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Evaluation of genotoxic potential throughout the upper and middle stretches of Adige river basin

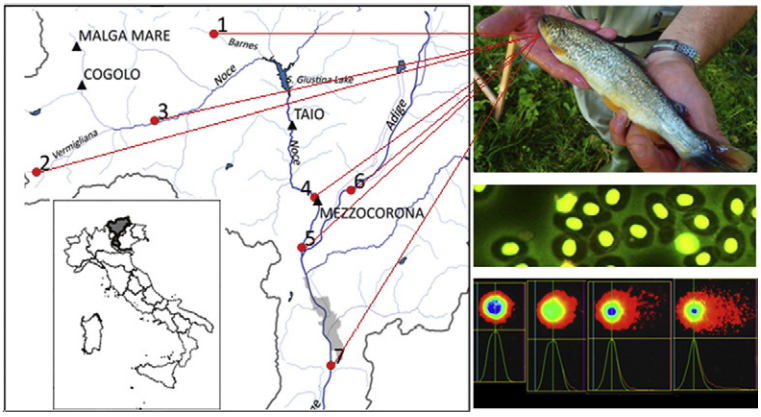
Margareta Kračun-Kolarević [a], Stoimir Kolarević [b,*], Jovana Jovanović [b], Vanja Marković [a], Marija Ilić [a], Predrag Simonović [b], Vladica Simić [c], Zoran Gačić [d], Elena Diamantini [e], Elisa Stella [e], Mira Petrović [f], Bruno Majone [e], Alberto Bellin [e], Momir Paunović [a], Branka Vuković-Gačić [b]

[a] University of Belgrade, Institute for Biological Research ¨Siniša Stanković¨, Belgrade, Serbia
[b] University of Belgrade, Faculty of Biology, Belgrade, Serbia
[c] University of Kragujevac, Faculty of Science, Kragujevac, Serbia
[d] University of Belgrade, Institute for Multidisciplinary Research, Belgrade, Serbia
[e] University of Trento, Department of Civil, Environmental and Mechanical Engineering, Trento, Italy
[f] Catalan Institute for Water Research, Girona, Spain

HIGHLIGHTS

- Genotoxicological survey was performed at 7 sites in the Adige river basin.
- Water samples were mutagenic in SOS/*umuC* assay on *Salmonella typhimurium* TA1535/pSK1002.
- Assays performed in fish blood indicated presence of genotoxicity in the Adige river basin.
- Comet assay showed the highest potential in discrimination of the sampling sites.
- Oxidative stress could be major contributor to observed DNA damage in specimens.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study a comprehensive genotoxicological survey throughout the upper and middle stretches of Adige river basin is presented. The study was carried out at 7 sites located along the Adige main course and one the most significant tributaries, the Noce creek, both presenting different levels of pollution pressure. To give an insight into the nature of the genotoxic activity we employed the battery of prokaryotic and eukaryotic assays. Mutagenicity in water samples was evaluated 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) were studied in blood cells of *Salmo cenerinus* Nardo, 1847 and *Salmo marmoratus* Cuvier, 1829. Within the applied bioassays, comet assay showed the highest potential for discriminating the sampling sites which are under lesser extent of pressure (sampling sites 1-Barnes at Bresimo and 4-Noce downstream S. Giustina) from the sites under high pressure (sampling sites 5-Noce at Mezzolombardo and 6/7-Adige upstream and downstream municipality of Trento). Significant correlation

* Corresponding author.
E-mail address: stoimirk@bio.bg.ac.rs (S. Kolarević).

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between the standard and Fpg - modified comet assay indicated that oxidative stress could be a major contributor to observed DNA damage in collected specimens.

1. Introduction

The Adige is the second longest river in Italy with a length of 410 km and a total contributing drainage area of about 12,000 km^2. A comprehensive overview of the hydrological and chemical stressors in the Adige river basin is presented in the work by Chiogna et al. (2016), to which we refer for further details. Available information indicates that some parts of the catchment are subject to pressures related to domestic, agricultural and industrial uses. In particular, locations characterized by significant chemical pollution have been identified in the proximity of wastewater treatment plants and return flows originating from agricultural areas (Caserini et al., 2004; Repice et al., 2013). Presence of persistent organic pollutants originating from melting glaciers has also been identified (Villa et al., 2006a, 2006b). However, quantification of pollutants provides only limited information on the substances present in the environment and gives no information on the relationship between contaminant exposure and biological effects in aquatic organisms; therefore a proper evaluation of the impact of pollutants by biomarkers becomes of relevant interest (Valavanidis et al., 2006; Jha, 2008; Fazio et al., 2012; Fazio et al., 2013; Aliko et al., 2015; Bianchi et al., 2015; Chromcova et al., 2015; Pagano et al., 2016). Directive 2000/60/EC of the European Parliament lays down a strategy against the pollution of water which involves the identification of priority substances among those that pose a significant risk to the aquatic environment with effects such as acute and chronic toxicity in aquatic organisms, accumulation of pollutants in the ecosystem and loss of habitats and biodiversity. In identifying priority hazardous substances the directive suggests the precautionary principle, relying in particular on determination of potentially adverse effects and on a scientific assessment of the risk (European Commission, 2000). Many of the substances listed in the Directive (European Commission, 2013) may interfere with DNA molecules of aquatic animals exhibiting genotoxic potential. As a consequence, occurred DNA damage might be reflected not only at the level of specimen but also at the level of whole ecosystem. Detection and quantification of genotoxic potential can be performed on different levels. Genotoxic pollutants can induce DNA damage within direct interaction with DNA molecule, or indirectly throughout the reactive oxygen species. Exposure to genotoxic pollutants in the environment is usually manifested as occurrence of single and double strand brakes or appearance of oxo-purines/oxo-pyrimidines which can be detected by comet assay (Collins, 2004). Afore mentioned DNA damage can have a different faith: it can be eliminated by repair mechanisms or programmed cell death or it can be promoted in permanent DNA damage which is manifested as structural and numerical chromosomal aberrations. This type of DNA damage is recognized as a biomarker of effect and most commonly is assessed by micronucleus assay (Jha, 2008). To the authors knowledge, currently there is no data on the genotoxic potential in the Adige river basin.

