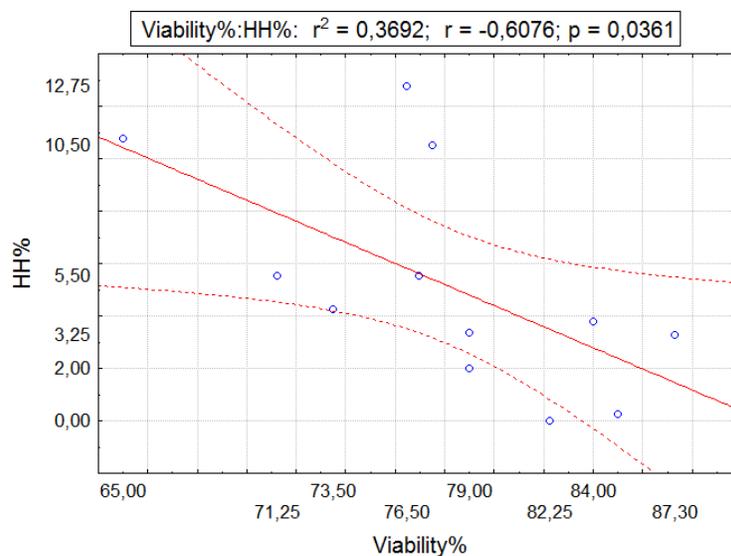
Fig. 4.7 Correlation between the cell viability (%) and frequency of hedgehogs (HH%); full

line - regression line; dashed line - 95 confidence level

4.2.7 Assessment of the DNA damage in blood cells

Alkaline comet assay was performed for the assessment of DNA damage while Fpg
-modified comet assay was performed for the assessment of oxidative stress.

4.2.7.1 Alkaline comet assay

Data obtained by using the alkaline comet assay showed variation of DNA damage within the studied sites (**Fig 4.8**). The sites with the highest levels of DNA damage were Litija, Vrhovo, Jasenovac and Šabac 2. Impact of wastewater discharges was evident at the site Šabac 2 which had significantly higher TI% values in comparison with upstream situated Šabac 1.

Relation between the data obtained in the standard comet assay, HH% and cell viability was investigated. There was neither significant correlation between the level of DNA damage and HH% (r = 0.20; p = 0.53) nor DNA damage and cell viability (r = -0.36; p = 0.25).
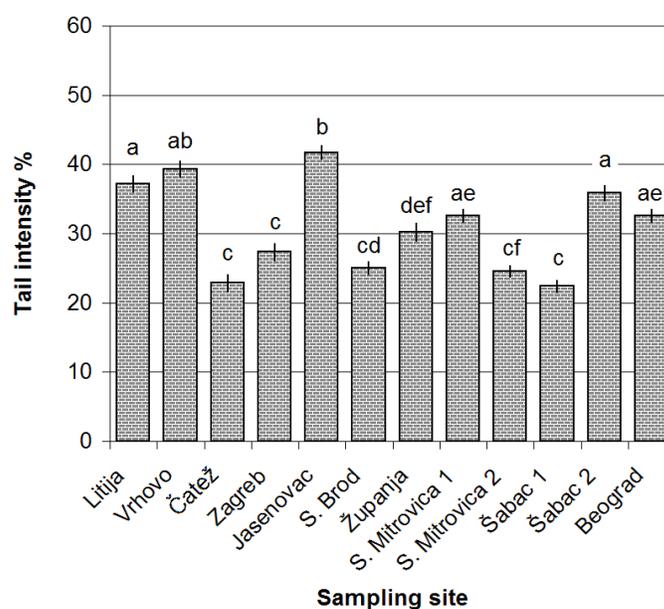
Fig 4.8 The values of tail intensity % obtained in standard alkaline comet assay in blood cells of minnow specimens; values are represented as mean ± SE; different letters denote significant differences among studied sites (p < 0.05)

4.2.7.2 Fpg – modified comet assay

Data obtained in Fpg - modified assay are summarized in the **Table 4.4**. TI% values from slides exposed to buffer only were within the same range as values from the standard alkaline comet assay; furthermore, significant positive correlation between the values was observed (**Fig 4.9A**).

Table 4.4 Fpg - modified comet assay, tail intensity values for buffer and Fpg - exposed slides and net contribution of 8-oxoG sites (mean ± SE); different letters denote significant differences among studied sites ($p < 0.05$)

Site	Fpg enzyme	Buffer only	Net 8-oxoG
Litija	56.7 ± 1.2^{ad}	38.2 ± 1.2^{a}	18.5
Vrhovo	47.7 ± 1.2^{b}	35.5 ± 1.1^{a}	12.2
Čatež	51.4 ± 1.6^{ab}	22.7 ± 1.3^{b}	28.7
Zagreb	29.6 ± 1.2^{cf}	18.2 ± 1.0^{c}	11.4
Jasenovac	58.1 ± 1.1^{d}	37.9 ± 1.1^{a}	20.2
S. Brod	34.4 ± 1.0^{ce}	28.5 ± 1.0^{d}	6.0
Županja	38.1 ± 1.2^{e}	25.2 ± 1.3^{bd}	12.9
S. Mitrovica 1	50.9 ± 1.1^{ab}	25.6 ± 1.1^{bd}	25.3
S. Mitrovica 2	26.4 ± 1.0^{f}	17.2 ± 0.9^{c}	9.3
Šabac 1	31.5 ± 1.0^{cef}	20.0 ± 1.0^{bc}	11.5
Šabac 2	51.4 ± 1.2^{ab}	35.6 ± 1.1^{a}	15.8
Belgrade	51.2 ± 1.3^{ab}	24.3 ± 1.1^{b}	26.9

In all cases digestion with Fpg - enzyme led to a significant increase of DNA damage in comparison with corresponding standard alkaline and buffer treated control. Significant correlation was observed between the mean TI% values obtained from standard alkaline comet assay and slides exposed to Fpg – enzyme (**Fig 4.9B**).
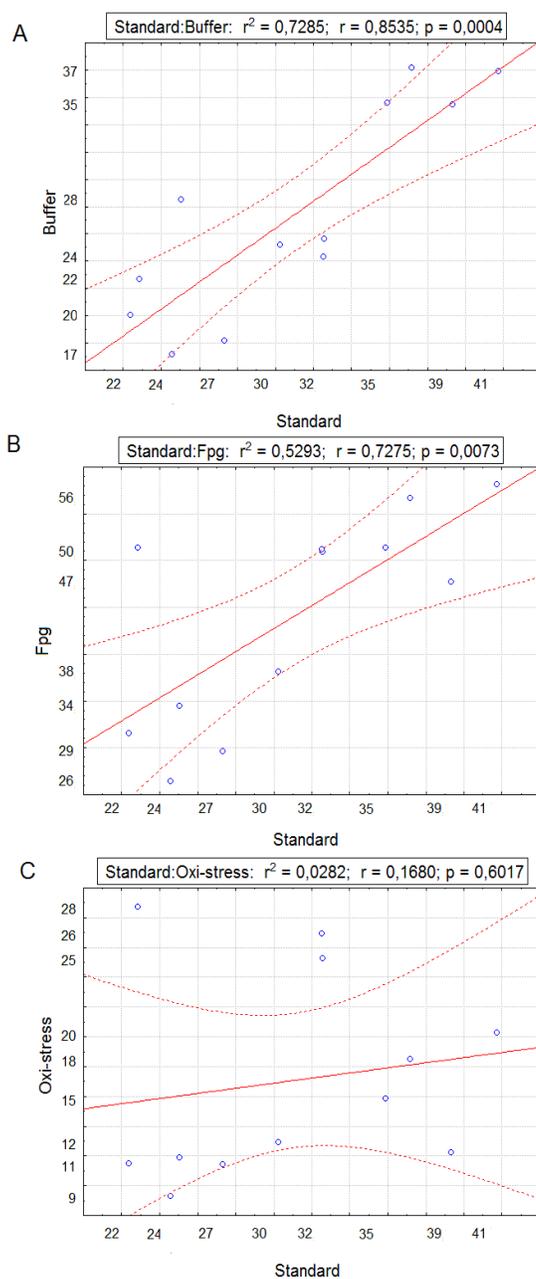
Fig 4.9 Correlation between the levels of DNA damage obtained in different assays
Correlation of the values obtained in standard comet assay and (A) buffer exposed slides,
(B) Fpg exposed slides and (C) net 8-oxoG sites; full line - regression line; dashed line - 95
confidence level

The highest net contribution of 8-oxoG sites was detected in specimens from Čatež, S. Mitrovica 1 and Belgrade (**Table 4.4**). When plotting the values of TI% obtained in the standard alkaline comet assay and the values on net contribution of 8-oxoG sites in DNA damage, significant correlation was not observed (**Fig 4.9C**).

