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Assessment of the genotoxic potential along the Danube River by application of the comet assay on haemocytes of freshwater mussels: The Joint Danube Survey 3

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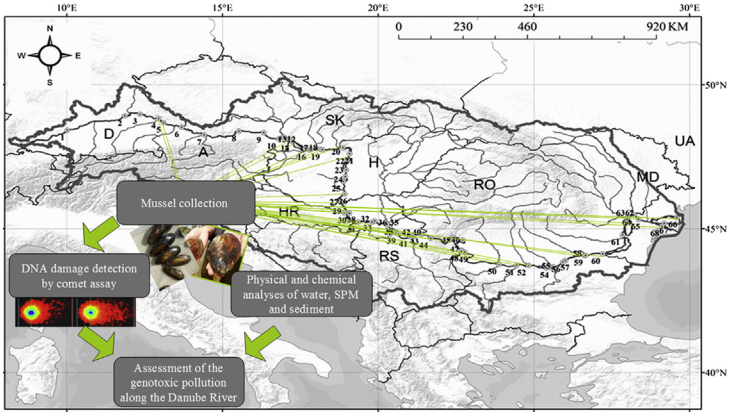
HIGHLIGHTS

- The level of genotoxic pollution was assessed along the Danube River during JDS3.
- Comet assay was used to study the level of DNA damage in freshwater mussels.
- The highest levels of DNA damage were recorded in the section VI (Panonnian Plain).
- DNA damage correlated with concentrations of hazardous priority substances.
- *Unio* sp. was more sensitive to the Danube River pollution comparing to *S. woodiana*.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study we assessed the level of genotoxic pollution along the Danube River by measuring the level of DNA damage in the haemocytes of freshwater mussels of *Unio* sp. (*Unio pictorum*/*Unio tumidus*) and *Sinanodonta woodiana*. The comet assay was used for the assessment of DNA damage. The research was performed on 34 out of 68 sites analysed within the Joint Danube Survey 3 — the world's biggest river research expedition of its kind in 2013. During research, 2285 river kilometres were covered with an average distance of 68 km between the sites. The complex data set on concentrations of various substances present in water, suspended particulate matter and sediment on investigated sites gave the opportunity to identify the groups of xenobiotics which mostly affect the studied biomarker — DNA damage. The highest levels of DNA damage were recorded in the section VI (Panonnian Plain), which is under the impact of untreated wastewater discharges. Both positive and negative influences of the large tributaries on the level of genotoxicity in the Danube River were evident. Significant

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correlation in response was detected between the studied species of freshwater mussels. The level of DNA damage in mussels correlated with concentrations of compounds from the group of hazardous priority substances (polycyclic aromatic hydrocarbons), persistent organic pollutants (dioxins) and emerging pollutants (Oxazepam, Chloridazon-desphenyl).

1. Introduction

The Joint Danube Survey 3 (JDS3) was the world's biggest river research expedition of its kind in 2013. This is the third expedition in a series carried out every six years on the Europe's second largest river — JDS1 was in 2001 and JDS2 in 2007 (http://www.danubesurvey.org). The main objectives of JDS3 were to collect information on more than 800 chemical, biological, microbiological, ecotoxicological and radiological parameters in the Danube River Basin (most of them not covered in the ongoing monitoring practise) and to provide the data obtained from a single source that is comparable for the entire river. Besides the various chemical and biological indicators of the water quality, ecotoxicity was one of the topics introduced in the previous survey — JDS2. Toxic effects of collected sediment samples to *Lemna minor* and *Vibrio fisheri* were detected in *ex situ* experiments (Tothova, 2008). Unlike the toxic effects which are usually manifested relatively shortly after the exposure, genotoxic effects become evident after a prolonged period, with significant biological consequences at the cellular, organ, whole animal and finally community and population levels (Jha, 2008). Genotoxic agents in the environment can induce various DNA lesions, such as strand breaks, modified bases, DNA–DNA crosslinks and DNA–protein cross-links, which may block replication and transcription, potentially leading to cell death, or may give miscoding information, generating mutations (Knežević-Vukčević et al., 2007). Depending whether the lesions occur in somatic or germ cells, the consequences can be evident in current or following generations. Therefore, knowledge of the extent of the genotoxic pollution along the Danube River is crucial for the assessment of the ecosystem quality but also for the assessment of the risk to human health that genotoxic pollution can pose via food chain or different water exploitations.