Fish have been proved to be reliable bioindicators in our previous ecogenotoxicological studies (Sunjog et al., 2012, 2013, 2014). As fish take the top level of the food web in freshwater ecosystems, they may be considered as amplifiers of genotoxic substances present in organisms at lower trophic levels (Aborgiba et al., 2016). Trout species have been commonly employed for detection of genotoxicity *in situ* (Belpaeme et al., 1996; Mitchelmore and Chipman, 1998; Inzunza et al., 2006; Altinok et al., 2012; Dogan et al., 2011).

Having in mind afore mentioned the primary goal of our study was therefore to evaluate the genotoxic potential in the Adige river basin. To give an insight into the nature of genotoxic activity we employed the battery of prokaryotic and eukaryotic assays. Mutagenicity in water samples was evaluated 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) were studied in blood cells of *Salmo cenerinus* Nardo, 1847 and *Salmo marmoratus* Cuvier, 1829. Possible influence of cytotoxicity was taken into consideration and therefore cell viability was studied based on the membrane stability. The study was carried out at 7 sites located in the Adige river basin, which were chosen in compliance with the goals of GLOBAQUA project (Navarro-Ortega et al., 2015) dealing with the effects of multiple stressors on biodiversity and functioning of aquatic ecosystems.

2. Materials and methods

2.1. Sampling area

Locations of sampling sites are indicated in Fig. 1, while the list of analyzed parameters is summarized in Table 1. Basic physical and

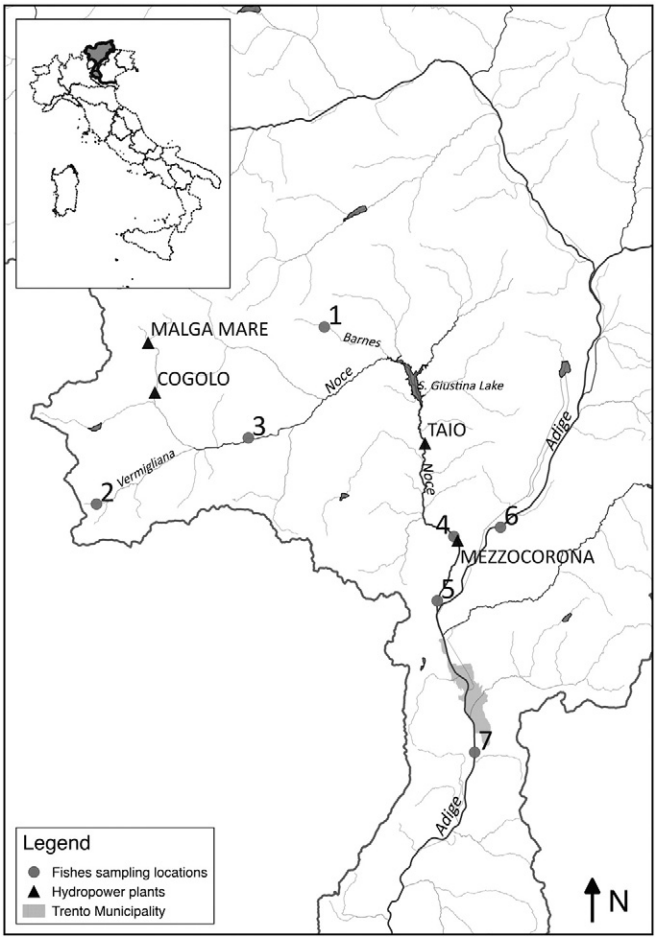

Fig. 1. Fish sampling locations in Adige river basin. The upper left inset shows the location of the Adige basin within the Italian territory.

Table 1
Overview of the parameters analyzed within the Adige river basin survey.