4.2.9 Micronucleus

The highest frequency of MN was detected in specimens collected at the sites Vrhovo and Zagreb (**Fig 4.10**). However, there was no significant difference in MN frequency among the investigated sites.
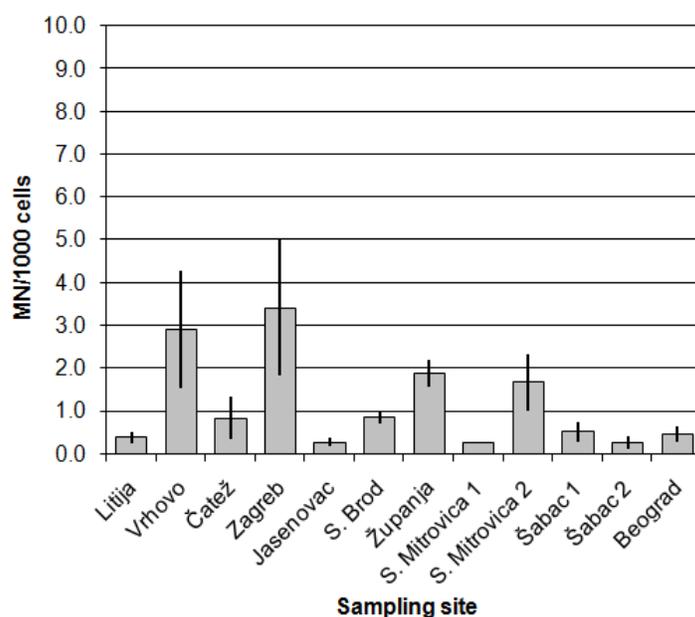
Fig 4.10 The frequency of MN in fish blood samples, values are represented as mean ± SE; significant difference among studied sites was not observed

4.2.10 Correlation of genotoxicological parameters and physico-chemical and chemical parameters measured in water and fish

When plotting data obtained in comet assay and micronucleus assay against the data on physico-chemical parameters measured at the sites, significant correlation was observed only between the TI% values in alkaline and Fpg-modified comet assay and the concentration of NO_2 in the water (**Table 4.5**).

Table 4.5 Correlation between the monitored genotoxicological parameters and physico-chemical parameter as the sites; marked correlations are significant ($p < 0.05$)

Assay	parameter	pH	t (C°)	conductivity (µS)	TDS (ppm)	O_2 (%)	O_2 (mg/L)	NO_2	NO_3
Comet	r	-0.04	-0.36	0.03	0.06	-0.53	-0.36	**0.66**	-0.51
	p	0.910	0.271	0.920	0.872	0.090	0.282	0.026	0.108
Fpg - comet	r	0.03	-0.29	-0.13	-0.11	-0.24	-0.14	**0.63**	-0.15
	p	0.935	0.391	0.697	0.756	0.479	0.681	0.036	0.670
8-oxoG sites	r	-0.06	0.07	-0.27	-0.25	0.12	0.09	0.16	0.22
	p	0.868	0.832	0.422	0.452	0.721	0.790	0.641	0.516
MN	r	0.52	-0.06	0.23	0.23	0.34	0.37	-0.35	-0.25
	p	0.098	0.859	0.488	0.493	0.302	0.261	0.290	0.452

Correlation analyzes between the results of the genotoxicological tests and data on concentrations of metals and metalloids are given in the **Table 4.6**. Significant positive correlation was observed between the DNA damage detected in alkaline and Fpg comet assay and concentration of Zn and between the results of the alkaline comet assay and concentration of Se.

When plotting the data obtained in genotoxicological tests and data on concentration of metals and metalloids in fish tissue, significant positive correlation was observed only between the values of TI% in alkaline comet assay and concentration of Hg in tissue ($r = 0.75$, $p = 0.013$). Significant negative correlation was observed between the frequency of 8-oxoG sites and concentration of Cu ($r = -0.66$, $p = 0.038$) (**Table 4.7**).

4.2.11. Ranking of the sites by IBR

The final IBR values as well as ranking for each studied marker are graphically presented in **Fig 4.11**. The site Šabac 1 had the lowest IBR rank (1.03) while the Županja and Vrhovo had the highest ranks (8.15 and 7.97, respectively) (**Table 4.8**).

Table 4.6 Correlations between the monitored genotoxicological parameters and concentration of metals and metalloids in water; marked correlations are significant ($p < 0.05$)

Assay	parameter	Cr (ng/mL)	Mn (ng/mL)	Fe (ng/mL)	Co (ng/mL)	Cd (ng/mL)	As (ng/mL)	Cu (ng/mL)	Ni (ng/mL)	Pb (ng/mL)	Zn (ng/mL)	Se (ng/mL)	Hg (ng/mL)*	MPI
Comet	r	-0.17	0.38	0.14	0.31	0.39	-0.12	0.29	-0.24	0.36	**0.71**	**0.78**	-0.16	0.49
	p	0.653	0.316	0.713	0.415	0.299	0.763	0.450	0.537	0.339	0.031	0.013	0.680	0.183
Fpg - comet	r	-0.19	0.54	0.12	0.09	0.46	-0.17	0.28	-0.30	0.16	**0.72**	0.59	0.29	0.50
	p	0.629	0.136	0.757	0.810	0.210	0.654	0.470	0.434	0.684	0.028	0.094	0.441	0.167
8-oxoG sites	r	-0.13	0.60	0.15	-0.18	0.56	-0.03	0.40	-0.31	-0.03	0.57	0.21	0.52	0.48
	p	0.743	0.087	0.710	0.646	0.115	0.945	0.289	0.414	0.932	0.111	0.596	0.155	0.192
MN	r	0.44	-0.54	-0.13	0.62	-0.28	-0.53	-0.05	-0.18	0.72	-0.13	-0.30	-0.13	-0.31
	p	0.230	0.136	0.748	0.078	0.457	0.141	0.897	0.639	0.027	0.734	0.433	0.749	0.421

Table 4.7 Correlations between the monitored genotoxicological parameters and concentration of metals and metalloids in fish tissue; marked correlations are significant ($p < 0.05$)