The major goal of this study was to assess the level of genotoxic pollution along the Danube River by employment of the comet assay on the haemocytes of freshwater mussels of *Unio* sp. (*Unio pictorum* and *Unio tumidus*) and *Sinanodonta woodiana*. Mussels became favourable organisms for the detection of the xenobiotics in the environment (Roméo et al., 2003; Andral et al., 2004; Amiard et al., 2006). In our previous studies, we have demonstrated the applicability of freshwater mussels *Unio* sp. and *S. woodiana* in the detection of genotoxic potential *in situ* and *ex situ* (Kolarević et al., 2013; Vuković-Gačić et al., 2014; Gačić et al., 2014). While the both species of *Unio* genus are autochthonous for the Danube River Basin, *S. woodiana* is recognised as a highly invasive alien species which became also widely distributed throughout the Danube River Basin (Csányi and Paunovic, 2006; Paunovic et al., 2006; Tomović et al., 2012). Therefore, we also wanted to compare the *in situ* response of investigated autochthonous (*Unio* sp.) and alochthonous (*S. woodiana*) species. The complex data set on concentrations of various substances present in water, suspended particulate matter and sediment on investigated sites gave us the opportunity to identify the group of xenobiotics which mostly affect the studied biomarker — DNA damage.

2. Material and methods

2.1. Study area

The specimens of freshwater mussels were collected in the period of six weeks during August and September 2013. The research was performed on 34 out of 68 sites analysed within the JDS3. During research 2285 river kilometres were covered with an average distance of 68 km between the sites. Data regarding sampling sites is shown in Table 1 while the map of all JDS sampling sites is presented in Fig. 1. The sites are organized as the Danube River sections with borders suggested by Robert et al. (2003) based on the typology developed in accordance with EU Water Framework Directive for the Danube River.

Data regarding the concentrations of various pollutants in water, suspended particulate matter (SPM) and sediment in samples collected at the same date when the specimens of mussels for genotoxicological analyses were collected can be found in final scientific report of the JDS3 of the International Commission for the Protection of the Danube River — ICPDR (available at http://www.icpdr.org/main/jds3-final-scientific-report-available).

The reference site was chosen by the "best available site" concept as it was practically impossible to find a pristine site on the river. The criteria for choosing the reference site were that the site should not be under the high pollution pressure and that it should be situated on the river itself and not on the tributary. Therefore, we were focused on two particular nature protected areas of the Danube River. The site JDS44, situated in the National park "Djerdap" (Serbia), was used as the reference site for the specimens of *Unio* sp., while the site JDS27 situated in the National park "Drava–Duna" (Hungary) was used as reference for the specimens of *S. woodiana*.

2.2. Collection of the specimens

The specimens of *Unio* sp. were collected from 31 sites while the specimens of *S. woodiana* were collected from 15 sites. At each site, adult specimens of *Unio* sp. (5–7 cm shell lengths) and *S. woodiana* (7–15 cm shell length) were collected from 2–5 m water depth by diving, benthological hand net or dredge. The specimens were immediately transported to the laboratory of the research ship Argus and stored into cooling boxes. Within 2 h from sampling, haemolymph was collected from the adductor muscle and the cell suspension was prepared by a procedure described in details by Gačić et al. (2014). The number of individual specimens analysed for each site is given in Table 1.

2.3. Comet assay

The comet assay, also known as the single cell gel electrophoresis (SCGE), is a sensitive and rapid technique for the detection of DNA damage in individual cells. It is based on the migration of denatured DNA during electrophoresis, in which damaged nuclei form comet-like shapes. Comet assay has been accepted as one of the major tools for assessing pollution related genotoxicity in aquatic organisms (Dixon et al., 2002).

The comet procedure was performed under yellow light as described by Singh et al. (1988) with slight modifications. 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 °C). The slides were held in freshly made cold (4 °C) lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1.5% Triton X-100, pH 10) for 3 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

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