Parameter	SOS/*umuC*	Genotoxicity (standard and Fpg - comet assay and micronucleus)		Weight (g)	Length (cm)	Condition index
Stretch	Water	Fish	N of specimens	Fish	Fish	Fish
1-Barnes at Bresimo	+	*S. cenerinus*	4	84.5 ± 12.6	21.6 ± 0.9	0.8 ± 0.1
2-Vermigliana at Vermiglio	+	*S. cenerinus*	4	133.3 ± 39.9	23.0 ± 2.2	1.1 ± 0.1
3-Noce at Mezzana	+	*S. cenerinus* + *S. marmorata*	4 + 2	137.2 ± 25.8	23.9 ± 1.0	1.0 ± 0.1
4-Noce downstream S. Giustina	+	*S. cenerinus*	3	72.0 ± 23.4	21.0 ± 6.2	0.91 ± 0.5
5-Noce at Mezzolombardo	+	*S. cenerinus* + *S. marmorata*	3 + 2	127.6 ± 162.3	26.0 ± 16.4	1.2 ± 0.2
6/7-Adige upstream and downstream municipality of Trento	+	*S. marmorata*	6	121.3 ± 86.9	22.4 ± 5.1	0.9 ± 0.1

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

2.2. 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 water samples filtrated through 0.2 μm pore size filters using the protocol described by Žegura et al. (2009). Briefly, the treatment was performed for 2 h at 37 °C in incubation mixtures composed of 180 μL of water sample, 20 μL of 10 × TGA and 70 μL of bacterial culture of *S. typhimurium* TA1535/pSK1002 in exponential phase or in the case of metabolic activation 180 μL water sample, 20 μL 10 × TGA with cofactors and 70 μL of S9 bacterial culture mixture prepared as described in the ISO standard ISO/CD: 1382900 (ISO, 2000). Prepared aroclor induced male rat liver S9 fraction was purchased from Moltox, USA. Positive controls included 4-NQO (Sigma-Aldrich USA, final concentration 0.5 μg/mL) and benzo[*a*]pyrene (Sigma-Aldrich USA, final concentration 10 μg/mL). Sterile bidistilled water was used as the negative control. Incubation mixtures were diluted 10 times, incubated for 2 h and the bacterial growth rate was determined by measuring absorbance at 600 nm at microtiter plate reader (Thermo scientific USA, Multiskan FC). β-Galactosidase activity was determined using o-nitrophenyl-β-D-galactopyranoside - ONPG (Sigma-Aldrich, USA) as a substrate for 20 min at 25 °C. 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 <0.75, that represents 25% inhibition of biomass, is considered to be an indication of cytotoxicity. Induction ratio (IR) was calculated by the formula: sample OD405/control OD405*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.

2.3. Fish specimen collection

Specimens of *Salmo cenerinus* Nardo, 1847 and *Salmo marmoratus* Cuvier, 1829 were collected by electro-fishing (Aquatech DC electro fisher IG 1300, 2.6 kW, 80–470 V). Condition factor was calculated according to formula (Bervoets and Blust, 2003): $CF = W/L^3 \times 100$, where W is weight (g) and L is total length of fish (mm) as indicated in Table 1.

2.4. Blood sample collection and preservation

Blood samples were cryopreserved based on methodology described in Akcha et al. (2003) with slight modifications. Immediately after sampling, fish were anesthetized with clove oil prior to dissection. Blood was collected directly from the heart with 3 mL syringes (21 G needle rinsed with sodium heparin) and one drop of blood was diluted 20× in 4 °C cooled medium (RPMI 1640 (PAA, Austria) supplemented with 25% FBS (PAA, Austria)). Cryoprotective agent was then added (DMSO (Sigma-Aldrich, USA) final concentration 20%) and samples were immediately frozen in liquid nitrogen until the analysis (up to 3 weeks). For Fpg enzyme calibration (Section 2.6) additional samples were taken from the sites which were expected to be under less anthropogenic impact (sites 1 and 2).

Prior to Adige river basin survey, the effects of cryopreservation on cell viability and the level of DNA damage were assessed in preliminary experiments in 4 specimens of freshwater bream *Abramis brama* collected at the site situated in the Danube River near Belgrade.

2.5. Comet assay

Preserved blood samples were taken from liquid nitrogen and immediately thawed at 21 °C in water bath. Cell viability was assessed by acridine orange/ethidium bromide differential staining described in details in Gačić et al. (2014). Samples were diluted in 1xPBS to obtain approximately 50,000 cells/mL. For each sample three slides were prepared: one for alkaline and two for Fpg – modified comet assay.

The comet procedure was performed under yellow light as described in Kolarević et al. (2013) with slight modifications. 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 °C). 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. Denaturation was performed in cold (4 °C) alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min followed by electrophoresis with a voltage gradient 0.75 V/cm and amperage 300 mA for 20 min at (4 °C). Afterwards, neutralization 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) and examination with a fluorescence microscope (Leica, DMLS, Austria, under magnification 400×, 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.

2.6. Fpg – modified comet assay

For each sample, two slides were prepared for Fpg - modified assay as described in Section 2.5, one for Fpg buffer and one for the Fpg enzyme. After 1 h 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 Fpg 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 Fpg enzyme 45 μL of 300× 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

Table 2
Data on physical and chemical parameters measured in water at the sampling sites.

Site	pH	T (C°)	Conductivity (μS)	TDS (ppm)	O_2 (%)	O_2 (mg/L)	NO_2 (mg/L)	NO_3 (mg/L)	NH_4 (mg/L)	PO_4 (mg/L)
1- Barnes at Bresimo	7.8	9.6	108	55	87.7	9.2	0.01	1.40	0.01	0.07
2-Vermigliana at Vermiglio	8.1	15.3	163	83	85.6	7.1	0.01	2.36	0.01	1.13
3-Noce at Mezzana	7.7	9.5	97	47	91.5	9.6	0.03	0.78	0.05	18.66
4-Noce downstream S. Giustina	8.5	15.8	204	101	103.3	9.7	0.02	2.34	0.01	1.30
5-Noce at Mezzolombardo	9.4	17.3	203	101	100.4	9.3	0.01	1.82	0.02	1.30
6-Adige upstream municipality of Trento	7.9	13.6	180	88	91.4	8.8	0.03	1.20	0.05	1.22
7-Adige downstream municipality of Trento	8.2	15.7	232	114	99.2	9.7	0.02	2.96	0.31	1.20

were removed and slides were subjected to denaturation step as for the standard comet assay protocol described in the Section 2.5. The level of oxidative stress for each sample was evaluated by subtraction of the mean TI% value obtained from slide exposed to buffer only from the mean TI% value obtained from the slide exposed to Fpg enzyme.