Assay	parameter	Al	As	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Sn	Zn
Comet	r	0.00	-0.60	-0.12	-0.13	0.62	0.39	0.27	**0.75**	0.32	-0.17	-0.31	0.44	0.51	0.38
	p	0.993	0.066	0.751	0.730	0.057	0.268	0.453	0.013	0.363	0.642	0.389	0.202	0.129	0.278
Fpg - comet	r	-0.26	-0.09	-0.32	-0.33	-0.23	-0.17	-0.22	0.50	-0.08	-0.45	-0.15	0.09	0.22	0.00
	p	0.472	0.814	0.360	0.347	0.526	0.643	0.550	0.145	0.836	0.192	0.677	0.815	0.546	0.987
8-oxoG sites	r	-0.40	0.44	-0.17	-0.34	-0.22	**-0.66**	-0.54	-0.05	-0.48	-0.47	0.23	-0.33	-0.16	-0.48
	p	0.252	0.202	0.634	0.340	0.543	0.038	0.105	0.893	0.159	0.171	0.524	0.354	0.659	0.161
MN	r	0.13	-0.15	0.62	0.19	0.51	0.54	0.54	-0.06	0.13	0.16	0.34	0.62	0.27	0.27
	p	0.726	0.673	0.055	0.600	0.135	0.108	0.109	0.870	0.720	0.665	0.339	0.058	0.455	0.454
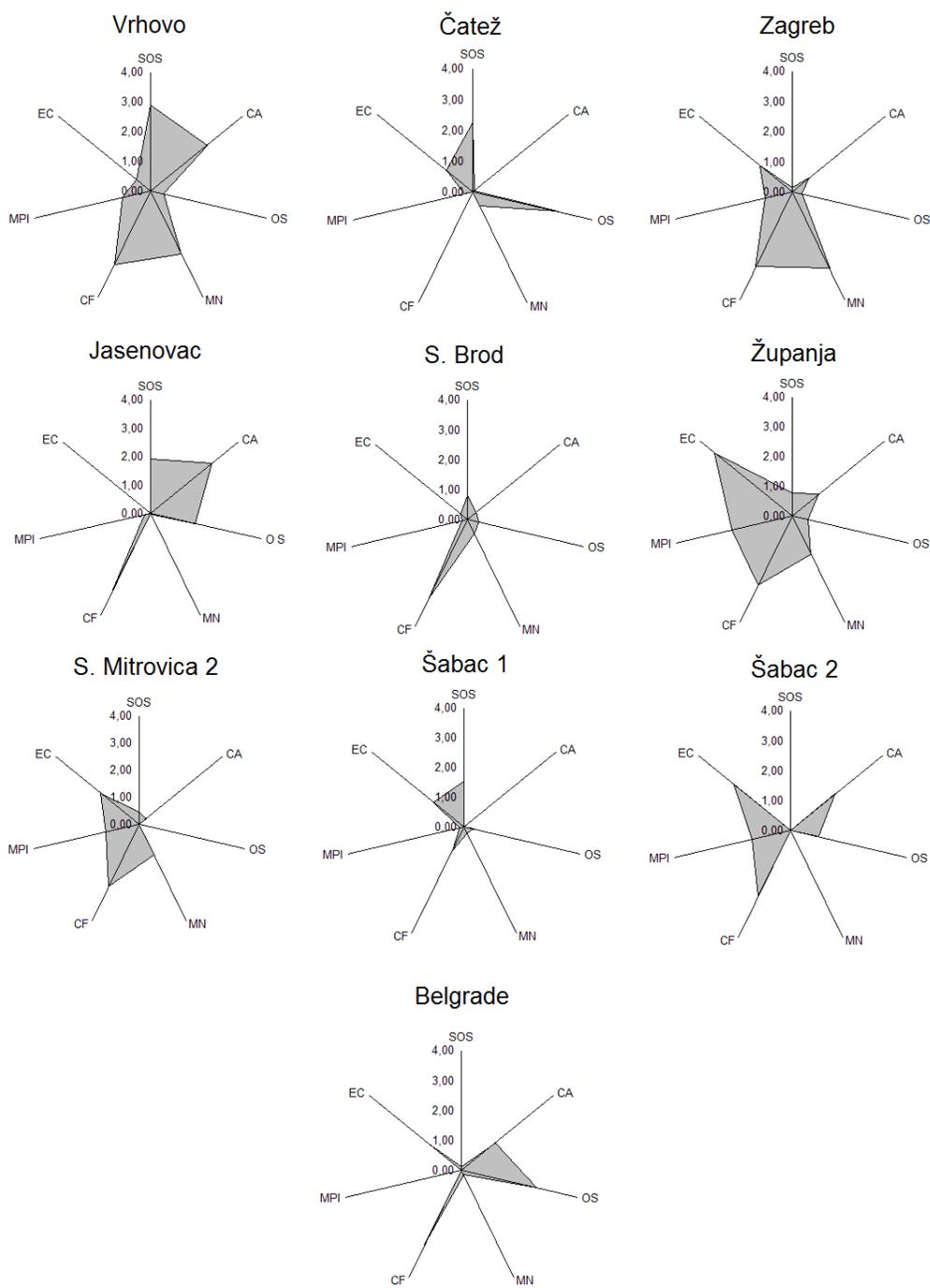
Fig. 4.11 Graphical presentation of IBR

Table 4.8 Integrated Biomarker Response (IBR) ranking of the studied sites

	Vrhovo	Čatež	Zagreb	Jasenovac	S. Brod	Županja	S. Mitrovica 2	Šabac 1	Šabac 2	Belgrade
SOS/*umuC* (+S9)	2.87	2.23	0.14	1.92	0.78	0.78	0.46	1.54	0.00	0.12
CA	2.44	0.07	0.71	2.78	0.38	1.13	0.32	0.00	1.94	1.46
OS	0.43	2.82	0.32	1.59	0.38	0.53	0.00	0.32	0.95	2.57
MN	2.36	0.52	2.80	0.02	0.53	1.43	1.25	0.22	0.00	0.18
CF	2.75	0.00	2.75	2.98	2.93	2.59	2.56	0.87	2.45	2.78
MPI	0.96	0.30	0.92	0.23	0.18	2.08	1.27	0.15	1.33	0.00
EC	0.59	1.11	1.38	0.00	0.29	3.35	1.79	1.33	2.45	1.41
IBR	**7.97**	**1.80**	**5.03**	**4.11**	**1.17**	**8.15**	**3.78**	**1.03**	**3.25**	**1.98**

DISCUSSION

4. Discussion

In this study we have performed comprehensive genotoxicological survey along the Sava River. Considering the amount of work on site and size of the research area, it was necessary to perform the preliminary research which would provide logistics for organization of the survey. Therefore we have selected the site situated nearby the city of Belgrade to carry out preliminary experiments.

4.1. Preliminary research – the Duboko site

Based on the results of previous study we have selected the Duboko site (Vuković-Gačić et al., 2014). Selected site is under the impact of two major sources of pollution: the coal processing power plant, with related fly ash disposal fields, and the wastewaters originating from the town Obrenovac. Therefore, we were focused on parameters which could provide information regarding the source of pollution.

On one side we have selected microbiological indicators of water quality and on the other as indication of industrial pollution we were focused on concentration of metals.

For assessment of **faecal pollution** we have investigated coliforms, enterococci and *C. perfrigens*. Coliform bacteria, especially *E. coli* and intestinal enterococci are considered the backbone of faecal pollution monitoring and are the most used indicator organisms. The use of total coliforms is almost abandoned in monitoring techniques, due to the fact that they are wide spread and may originate from the environment (Kolarević et al., 2011). *E. coli*, a widely used faecal indicator bacteria, is the most abundant among the other coliforms which constitute the intestinal flora of warm-blooded animals and its presence in water is primarily associated with faecal pollution (Rompré et al., 2002). Surface waters are not natural environment for enterococci so their presence is a secure sign of faecal pollution (Cabral, 2010). Thus, more precise indicators of the faecal pollution presence are faecal (thermotolerant) coliforms and intestinal enterococci (faecal streptococci), especially in monitoring the recreational water quality. *C. perfringens*, faecal bacteria which form spores is proved to be an important organism for detection of remote and persistent faecal

pollution or pollution originating from the past. This sulphite-reducing, anaerobic bacterium is found in the large intestine and represent around 0.5% of faecal microflora. It is suggested that *C. perfringens* should be used for monitoring the fate of pathogens after they are released in the aquatic environment (Bitton, 2005).

At the investigated site presence of faecal pollution was evident during both sampling seasons. Number of *E .coli* and enterococci indicated critical level of pollution. Observed findings are in compliance with the results obtained previously (Vuković-Gačić et al., 2014). Similar level of pollution was detected in both seasons based on the numbers of total coliforms and *C. perfringens*.

Concentration of metal and metalloids has been monitored as indication of **industrial pollution**. Moreover, based their concentration, pollution can be traced to ash disposal field of power plant "Nikola Tesla" according to the study study of Kostiç et al. (2012). In the study Sunjog et al. (2014) performed on European chub (*Squalius cephalus*) detected genotoxic pollution in the Kolubara River Basin nearby the Duboko was most likely related to surrounding coal processing facilities. Concentrations of metals have been shown to be important metadata for interpretation of ecogenotoxicological results (Kolarević, 2014; Sunjog, 2016; Kračun-Kolarević, 2017).

As for the faecal pollution, similar pollution level based on metal concentration was noticed during both sampling seasons. The only noteworthy difference was observed for concentration of Zn, which was 4 times higher in winter.

4.1.1 Differential DNA damage response of selected organisms

Preliminary research was carried out in organisms belonging to different trophic levels. The study was performed on *U. tumidus* as the representative of mussels and two bream species as the representatives of fish. In our previous study, we have demonstrated the applicability of comet assay in haemocytes of *U. tumidus* for detection of genotoxic potential *in situ* (Vuković-Gačić et al., 2014), while ecotoxicological studies indicated that bream specimens could be used for biomonitoring, as they show accumulation of pollutants

and physiological and molecular responses to pollution in the environment (Pavlov et al., 1992; Jedamskigrymlas et al., 1995; Jovičić et al., 2014). Moreover, our study performed in freshwater bream (*Abramis brama*) indicated high sensitivity of this organism in detection of genotoxic potential *in situ* (Kostić et al., 2016). It was important to us to investigate the level of the response of organisms in relation to different sampling season. Previously, it was demonstrated that seasonal differences exist in response of both freshwater mussels and fish. The study of Kolarević et al. (2013) indicated possible increased sensitivity of mussels (*Sinanodonta woodiana*) during summer season mainly driven by water temperature. Similarly, our study performed on freshwater bream also suggested seasonal variation of response where the highest response was detected in summer (Kostić et al., 2016).

Indeed, for both organisms, response in sense of the level of DNA damage was much higher in summer season. In winter season, response of the mussels and fish was within the similar range, while in summer, response was much higher in fish. Observed differential response in the studied organisms was expected taking into consideration the level of exposure of each of the selected species in terms of mobility, nutrition, filtration rate and defense mechanisms. Although there are no ecogenotoxicological studies dealing with comparative response among the species *in situ*, the results of numerous *ex situ* studies are in favour of observed differences. The values of LC_{50} detected in toxicological studies, or LOEC (the lowest observable effective concentration) values in ecogenotoxicological studies dealing with the various pollutants, differ significantly depending on the chosen bioindicator. For instance, the study of Labrot et al. (1999) demonstrated that LC_{50} values obtained after acute exposure to Pb differ in few orders of magnitude for mussels compared to fish. Further on, the study of Kim and Hyun (2006) performed on carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*), and clam (*Spisula sachalinensis*), clearly demonstrated differential response of selected species during *ex situ* exposure to direct-acting mutagen, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), and an indirect mutagen, benzo[*a*]pyrene. In the mentioned study response of the organisms was examined by application of the comet assay and formation of micronuclei. The situation is even more

complex in studies performed *in situ*, considering that animals are exposed to mixture of substances. In our paper Aborgiba et al. (2015) we have indicated that the source of pollution has strong influence on the level of response in selected species.

4.1.2 Cryopreservation

One of the major concerns in organization of the Sava River Survey was the performing the cell viability assay and comet assay directly upon sampling. Organization of such kind of field laboratory would be rather difficult and other strategies have been taken into consideration. Best solution has been seen in cryopreservation as this methodology has been already successfully used in our laboratory on fish blood samples (Kračun-Kolarević et al., 2016). Major issue which might occur within cryopreservation is reduction of cell viability which might influence the results of the comet assay. Therefore we have studied the effects of cryopreservation on few events: cell viability, DNA damage and occurrence of highly damaged nuclei or so called hedgehogs which by some authors might occur as the result of apoptosis (Olive et al., 1993; Hartmann and Speit, 1997; Collins, 2004).

Regarding the invertebrates, literature data that deals with cryopreservation of haemolymph is scarce. We have decided to follow protocol of the study Kwok et al. (2013) performed on marine mussels (*Mytillus* sp.) modified based on the finding of the study of Gačić et al. (2014) regarding the establishing the primary culture of haemocytes. Our results indicated that cryopreservation reduces viability of haemocytes for 20% which results in increase of DNA damage and frequency of the hedgehogs.

Situation was much better for fish blood samples which were also cryopreserved by immediate immersion in liquid nitrogen. This kind of sample processing is indicated as reliable for assessment of DNA damage by numerous studies (Visvardis et al., 1997; Duthie et al., 2002; Recio et al., 2012, Kračun-Kolarević et al., 2016). Prior to the survey we have performed preliminary analyses in the blood samples of bleak. We have decided to shift from bream to minnow species as more appropriate considering that the findings of the study Simonović et al. (2015) indicated that minnow species can be found in upper, middle and lower stretch of the Sava River. The results indicated that cryopreservation reduces cell

viability while the level of DNA damage was within the same range as in the fresh sample which is in compliance with the results of Akcha et al. (2003). However, considering that cryopreservation affects cell viability, we wanted to investigate possible interference of apoptosis on the comet assay results in samples collected during survey. The significant correlation was observed between the level of cell viability and hedgehogs frequency. However, it is important to emphasize that TI% values obtained in comet assay did not show a correlation with the cell viability or the frequency of hedgehogs and therefore data on these parameters were studied independently.

4.1.3 Outcomes of the preliminary research

To summarize, outcomes of the preliminary research gave us clear instructions to be followed for the Sava River Survey. Based on the results we have chosen summer season for period in which the survey should be carried. Considering higher response in comparison with mussels, fish were favored as organism for bioindication. This choice was supported by the results of cryopreservation which was more successful in fish blood in comparison with mussel's haemolymph. Finally, additional drawback in usage of mussels in the Sava River Survey was absence of these organisms in upper stretch based on literature data (Lucić et al., 2015). Besides the comet assay which assess the level of DNA damage as biomarker of exposure, additional tests should be included which will give wider image of detected genotoxic potential.

4.2 The Sava River Survey

Having in mind aforementioned we have decided to perform the Sava River Survey in September 2015. We have chosen 12 sites used in routine monitoring program of the countries in the basin. Physico-chemical, chemical and microbiological data were used for assessment of pollution pressures at the sites. Fish species *A. alburnus* and *A. bipunctatus* were used as bionindicators. Mutagenic potential in water samples was assessed in prokaryotic system by SOS/*umuC* in *S. typhimurium* 1535 pSK1002. Comet assay and Fpg-modified assay and micronucleus test were performed in cryopreserved fish blood samples.

When constructing the battery of bioassays we were focused on types of assays which have already been employed earlier in ecogenotoxicological studies of the Sava River (Klobučar et al., 2003, 2012; Kopjar et al., 2008; Pavlica et al., 2001; Vuković-Gačić et al., 2014, 2015; Aborgiba et al., 2015; Krča et al., 2007). Also, we have used experience gained during the Joint Danube Survey 3 – JDS3 (Kirschner et al., 2015; Deutschmann et al., 2016; Kolarević et al., 2016; Kittinger et al., 2015) for updating the assay list as usage of the same methodology would enable comparison of water quality of the Sava and Danube in further research. As the assays employed in the study showed different sensitivity in detection of the stressors at investigated sites we have finally employed IBR approach to identify the most critical spots on the river but also to recommend which site has potential to be a reference site for Sava River.