2.7. Calibration of Fpg – modified comet assay

As indicated by the manufacturer, the final concentration of Fpg enzyme to be used must be optimized for particular cell line to maximize the difference in comet size between the cells exposed to oxidizing agent treated with Fpg and those exposed to reaction buffer only. Prior to comet assay analyses, optimum concentration of Fpg enzyme was determined based on guidelines of Collins (2004). First, the level of DNA damage was assessed in additional samples taken from sites 1 and 2. The specimen with the lowest level of DNA damage was used for Fpg enzyme calibration. Briefly, cryopreserved sample was diluted in 1xPBS to obtain approximately 50,000 cells/mL. The suspensions were centrifuged (2000 rpm, 10 min, 4 °C), the supernatants were discharged and pellets were suspended in of 1xPBS only (for control) or 1xPBS with final concentrations of H_2O_2 50 and 200 μM. Treatment was performed for 10 min at 4 °C in dark and the suspensions were washed with 1xPBS. The rest of procedure is carried out as indicated in Sections 2.5 and 2.6. Fpg enzyme was 300×, 3,000× and 30,000× diluted in buffer and applied on the slides.

2.8. Micronucleus assay

Slides for micronucleus assay were prepared as described in Štraser et al. (2011) with slight modification. Approximately 1 mL of cryopreserved sample was fixed with acetic acid/methanol (1:3 ratio). Suspensions were centrifuged at 1000 rpm for 5 min and pellets were washed twice with fixative. For each sample, 100 μL of fixed cell suspension was dispersed on two clean microscope slides. The slides were air dried for 24 h, stained with acridine orange 25 μg/mL and examined at 1000× magnification. From each slide at least 2000 cells with intact membrane was examined (4000 per specimen).

Nuclear abnormalities studied were micronuclei (MNi) – oval non-refractile nuclear body with a size between 1/16 and 1/3 of the mean diameter of the main nuclei, not connected or overlapping with main nuclei and BUDs - nuclear abnormalities which resemble MN that have a wide and obvious nucleoplasmatic connection with the main nucleus based on the criteria defined in Fenech et al. (2003). Cells with highly fragmented nuclei were not included into scoring to avoid possible interference of apoptosis. Additionally, cells with two nuclei were marked as binucleated (BN) which was also considered as a nuclear abnormality (Çavaş and Ergene-Gözükara, 2005).

2.9. Statistical analyses

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. Results

3.1. Basic physical-chemical parameters measured in water at the sampling sites

At the sampling sites, temperature varied from 9.5 to 17.3 °C (Table 2). At the majority of sampling sites, pH was mildly alkaline. The highest concentration of NO_3 and NH_4 was recorded at the site 7-Adige downstream municipality of Trento. The highest concentration of PO4 was detected at the site 3-Noce at Mezzana.

3.2. Mutagenic potency of water samples - SOS/umuC

The results of the assay indicated mutagenic potential in water samples at 5 out of 7 sites but without metabolic activation (Table 3). Only at the site 3-Noce at Mezzana threshold value of 1.5 was breached in both cases – with and without S9.

Table 3
The results of SOS/*umuC* test (mean ± SD).

	Without S9		With S9	
Sites	G	IR	G	IR
1-Barnes at Bresimo	0.88 ± 0.06	**1.81 ± 0.27**	1.02 ± 0.03	1.35 ± 0.18
2-Vermigliana at Vermiglio	0.92 ± 0.06	**1.9 ± 0.16**	1.02 ± 0.04	1.35 ± 0.12
3-Noce at Mezzana	0.92 ± 0.03	**1.78 ± 0.25**	0.96 ± 0.02	**1.81 ± 0.24**
4-Noce downstream S. Giustina	0.88 ± 0.07	1.20 ± 0.30	1.22 ± 0.30	0.83 ± 0.23
5-Noce at Mezzolombardo	0.92 ± 0.04	**1.58 ± 0.20**	0.99 ± 0.02	1.21 ± 0.05
6-Adige upstream municipality of Trento	0.92 ± 0.19	**1.85 ± 0.16**	1.00 ± 0.06	1.37 ± 0.21
7-Adige downstream municipality of Trento	0.89 ± 0.18	1.18 ± 0.15	1.04 ± 0.13	1.07 ± 0.18
Positive control 4-NQO/B(a)P	0.54 ± 0.07	**18.37 ± 4.07**	0.84 ± 0.01	**1.8 ± 0.05**

G-growth ratio, IR-induction ratio; bold values exceeded threshold value 1.5.