4.2.1 Indication of presence of wastewaters

The whole Sava River Basin is receiving high amounts of untreated or improperly treated wastewaters originating from the various size settlements that lie on the banks of the Sava River and its tributaries (Kapetanović et al., 2015). Therefore, our primary goal was identification of hotspots of contamination with wastewaters as possible sources of genotoxic pollution. Same as in the preliminary research for assessment of **faecal pollution** we have investigated coliforms, enterococci and *C. perfrigens*. We have observed variation in water quality along the river where the sites Županja, S. Mitrovica 2 and Šabac 2 are recognized as hotspots of faecal pollution. Moreover, wastewaters discharge points were found at the sites Zagreb (WWZ), S. Mitrovica (WWSM) and Šabac (WWS). Samples collected from the WW discharge points at the sites S. Mitrovica and Šabac indicated excessive faecal pollution affecting water quality at downstream situated sites (S. Mitrovica 2 and Šabac 2 respectively). Low water level during sampling facilitated detection of the outlets which could not have been identified within previous survey of the river performed by our research group in 2014 (Vrzel et al., 2016). As an indication of wastewater effluents, we have decided to use the numbers of *E. coli* in water. The results of the JDS3 confirmed that this parameter and concentration of caffeine in the Danube River are the most reliable indicators of contamination related to wastewaters (Bahlmann et al., 2015; Kirschner et al.,

2015). It was quite worrying fact that the long term monitoring data available for the impacted sites shows persistent pollution at few year bases (Vrzel et al., 2016).

Besides the identification of the hotspots of faecal pollution it was also of utmost importance to identify the source of pollution as the source of pollution could be crucial for interpretation of genotoxicological data. Also, specific differentiation between sources of fecal contamination is of importance because the risk to humans is usually considered higher from human faecal contamination (sewage) than from animal faecal contamination. New approaches based on PCR quantification and biomarker analysis are used to track microbiological sources of contamination. The human-associated fecal marker BacHum (Kildare et al., 2007) and a recently modified version of the HF183II (Green et al., 2014) are used to track human associated faecal pollution, while the ruminant-associated BacR qPCR assay (Reischer et al., 2006) and the pig-associated Pig2Bac qPCR assay (Mieszkin et al., 2009) are the methods for detecting animal faecal pollution sources.

Therefore, DNA samples extracted from water samples collected at the sites were sent to Technical University in Vienna, Austria for the Microbial source technique (MST) analysis. This quantitative PCR (qPCR)-based assay used for the analysis of general-, human-, wastewater-, or animal-associated genetic *Bacteroidetes* faecal markers have gained increasing popularity in the field of faecal pollution analysis and microbial source tracking during recent years (Mayer et al., 2016). The human-associated faecal markers BacHum and HF183II were detected in all samples while the ruminant-associated BacR and the pig-associated Pig-2-Bac markers were not detected in any of the samples. Significant correlation was observed between the standard indicators (coliforms and enterococci) and human associated faecal markers. This is consistent with previous studies that support the fact that faecal pollution based on qPCR quantification can be performed with at least equal precision compared with traditional ISO-based cultivation techniques (Stapleton et al., 2009; Mayer et al., 2016). The lowest correlation was observed with numbers of *C. perfringens*. Among the indicators studied, CP numbers show the lowest degree of variation along the river, as reported earlier (Byamukama et al., 2005).

To sum up, our results indicated presence of faecal pollution hotspots along the Sava River, pollution source is human related and the longevity of pollution at those sites is persistent.

4.2.2 Indication of possible industrial pollution

Presence of industry related pollution was assessed based on available data on concentration of metals and metalloids in water and fish. Since the concentrations of these parameters in water were quite low, we were focused on concentrations measured in tissues as more relevant. Recent studies regarding metals and metalloids contamination in the Sava River were based on the European chub (*Squalius cephalus*) (Marijić et al., 2012, 2013; Dragun et al., 2015). We have used the data available for bleak as this fish was used for estimation of metals and metalloids in other studies (Uysal et al., 2009; Petkovšek et al., 2012; Mercai et al., 2014). The highest industrial pollution based on MPI was observed for the Županja sampling site and lowest for the Belgrade sampling site. This result is in accordance with Dragun et al. (2009) who previously reported that Sava River water reflects a certain anthropogenic impact in the Croatian section of the river while. On the other hand, Vuković et al. (2011) stated that the industrial activity in Serbia slowed down during the past two decades, which is the reason for the weakly noticeable anthropogenic input of heavy metals in the Sava River system (water and sediment) from the nearby environment.

Although we have assessed the presence of wastewaters by two aspects (indicators of faecal pollution and industrial pollution) it is important to emphasize that significant correlation was observed between the numbers of *E. coli* and MPI clearly showing that at the majority of the sites pollution from a single source prevails.

When making the parallel between the data on metal accumulation in tissue and the data from genotoxicological assays, we would emphasize that the positive correlation was observed between the concentration of mercury and TI% values. Mercury is listed as a priority substance by Directive 2008/105/EC whose genotoxic potential in aquatic environment is well known (Pereira et al., 2010). Negative correlation was detected

between the oxidative stress and concentration of copper which is understandable as copper can have a protective effect as a constitutive of superoxide dismutase (Gaetke and Chaw, 2003). Correlations which were not significant were not taken into consideration because of the possible causative effect.

4.2.3 Assessment of genotoxic potential along the river

4.2.3.1 An overview of the selected bioassays

The dataset obtained in our study represents only the so called snapshot of the current status of the river; therefore, we have chosen bioassays which would enable detection of the effects of recent genotoxic pollution and the effects of the prolonged exposure to pollution as well.

4.2.3.1.1 Mutagenicity of water samples

The SOS/*umuC* was included in our research as the study of Žegura et al. (2009) and Kittinger et al. (2013) indicated the assays high potency in detection of genotoxic potential in wastewaters and surface waters. We have assumed that the assay would provide preliminary screening of genotoxic potential, but the induction ratio has not exceeded value 1.5 (threshold value) in any of the investigated samples. Similarly, in the study of Kittinger et al. (2015) performed on the water samples of the Danube River, only four sites out of 68 investigated within the JDS3 showed mutagenic potential, indicating either low sensitivity of the assay or low genotoxic potential of water.

4.2.3.1.2 Genotoxic effects in fish

As the SOS/*umuC* provides only information on genotoxic potential of water sampled in particular moment, we have decided to evaluate the effect of prolonged exposure in aquatic animals inhabiting the studied sites. In our previous research we have shown that various fish species can be used as reliable bioindicators for the detection of genotoxic pollution (Sunjog et al., 2012, 2014). In our current study spirlin and bleak were the most convenient option due to reasons indicated before and moreover, during the JDS3

we managed to cover over 2,000 rkm of the Danube with bioassays in blood of bleak (Deutschmann et al., 2016).

Comet assay was employed to detect DNA damage occurred recently; this method is the most commonly used for assessment of pollution related genotoxicity in aquatic organisms (Dixon et al., 2002). We have introduced Fpg - modified comet assay which additionally detects the DNA damage caused by oxidative stress in specimens as the oxidative stress is identified as the major contributor to DNA damage in the majority of studies dealing with aquatic environments. Generally, the mode of action of priority substances in environment is based on generation of reactive oxygen species (Mitchelmore and Chipman, 1998). As we have not observed correlation between the standard comet assay and frequency of 8-oxoG sites we can speculate that oxidative stress is not the only or major contributor of the detected gentoxic potential. Finally, micronucleus assay was used for the detection of permanent damage indicating presence of potential clastogenic and/or aneugenic agents. Consequently, the standard and Fpg-modified comet assay showed higher potential in differentiation of the sites based on genotoxic potential in comparison with micronucleus assay and SOS/*umuC* test.

4.2.3.2 Genotoxic potential along the river and overview of the literature data

4.2.3.2.1 The upper Sava (Slovenia)

In the Slovenian stretch of the river, the highest genotoxic potential was detected at the site Vrhovo. At this site, we have observed a significant increase of TI% values in comparison to the reference site accompanied with the highest MN frequency. Increased induction ratio in SOS/*umuC* also points to possible mutagenicity. This site was not identified as a hotspot of faecal pollution, but we have noticed metal contamination by the highest concentrations of Cr, Cu and Zn in fish tissue, when compared to other sites. Moreover, the IBR rank of the site was among the highest. The study of Källqvist et al. (2008) indicated that pore-water samples from the site Vrhovo had several fold higher toxic effect (algae growth inhibition test) in comparison with other sites on the Sava River. Moreover, the study of Milačič et al. (2010) indicated that sediment at this site contains

high concentrations of metals (Ni, Zn, Cu, Cd) which can have considerable genotoxic potential (Kitchin and Ahmad, 2003).

Going further downstream, the situation is completely different at the site Čatež. In comparison with the reference site, there was no difference in the level of DNA damage measured by micronucleus or comet assay. This site caught our attention as a possible reference site on the basis of available genotoxicological literature data. As mentioned before, the section of the river stretch from the Slovenian-Croatian border to the confluence of the Una River is the most studied part. In the study of Pavlica et al. (2011) the area nearby the site Čatež (a few km downstream) was used as a reference site for the assessment of genotoxicity along the Sava River by bioassays performed in European chub (*Squalius cephalus*). The same group of authors also confirmed low level of genotoxic pollution in the mentioned area in their study performed on zebra mussel *Dreissena polymorpha* (Klobučar et al., 2003). However, at this site we have detected the highest level of oxidative stress which can be linked to the highest concentration of As measured in tissue (Kitchin and Ahmad, 2003).

4.2.3.2.2 The middle Sava (Croatia)

Zagreb (750,000 inhabitants) is the largest urban settlement situated in the middle stretch of the Sava River. In previous studies performed in the Croatian stretch of the river, major focus was placed on this site as a greatest source of pollution. The studies of Klobučar et al. (2003, 2012) and Pavlica et al. (2011) indicated presence of genotoxic pollution by comet and micronucleus assay in various aquatic organisms (fish, crayfish, mussels). Our results indicated that environmental quality of water has improved when compared to the data obtained in the period prior to implementation of wastewater treatment facility. None of the applied bioassays has indicated increase of genotoxic potential in comparison to upstream situated Čatež. By the numbers of *E. coli*, this site was not recognized as a hotspot of faecal pollution, but still the highest concentrations of Cd and Pb in fish tissue were recorded there.

Surprisingly, among the studied sites, the highest values of TI% were detected at the site Jasenovac. The site is characterized by low level of faecal pollution but with the highest concentration of mercury in the fish tissue. In the middle section, the site with the highest rank by IBR value was Županja. The site was identified as hotspot of faecal pollution with the highest value of MPI (the highest concentration of Al, Fe, Ni, Mn, Se). Comparing to the site with the lowest IBR value (Šabac 1), significant increase of DNA damage measured by the standard comet assay was observed. Observed genotoxic potential is in compliance with data of our previous study performed on mussels (*Unio* sp.) on the site situated close to Županja (2 km upstream) (Vuković-Gačić et al., 2015).

4.2.3.2.2 The lower Sava (Serbia)

In Serbian stretch we have investigated the impact of the largest settlements situated on the river banks: Sremska Mitrovica (40,000 inhabitants), Šabac (50,000 inhabitants) and Obrenovac (50,000 inhabitants). Data of our previous study indicated presence of genotoxic potential at these sites (Vuković-Gačić et al., 2015). In this section, town Šabac had the highest impact on water quality. While the site situated upstream (Šabac 1) had the lowest IBR value among all studied sites, situation was quite different at the site downstream the wastewater outlets (Šabac 2) which was identified as a hotspot of faecal pollution with evident indications of genotoxic potential by both standard and Fpg - modified comet assay. The site Belgrade is situated upstream of the urban area of Belgrade city. This site is mainly under the impact of upstream situated city Obrenovac and associated settlements. At this site we have detected a significant increase of DNA damage measured by TI% and increased levels of oxidative stress in comparison with the reference site. These findings are in compliance with our previous studies performed on mussels, worms and fish collected in this area (Vuković-Gačić et al., 2013; Aborgiba et al., 2016).

This study provides a valuable and complex set of data on genotoxic potential of the Sava River obtained from a single source which enables detection of the effects of genotoxic pollution on different levels. Result indicated differential sensitivity of applied bioassays in detection of genotoxic pressure. The standard and Fpg-modified comet assay

showed higher potential in differentiation of the sites based on genotoxic potential in comparison with micronucleus assay and SOS/*umuC* test. Our data represent snapshot of the current status of the river which indicates the presence of genotoxic potential along the river which can be traced to the deterioration of quality of the Sava River by communal and industrial wastewaters.

CONCLUSIONS

6. Conclusions

Based on the results of this thesis following conclusions can be drawn:

The outcomes of the preliminary research at the site Duboko:

- The results of the preliminary study were essential for the organization of the whole river survey.

- Physico-chemical, chemical and microbiological indicators measured at the Duboko site showed a similar level of pollution during both sampling seasons. According to the national legislation water quality at the selected site was within classes III and IV. Numbers of *E .coli* and enterococci indicated critical level of pollution.

- Freshwater mussels and fish showed response to presence of genotoxic pollution at the site and both organisms showed a higher response to pollution in the summer season suggesting that a period of year with higher temperatures should be considered for execution of the whole river survey.

- Having in mind the size of the study and sampling schedule, cryopreservation of tissues needed for genotoxicological analyses was taken into consideration. Negative effects of cryopreservation on cell viability and DNA damage were more evident in mussels in comparison with fish.

- Taking into account these results together with the distribution of used organisms throughout the Sava River Basin, fish species should be chosen for the Sava River Survey.

The outcomes of the whole river survey:

- According to the national legislation water quality based on basic physico-chemical parameters at selected sites varied within II and III class.

- The data revealed that the concentrations of metals in water were low in comparison to values characteristic for the Sava River, based on available literature.

- The metal pollution index indicated that the least polluted site was Čatež while the highest concentrations of metals were detected in Belgrade. Only As and Ni had an increasing trend in concentration values in the lower stretch of the river when compared to the upper stretch.

- The highest concentrations and number of metals were recorded in specimens of fish from Županja sampling site while the lowest concentrations of metals and metalloids were determined at the Belgrade sampling station. Subsequently the highest MPI was calculated for Županja sampling station and the lowest for the Belgrade sampling station.

- Microbiological indicators showed that the majority of the river samples were slightly and moderately polluted (class I and II).

- Wastewaters discharge points were found at the sites Zagreb, S. Mitrovica and Šabac. Samples collected from the WW discharge points at the sites S. Mitrovica and Šabac indicated excessive faecal pollution affecting water quality at downstream situated sites. Therefore, the sites Županja, S. Mitrovica 2 and Šabac 2 are recognized as hotspots of faecal pollution.

- Significant correlation was observed between the numbers of *E. coli* and MPI clearly showing that at the majority of the sites pollution from a single source prevails.

- In the tested water samples cytotoxic and mutagenic effects were not recorded in any case.

- The most appropriate fish species for carrying out the research along the Sava River were minnow species (*A. alburnus* and *Alburnoides bipunctatus*)

- Average cell viability in cryopreserved fish blood samples was about 80% with significant negative correlation observed between the cell viability and HH frequency.

- The sites with the highest levels of DNA damage in alkaline comet assay were Litija, Vrhovo, Jasenovac and Šabac 2. Impact of wastewater discharges was evident at the site Šabac 2 which had significantly higher TI% values in comparison with upstream situated Šabac 1.

- In all cases digestion with Fpg - enzyme led to a significant increase of DNA damage in comparison with corresponding standard alkaline and buffer treated control. Significant correlation was observed between the mean TI% values obtained from standard alkaline comet assay and slides exposed to Fpg – enzyme

- The highest frequency of MN was detected in specimens collected at the sites Vrhovo and Zagreb

- Based on the Integrated Biomarker Response the site Šabac 1 had the lowest IBR rank while the Županja and Vrhovo had the highest ranks.