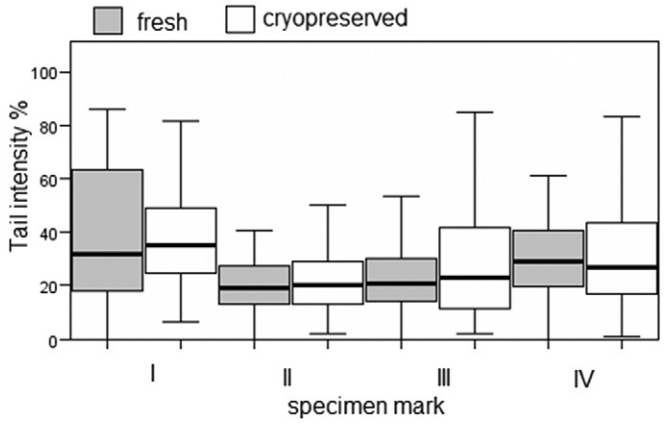

Fig. 2. The effects of cryopreservation of blood samples of 4 freshwater bream specimen on the DNA damage level (Median, Box 25%–75%, Whisker non-outlier range).

3.3. Effects of cryopreservation – preliminary investigation

The effects of cryopreservation on cell viability and DNA damage induction were assessed in preliminary experiments performed on 4 specimens of freshwater bream collected from the Danube River. The impact of cryopreservation on cell viability was not observed and viability remained higher than 90% in all cases (data not shown). The level of DNA damage in cryopreserved samples was within the similar range as in the fresh samples. Significant difference between the fresh and cryopreserved samples was not observed (Fig. 2).

3.3.1. Assessment of cell viability in cryopreserved samples from the Adige river basin

Cell viability was also assessed in each sample collected from the Adige river basin immediately after thawing. As indicated in the Table 4, viability was higher than 80% in all samples. From the Table 4, it can also been seen that there was no significant variation in cell viability or HH frequency among the investigated sites. When plotting the cell viability with HH frequency there was no significant relations ($r = -0.03$, $p = 0.87$).

3.4. Assessment of genotoxicity

3.4.1. Assessment of DNA damage – standard comet assay

As indicated in Fig. 3 the highest values of DNA damage were recorded at the sites 5 and 6/7 (TI% was 43.3 ± 1.3 and 47.6 ± 1.2 respectively) while the lowest level of DNA damage was recorded at the site 1 (27.4 ± 1.1).

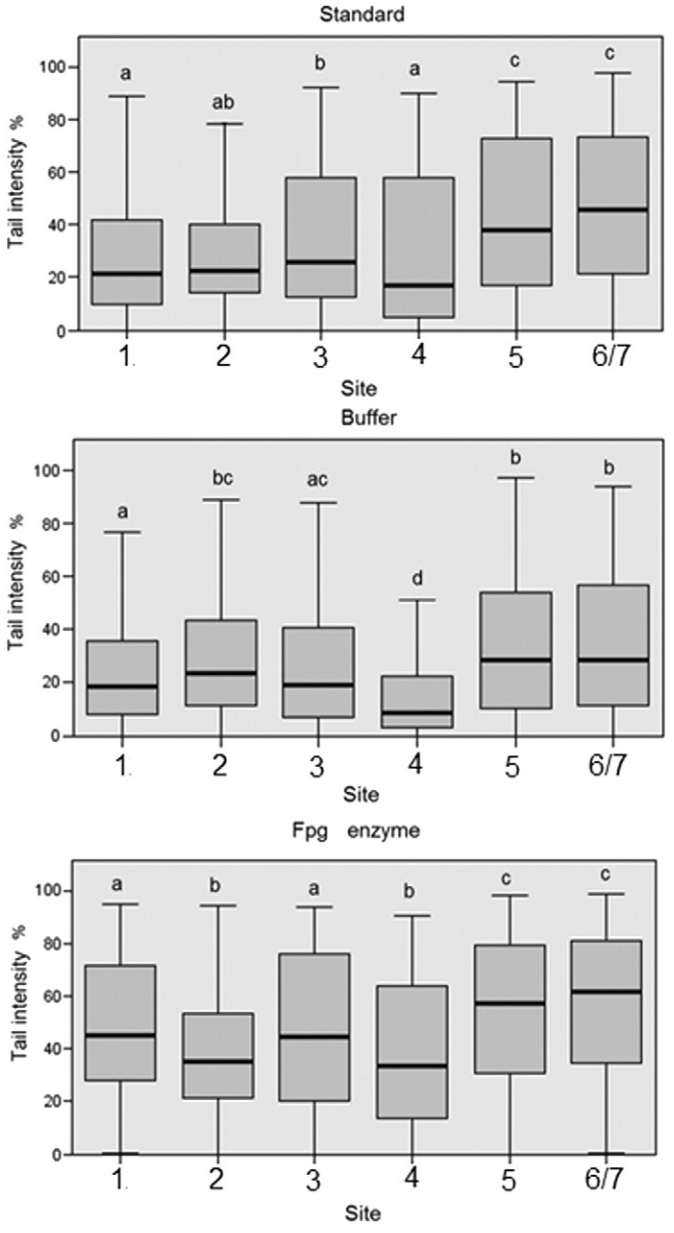

Fig. 3. Values of tail intensity % obtained in fish blood cells in standard comet assay and Fpg - modified assay after exposure to buffer only and Fpg enzyme (Median, Box 25%–75%, Whisker non-outlier range); different letters denote significant differences among studied sites ($p < 0.05$).