- This study provides a valuable and complex set of data on genotoxic potential of the Sava River obtained from a single source which enables detection of the effects of genotoxic pollution on different levels.

- Result indicated differential sensitivity of applied bioassays in detection of genotoxic pressure. The standard and Fpg-modified comet assay showed higher potential in differentiation of the sites based on genotoxic potential in comparison with micronucleus assay and SOS/*umuC* test.

- This study represent a snapshot of the current status of the river which indicates the presence of genotoxic potential along the river which can be traced to the deterioration of quality of the Sava River by communal and industrial wastewaters.

LITERATURE

7. Literature

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SUPPLEMENTARY
MATERIAL

Supplementary material 1

Table 1 Values of basic physico-chemical parameters measured in water at the sampling site Duboko (Data Source - IBISS)

Month	T (C°)	O_2 (mg/L)	O_2 (%)	pH	Cond. (μS)	NO_2 (mg/L)	NO_3^- (mg/L)	NH_4^+ (mg/L)	PO_4^{3-} (mg/L)
Winter	8.8	11.3	99	8.5	355	0.063	5.46	1.532	0.32
Summer	21.6	9.7	109	8.6	x	0.060	1.46	0.534	0.22

Table 2 Concentrations of heavy metals and metalloids in water samples at the site Duboko (Data source - Serbian Environmental Protection Agency)

	Concentrations of metals and metalloids in water in µg/L																			
	Total												Dissolved								
Season	Fe	Mn	Zn	Cu	Cr	Pb	Cd	Ni	Al	Co	As	B	Fe	Mn	Zn	Cu	Cd	Ni	Al	As	B
Winter	524.4	30.4	15.9	5.4	2.0	1.6	0.06	2.0	358.4	0.6	1.3	19.3	107.6	12.9	7.6	1.9	0.04	0.8	122.75	1.2	19.3
Sumer	402.5	67.6	10.4	5.1	1.6	2.7	0.06	3.9	276.1	1.2	2.2	26.2	72.2	26.7	1.8	1.1	0	2.1	59.3	1.7	18.9

Supplementary material 2

Table 1 Physico-chemical parameters measured in water at sampan sites along the Sava River. Values represent average of 5

measurements. Data provided by IBISS, Belgrade, Serbia

	pH	t (C°)	conductivity (µS)	TDS (ppm)	O_2 (%)	O_2 (mg/L)	NO_2	NO_3	NH_4
Litija	7.6	17.7	425.6	213.0	87.8	8.2	0.13	2.42	<0.05
Čatež	7.8	20.2	425.0	217.0	86.6	7.6	0.03	2.38	0.086
Vrhovo	7.8	23.1	429.6	217.0	102.0	8.6	0.03	3.50	0.042
Zagreb	7.8	22.7	464.0	230.8	102.2	9.1	0.03	3.06	<0.05
Jasenovac	7.0	23.8	466.0	234.4	65.2	5.5	0.11	3.08	<0.05
S. Brod	7.2	24.7	467.6	236.0	91.2	7.5	0.03	3.80	<0.05
Županja	7.5	24.7	587.6	294.8	75.6	6.2	0.03	3.02	0.05
S. Mitrovica1	7.4	22.6	423.6	212.4	85.4	7.3	0.04	3.16	0.05
S. Mitrovica2	7.0	22.4	389.0	193.8	83.9	7.1	0.02	2.66	<0.05
Šabac 1	7.4	22.3	430.8	216.0	83.4	7.0	0.02	2.98	<0.05
Šabac 2	x	x	x	x	x	x	0.02	1.18	<0.05
Beograd	7.0	24.0	392.8	199.2	88.0	7.4	0.02	2.88	0.088

x-not measured

Table 2 Determination of soluble concentrations of elements in filtered (0.45 µm) water samples of the Sava River determined

by ICP-MS. Measurement uncertainty better than ± 2%. Data provided by Jožek Štefan Institute, Ljubljana, Slovenia

Sampling site	Cr (ng/mL)	Mn (ng/mL)	Fe (ng/mL)	Co (ng/mL)	Cd (ng/mL)	As (ng/mL)	Cu (ng/mL)	Ni (ng/mL)	Pb (ng/mL)	Zn (ng/mL)	Se (ng/mL)	Hg (ng/mL)*	MPI
Vrhovo	0.38	0.27	7.03	0.12	0.001	0.61	1.01	0.86	0.11	1.41	0.09	0.28	0.27
Čatež	0.29	0.39	1.40	0.08	0.001	0.57	0.90	0.47	0.06	0.83	0.07	0.46	0.20
Zagreb	0.27	0.34	2.93	0.12	0.005	0.69	1.21	0.51	0.11	1.02	0.07	0.29	0.27
Jasenovac	0.15	3.66	3.29	0.08	0.007	1.14	1.05	0.68	0.05	1.79	0.13	0.31	0.35
S. Brod	0.22	0.39	16.9	0.11	0.001	1.25	1.11	5.73	0.04	0.71	0.07	0.22	0.30
Županja	0.23	0.43	29.4	0.08	0.001	1.49	1.12	2.80	0.04	0.33	0.11	0.13	0.28
S. Mitrovica 2	0.28	0.26	1.59	0.04	0.001	1.51	0.90	1.38	0.02	0.60	0.08	0.34	0.19
Šabac 1	0.31	1.54	2.57	0.04	0.001	1.56	0.85	1.15	0.04	0.21	0.08	0.25	0.22
Belgrade	0.28	7.42	27.2	0.05	0.015	1.91	1.68	1.33	0.05	2.18	0.10	0.27	0.53

*Hg concentration was determined from the whole water sample

For the determination of the soluble element content in water samples from the Sava River, samples were filtered through a 0.45

µm filter and concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS). For the

determination of the total Hg concentration, all water samples were analyzed to prevent losses of dissolved gaseous Hg during

the filtrations.

Table 3 The average element concentrations in whole body composite (wbc) samples of *A.alburnus* and *A. bipunctatus* (marked with *) in mgkg^{-1} ww, and MPI values for each sampling station; Data provided by Faculty of Natural Sciences, Kragujevac, Serbia