3.4.2. Assessment of DNA damage – Fpg – modified comet assay

3.4.2.1. Calibration of Fpg enzyme. Based on titration experiments the optimal concentration of Fpg enzyme, which maximized the difference in comet size between the cells treated with Fpg and those exposed only to reaction buffer, was 300× dilution. In untreated group of cells, Fpg induced net increase of TI% of 8.6%, in group treated with 50 μM increase of 15.4% and in group treated with 200 μM increase of 6.3% (Fig. 4).

3.4.2.2. Fpg – modified comet assay. In all cases digestion with Fpg enzyme led to significant increase of DNA damage in comparison with corresponding standard alkaline and buffer treated control (Fig. 3). As for the standard comet assay, the highest values of TI% were detected in the specimens collected at the sites 5-Noce at Mezzolombardo and 6/7-Adige upstream and downstream Trento. The highest net contribution of 8-oxoG sites was detected in specimens from the sites 6/7 (Table 5).

Significant correlation was observed between the mean TI% values obtained from slides for standard alkaline comet assay and

Table 4
Cell viability (%) assessed by differential acridine orange/ethidium bromide staining and frequency of HH (%) shown as mean ± SD.

Sites	Cell viability %	HH frequency %
1-Barnes at Bresimo	90.3 ± 4.6	16.2 ± 10.2
2-Vermigliana at Vermiglio	89.8 ± 3.3	9.8 ± 8.0
3-Noce at Mezzana	85.2 ± 10.1	6.0 ± 7.7
4-Noce downstream S. Giustina	80.7 ± 3.2	3.8 ± 3.7
5-Noce at Mezzolombardo	86.4 ± 5.0	3.7 ± 3.5
6/7-Adige upstream and downstream municipality of Trento	85.5 ± 9.4	8.4 ± 5.8

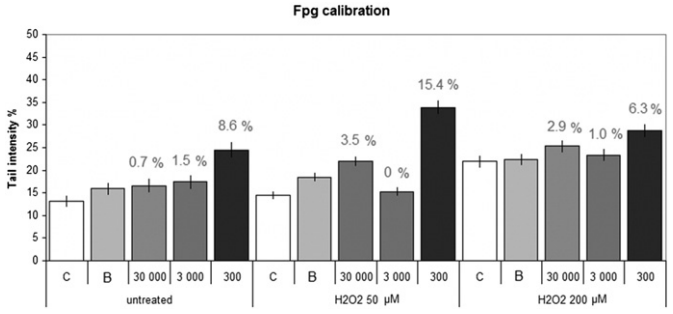

Fig. 4. Determination of the optimal concentration of Fpg enzyme (300×, 3,000× and 30,000× dilution) for detection of oxidized bases in untreated blood samples and samples exposed to H_2O_2 (50 and 200 μM); values are represented as mean ± SE; numbers above the columns indicate increase of mean TI% value in comparison with corresponding buffer exposed group; B - buffer, C - control.

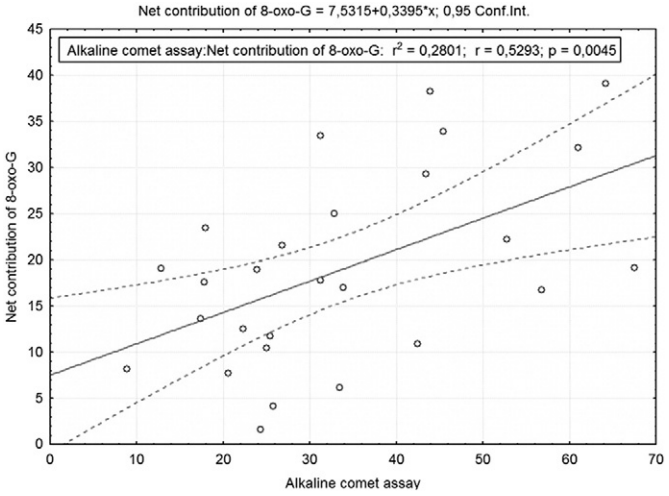

Fig. 5. Correlation between the levels of DNA damage obtained in standard comet assay and net 8-oxoG sites; full line - regression line; dashed line - 95 confidence level.

net contribution of 8-oxoG sites in DNA damage ($r = 0.53$, $p = 0.0045$) (Fig. 5).

3.5. Micronucleus

The highest frequency of MNi was detected in specimens collected at the site 3-Noce at Mezzana (Table 6). Significance was observed only when comparing this site to sites 2 and 6/7. There was no significant variation in frequency of BUDs and BNs among the studied sites. As shown in Fig. 6, significant correlation was observed between the frequency of MNi and BNs ($r = 0.73$, $p = 0$). Also there was no significant correlation between the data obtained in comet assay and micronucleus assay.

4. Discussion

Our study of genotoxic potential in the Adige river basin represents a "snapshot" of the current status of the basin with a battery of bioassays which had different potential in discrimination of the sites. Studying mutagenicity of water samples was included to evaluate the presence of genotoxicants at the moment of sampling while the assays performed in fish were expected to provide information on prolonged exposure to xenobiotics.