Sampling station/ Elements	Vrhovo *	Čatež *	Zagreb	Jasenovac	Slavonski Brod	Županja	Sremska Mitrovica	Šabac 1	Šabac 2	Belgrade
Al	5.49 ± 5.72	3.31 ± 0.18	2.92 ± 2.42	4.33 ± 3.26	2.08 ± 0.88	21.12 ± 28.08	13.49 ± 19.83	5.83 ± 3.76	10.15 ± 10.94	1.79 ± 0.93
As	0.008 ± 0.011	0.15 ± 0.06	0.12 ± 0.025	0.053 ± 0.037	0.13 ± 0.09	0.067 ± 0.059	0.051 ± 0.048	0.066 ± 0.069	0.065 ± 0.036	0.1 ± 0.086
Cd	0.008 ± 0.004	0.008 ± 0.002	0.1 ± 0.002	0.007 ± 0.001	0.016 ± 0.008	0.002 ± 0.002	0.0018 ± 0.0016	0.001 ± 0.001	0.008 ± 0.007	0.013 ± 0.008
Co	0.0009 ± 0.002	0.0007 ± 0.0006	0.001 ± 0.0007	0.0001 ± 0.0004	0.0008 ± 0.0004	0.02 ± 0.03	0.009 ± 0.016	0.002 ± 0.004	0.003 ± 0.007	0.0006 ± 0.0004
Cr	0.35 ± 0.13	0.17 ± 0.022	0.18 ± 0.045	0.224 ± 0.06	0.154 ± 0.024	0.25 ± 0.093	0.198 ± 0.109	0.17 ± 0.024	0.18 ± 0.024	0.18 ± 0.02
Cu	0.9 ± 0.33	0.48 ±0.05	0.67 ± 0.12	0.61 ± 0.15	0.68 ± 0.34	0.8 ± 0.39	0.76 ± 0.38	0.49 ± 0.072	0.78 ± 0.39	0.44 ± 0.14
Fe	21.5 ± 11.52	9.74 ± 3.2	14.5 ± 8.27	13.79 ± 5.05	11.44 ± 6.67	27.12 ± 25.45	19.08 ± 19.36	11.01 ± 3.43	14.88 ± 9.26	8.76 ± 3.44
Hg	0.08 ± 0.35	0.02 ±0.004	0.013 ± 0.003	0.102 ± 0.059	0.011 ± 0.003	0.028 ± 0.014	0.027 ± 0.019	0.041 ± 0.01	0.037 ± 0.027	0.011 ± 0.004
Mn	4.63 ± 2.49	1.37 ± 0.48	1.55 ± 0.71	2.85 ± 0.89	1.2 ± 0.47	5.34 ± 3.4	4.78 ± 3.73	3.37 ± 1.20	4.79 ± 2.9	1.73 ± 0.72
Ni	0.03 ± 0.018	0.02 ± 0.004	0.032 ± 0.033	0.027 ± 0.013	0.034 ± 0.01	0.155 ± 0.137	0.126 ± 0.096	0.047 ± 0.033	0.06 ± 0.03	0.025 ± 0.015
Pb	0.037 ± 0.042	0.11 ±0.02	0.17 ± 0.03	0.024 ± 0.027	0.1 ± 0.01	0.057 ± 0.076	0.056 ± 0.06	0.014 ± 0.014	0.077 ± 0.12	0.11 ± 0.26
Se	0.93 ± 0.17	0.24 ±0.06	0.34 ± 0.066	0.27 ± 0.052	0.11 ± 0.025	0.3 ± 0.06	0.27 ± 0.039	0.28 ± 0.021	0.28 ± 0.032	0.085 ± 0.04
Sn	0.028 ± 0.028	0.016 ±0.0007	0.016 ± 0.0008	0.023 ± 0.032	0.015 ± 0.002	0.04 ± 0.058	0.02 ± 0.013	0.009 ± 0.003	0.025 ± 0.026	0.016 ± 0.002
Zn	47.67 ± 9.73	24.74 ± 7.2	24.47 ± 6.6	28.37 ± 6.26	23.48 ± 4.82	36 ± 10.14	34.47 ± 5.98	35.42 ± 13.87	38 ± 11.48	23.09 ± 6.65
MPI	0.23	0.17	0.23	0.16	0.16	0.33	0.26	0.16	0.26	0.14

Assessment of concentration of metals and metalloids in fish tissue

Each individual separately was grinded in a Laboratory homogenizer Sterilmixer (International P.B.I. S.p.A.) and whole body composite (wbc) samples were weighed using an electronic balance (± 0.1 g) and stored at -20 °C prior to analysis.

Wbc samples were submitted to the Analytical Chemistry Laboratory within the Institute of Chemistry at the Faculty of Chemistry, University of Belgrade, Serbia, for chemical analysis. The element concentrations (Al, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Sn and Zn) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES), using a Thermo Fisher Scientific iCAP 6500 Duo ICP (Cambridge, United Kingdom) equipped with a RACID86 Charge Injector Device (CID) detector, concentric type nebulizer, quartz torch, and alumina injector. Fish samples (~1.5 g) were dried in a lyophilizer (Christ Alpha 2-4 LD, Harz, Germany), and then digested in an Advanced Microwave Digestion System (ETHOS 1, Milestone, Italy) using a mixture of 65% nitric acid and 30% hydrogen peroxide (Suprapur®, Merck, Darmstadt, Germany, 10:2, v/v) at 200ºC for 20 min. After cooling to room temperature and without filtration, the solution was diluted to a fixed volume (volumetric flask, 25 ml) with ultra-pure water with a conductivity of 0.055 µS/cm (Barnstead™ GenPure™ Pro, Thermo Scientific, Germany), before being analyzed by ICP-OES.

Blanks with no fish tissue were run with each batch of samples to monitor contamination by the reagents used. The standards for the instrument calibration were prepared on the basis of the multi-element (SS-Low Level Elements ICV Stock, 10 mg/L) and mono-element (Hg Calibration Stock, 10 mg/L Hg; Sn Calibration Stock, mg/L Sn) certified reference solutions ICP Standard (VHG Labs, Inc-Part of LGC Standards, Manchester, NH

03103 USA) and analyzed to support quality assurance and control. The muscle standard reference material (DORM-4; National Research Council of Canada) was digested in triplicate and analyzed to support quality assurance and control.

Mean values and standard deviations were calculated for each group and elements concentrations were expressed as $mgkg^{-1}$ wet weight (ww). Differences in mean concentrations of elements in fishes from different sampling stations were analyzed by non-parametric Kruskal-Wallis test. Post hoc inter-group comparisons of element levels (between pairs of sampling stations) were performed by the non-parametric Mann-Whitney test for 2 independent samples. Statistical analysis of data was carried out using SPSS 16.0 statistical package programs for Windows (SPSS Inc., Chicago, IL, USA).

The metal pollution index (MPI) was calculated to compare the total metal content in the different sampling stations using the following equation:

$$MPI = (cf_1 \times cf_2 \times cf_3 \times \cdots cf_n)^{\frac{1}{n}} \quad \text{Equation 1}$$

where Cf_n = concentration of the metal n in the sample.

Biography

Mustafa Salim Aborgiba was born 01.01.1964. in Zliten, Libya. He obtained bachelor diploma at the University of Zauia, Libya at the College of Basic Science in 1991. At the Faculty of Science of University Putra Malaysia he obtained master degree Master of Environment in 2004. Since 2004, he has been employed as University Staff Member at the Higher Institute students and Colleges. In period 2006 to 2008 he is working as Environmental Adviser at the Ministry of Environment in Libya while in period 2008 to 2012 he is also Environmental Expert accredited to the Judicial Courts in Libya. In 2013 he started Ph.D. studies at the Chair of Microbiology, Faculty of Biology, University of Belgrade dealing with the impact of genotoxic pollution on aquatic biota and assessment of the impact of faceal pollution in large lowland rivers. As author and co-author he has published so far 5 research papers of which 3 are published within the research for this dissertation. Also he participated at 2 national and 4 international conferences.

Изјава о ауторству

Потписани _____Mustafa S. Aborgiba_____

број уписа _____Б3048/2013_____

Изјављујем

да је докторска дисертација под насловом

Микробиолошки квалитет воде и детекција генотоксичног загађења различитих сектора реке Саве прокариотским и еукариотским тест системима" (енг. Microbiological quality of water and detection of genotoxic pollution in different sectors of the Sava River with prokaryotic and eukaryotic test systems)

- резултат сопственог истраживачког рада,
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Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора __Mustafa S. Aborgiba_____

Број уписа _____Б3048/2013_____

Студијски програм _____биологија_____

Наслов рада Микробиолошки квалитет воде и детекција генотоксичног загађења различитих сектора реке Саве прокариотским и еукариотским тест системима" (енг. Microbiological quality of water and detection of genotoxic pollution in different sectors of the Sava River with prokaryotic and eukaryotic test systems)

Ментор _____Проф. др Бранка Вуковић-Гачић, др Стоимир Коларевић_____

Потписани _____Mustafa S. Aborgiba_____ _____

изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског звања доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

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Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић" да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:
Микробиолошки квалитет воде и детекција генотоксичног загађења различитих

сектора реке Саве прокариотским и еукариотским тест системима" (енг.

Microbiological quality of water and detection of genotoxic pollution in different sectors of

the Sava River with prokaryotic and eukaryotic test systems)

која је моје ауторско дело.
Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.
Моју докторску дисертацију похрањену у Дигитални репозиторијум Универзитета у Београду могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.
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