For the study we selected sampling points which are under different types of pressure. Despite site 1- should present the lowest pollution impact, at this location and at site 2 (locations characterized by stream flow originating mainly from snow and glacier melting, Chiogna et al., 2014), a significant impact on DNA damage was observed. We attribute this to the possible presence of pollutants originating from glaciers meltwater (Villa et al., 2003; Villa et al., 2006a; Villa et al., 2006b), which has been recognized as a noteworthy source of genotoxic potential (Vasseur and Cossu-Leguille, 2006; González-Mille et al., 2010). In addition, at the sites 2 and 3 the presence of micropollutants released by waste water treatment plants located in proximity of important sky resorts might contribute to observed effects. Toxic and genotoxic potential of WWTP effluents are in fact well known as they commonly contain residues of pharmaceutical and personal care products as well as many other contaminants (Žegura et al., 2009). The site 4 is situated downstream the large storage reservoir S. Giustina Lake which may act as sink for contaminants. Sites 3 and 5 are also characterized by significant streamflow alterations induced by the presence of four large hydropower plants (see Fig. 1) located along the Noce tributary course (Bellin et al., 2016; Majone et al., 2016). Furthermore, site 5, which is located just before the confluence between Adige and Noce tributary, is at close distance to sites 6 and 7, and thus it is possible to assume that specimens inhabiting this part of the study area represent a single population. The major pressure is in this case related to water released from waste water treatments plants located in the municipality of Trento. In our previous studies we demonstrated genotoxic potential of municipal wastewater in various aquatic animals (Kolarević et al., 2013; Vuković-Gačić et al., 2014; Aborgiba et al., 2016; Kolarević et al., 2016). Also, the whole Trento province is known for an extensive viticulture activity. The study of Bony et al. (2008) emphasized genotoxic potential of commonly used vineyard pesticides in fish. One of the goals of GLOBAQUA project is to provide information on concentrations of xenobiotics in water, sediment and biota, and the sources of xenobiotics at the studied sites as currently there is no recent data on this topic (Navarro-Ortega et al., 2015).

The SOS/*umuC* has been included in our research as sensitive test for detection of genotoxic pollutants in wastewaters and surface waters as well (Žegura et al., 2006, 2009). For the sites close to Trento municipality we can conclude that the results are expected as those sites are situated in an area of the basin characterized by poor chemical status (Chiogna et al., 2016). Mutagenic potential detected at the sites 2 and 3 can be linked to WWTPs. For the site 1, the only possible explanation that can be provided at the moment is related to the release of POPs by melting from glaciers (Chiogna et al., 2016).

Table 5
Fpg - modified comet assay, tail intensity values for buffer and Fpg - exposed slides and net contribution of 8-oxoG sites (mean ± SE).

Sites	Buffer	Fpg enzyme	Net 8-oxoG
1-Barnes at Bresimo	24.5 ± 1.1	48.1 ± 1.3	23.6
2-Vermigliana at Vermiglio	29.2 ± 1.1	38.8 ± 1.1	9.5
3-Noce at Mezzana	26.5 ± 1.0	47.0 ± 1.3	20.5
4-Noce downstream S. Giustina	16.6 ± 1.1	37.9 ± 1.6	21.2
5-Noce at Mezzolombardo	34.1 ± 1.2	54.2 ± 1.2	20.2
6/7-Adige upstream and downstream municipality of Trento	35.1 ± 1.2	57.5 ± 1.1	22.4

Table 6
Frequency of MNi, BUDs and BN cells (‰) shown as mean ± SD.

Sites	MNi (‰)	BUDs (‰)	BNs (‰)
1-Barnes at Bresimo	6.8 ± 3.9^{ab}	5.0 ± 3.9	8.4 ± 3.8
2-Vermigliana at Vermiglio	3.0 ± 2.5^{b}	3.8 ± 2.0	2.6 ± 1.2
3-Noce at Mezzana	9.0 ± 7.6^{a}	4.5 ± 2.1	7.6 ± 7.9
4-Noce downstream S. Giustina	4.5 ± 1.6^{ab}	5.0 ± 2.6	2.7 ± 2.0
5-Noce at Mezzolombardo	4.5 ± 2.9^{ab}	3.2 ± 2.0	3.3 ± 1.8
6/7-Adige upstream and downstream municipality of Trento	1.8 ± 1.1^{b}	2.7 ± 1.0	3.2 ± 1.0

Different letters denote significant differences among studied sites ($p < 0.05$).

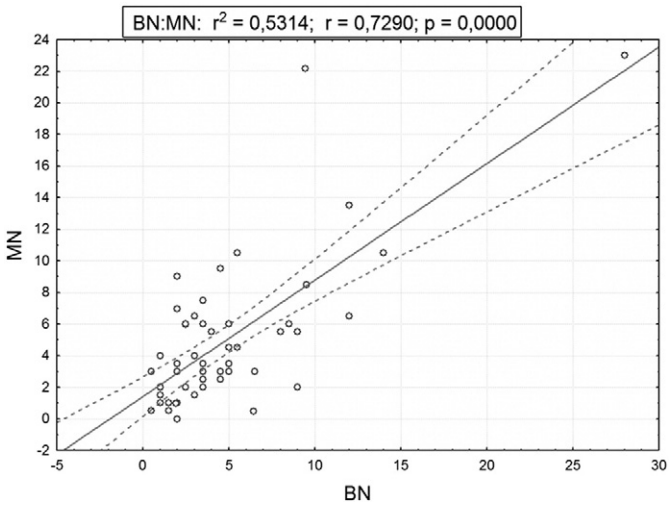

Fig. 6. Correlation between the frequency of MNi and BNs; full line - regression line; dashed line - 95 confidence level.

As the samples of fish blood could not be analyzed directly upon sampling, we had to include preservation of the tissue in our research. In available studies performed on fish blood authors either used short time preservation at 4 °C (Faggio et al., 2013) or applied cryopreservation for prolonged time storage. So far, cryopreservation of the fish blood samples has been used in few ecogenotoxicological studies which employ comet assay (Duthie et al., 2002; Akcha et al., 2003). When using such approach in handling the samples it is necessary to evaluate the cell viability after the thawing of the samples as cell viability lesser than 70% can interfere with the comet assay data in means of appearance of highly damaged apoptotic nuclei also known as HH (Liao et al., 2009). Assessment of cell viability is also very important as it can also be an indication of poor health status of the studied bioindicator (Pagano and Faggio, 2015). Our preliminary experiments on cryopreserved blood samples of freshwater bream indicated that there is no significant impact on cell viability and the level of DNA damage. Moreover we are confident that cryopreservation was not reflected on comet assay data considering the lack of relation between the cell viability and HH frequency in cryopreserved blood samples collected within the survey.

Based on the studies performed which employ comet assay in various trout species (Del Barga et al., 2006; Kim and Hyun, 2006; Dogan et al., 2011) we can see that baseline values on DNA damage obtained in trout specimens collected from the reference localities range up to TI% value 25. This indicates that the values in our study were increased at majority of sampling sites. Within the applied battery of bioassays, comet assay showed the highest potential in discrimination of the sampling sites which are under lesser extent of pressure (sampling sites 1-Barnes at Bresimo and 4-Noce downstream S. Giustina) from the sites under highest pressure (sampling sites 5-Noce at Mezzolombardo and 6/7-Adige upstream and downstream municipality of Trento). The higher sensitivity of comet assay in comparison with micronucleus test is in compliance of the study of Kim and Hyun (2006) performed on rainbow trout. To provide further information on nature of detected DNA damage we have introduced Fpg modified comet assay in our research.

Oxidative stress is the most commonly manifested by the appearance of oxidized bases, mainly 8-oxoG (Collins, 2004). Fpg enzyme is glycosylase isolated from *Escherichia coli* which is involved in excision repair (Boiteux et al., 1990). When included in the comet assay, Fpg enzyme converts 8-oxoG sites into strand brakes which can be later quantified as net contribution to observed DNA damage. Following the guidelines by Collins (2004) we have determined the optimum concentration of Fpg enzyme that had the highest potential in discrimination of the samples with the highest frequency of oxidized bases. Similarly as for alkaline comet assay, the highest TI% values were recorded in specimens collected at the sites 5 and 6/7. Interestingly, when plotting the data on TI% values from standard comet assay and net contribution of 8-oxoG sites in DNA damage for each specimen individually, significant correlation was observed. This indicates that oxidative stress could be major contributor to observed DNA damage in collected specimens. This is not surprising because the mode of action of priority hazardous substances in environment is based on generation of reactive oxygen species (Mitchelmore and Chipman, 1998; Bartoskova et al., 2013; Messina et al., 2014).

Finally, micronucleus assay was used to assess the presence of clastogenic and aneugenic effects. Available literature data on micronucleus employed in different trout species (Belpaeme et al., 1996; Sanchez-Galan et al., 1998; Sanchez-Galan et al., 1999; Ayllón et al., 2000; Rodriguez-Cea et al., 2003; Barga et al. 2006; Kim and Hyun, 2006; Andreikėnaitė et al., 2007) indicate that baseline frequency of MNi in specimens from reference/unpolluted area varies from 0.2–4‰. Based on this, we can state that only at the site 3 increased frequency of MNi has been observed. Generally, micronucleus assay had a low potential for discriminating between the sites. When comparing the data on MNi with the comet assay data we have not observed a significant correlation. It should be taken into consideration that the comet assay detects primary damage to the DNA molecule (Lee and Steinert, 2003; Frenzilli et al., 2009). Such damage is often not inherited by future cellular generations and, for this reason, cannot be detected by the micronucleus assay (Jha, 2008). Metabolic capacity, DNA repair efficiency, cell removal kinetics (replacement of dead or damaged cells) play significant role in MN expression (Bolognesi and Hayashi, 2011). Additionally, DNA damage detected in the comet assay in our case might have been driven mainly by oxidative stress and we can speculate that the detected damage is partly eliminated by repair pathways (Cooke et al., 2003) which could be an explanation for the lack of correlation between the assays.

5. Conclusions

This study represents the first complex survey of the genotoxic potential in the Adige river basin. Our data represent a “snapshot” of the current status of the river with an approach which enables detection of the effects of genotoxic pollution on different levels. Within the applied battery of bioassays, comet assay showed the highest potential in discrimination of the sampling sites which are under lesser extent of pressure (sampling sites 1-Barnes at Bresimo and 4-Noce downstream S. Giustina) from the sites under highest pressure (sampling sites 5-Noce at Mezzolombardo and 6/7-Adige upstream and downstream municipality of Trento). Significant correlation between the standard and Fpg modified comet assay indicated that oxidative stress could be major contributor to observed DNA damage in collected specimens.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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