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Assessment of DNA integrity in erythrocytes of *Cobitis elongata* affected by water pollution: the alkaline comet assay study

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A b s t r a c t . An assessment of DNA integrity in erythrocytes using the alkaline comet assay was carried out to estimate the impacts of water pollution on Balkan loaches (*Cobitis elongata* Heckel et Kner, 1858) inhabiting the Sava and Kupa Rivers, rivers of varying different water quality. The amount of DNA damage in cells was estimated from three different parameters: comet tail length as the extent of genetic material migration, tail intensity (% DNA in comet tail) and tail moment. The results suggest a genotoxicity of the aquatic environment in the Sava River and demonstrated significantly lower levels of DNA damage in fish captured from the Kupa River. This study confirmed that the comet assay, applied to fish erythrocytes, is a useful tool in determining potential genotoxicity of water pollutants. Although a good DNA damage pattern for Balkan loach was obtained, due to its global and regional conservation status, only restricted use of a small number of specimens per sampling site could be permitted.

Key words: Balkan loach, DNA integrity, comet assay, erythrocyte, water pollution

Introduction

Fish provide excellent material for the study of mutagenic or carcinogenic potential of water samples, since they can metabolize, concentrate, and store waterborne pollutants (A t e e q et al. 2005). Biomarkers able to detect oxidative stress and DNA fragmentation constitute early warning indicators assessable before more severe alterations in single organisms and the fish community occur.

The comet assay is a rapid and sensitive method for detecting primary DNA damage at the cell level. It combines a biochemical approach to detect DNA strand breaks and/or alkali labile sites with a single-cell approach typical for cytogenetic assays (C o l l i n s et al. 1997, L e e & S t e i n e r t 2003). This technique has already been successfully employed for monitoring DNA damage in laboratory and field studies with various fish species, both in freshwater and marine environments (D e v e a u x et al. 1997, B e l p a e m e et al. 1998, P a n d e y et al. 2006). Moreover, in some studies the impacts of reduced water quality on fish species were also investigated (L y o n s et al. 1999, S u m a t h i et al. 2001, A v i s h a i et al. 2002, M o r a e s d e A n d r a d e et al. 2004, W i n t e r et al. 2004).

Many have supported and demonstrated the relevance of fish erythrocytes for the comet assay, because fish blood, with 97% erythrocytes, ensures great homogeneity of cells for comet studies (T h e o d o r a k i s et al. 1994). Blood cells are constantly exposed to reactive oxygen species and provide a relatively non-invasive source of material for biomonitoring (T i a n o et al. 2000). In earlier comet assays, hepatocytes were also used (D e v e a u x

et al. 1997, Mitchelmore & Chipman 1998a, Risso-de Faverney et al. 2001). However, the use of blood cells is more advantageous since cell dissociation is not required. Blood is easily sampled and fish are returned to the water after collection, without the need for sacrificing them to conduct the study (Giacomini Lemos et al. 2005).

The Balkan loach (*Cobitis elongata* Heckel et Kner, 1858) is an autochthonous benthic fish found in many freshwaters throughout the Danube River basin of South-Eastern Europe (Croatia, Slovenia, Serbia, Montenegro and Romania) (Povž & Šumer 2003, Mrakovčić et al. 2006). In the Red Book of Freshwater Fish of Croatia, it is classified as a vulnerable species, while its global threat status is data deficient (Mrakovčić et al. 2006). The Balkan loach lives in relatively stationary populations and thus may reflect environmental conditions where the fish is caught. The species is common in its distribution area. However, in recent years it was surprisingly abundant in many polluted sites along the Sava River in Croatia, especially as juveniles.

Pollution causes changes to the structure of the fish community, and can be fatal to many species. It is known that rare and vulnerable species of fish are particularly threatened by long-term water pollution. An assessment of DNA integrity in their erythrocytes using the alkaline comet assay was conducted to estimate the impacts of water pollution on Balkan loaches inhabiting the Sava and Kupa Rivers, rivers of varying water quality. Data obtained could be also utilized to estimate the degree of genetic susceptibility, as well as to minimize threats to this endemic species and improve strategies for its protection in future.

Materials and Methods

Fish sampling was performed in September 2006. Loaches were collected by electrofishing at two sites in the Sava River, downstream (Site 1; Ivanja Reka; 45°46'36.93" N, 16°08'42.90" E; river km 687.2) and upstream of the City of Zagreb (Site 2; 45°49'42.58" N, 15°49'06.42" E; river km 717), as these sites are characterized by different levels of contamination. The Sava River is known to receive considerable amounts of anthropogenic inputs, including domestic waste discharge, municipal sewage discharge and industrial effluents considered to significantly contribute to high levels of water and sediment contamination. Fish were also collected from the Kupa River (Site 3, 45°38'37.20" N, 15°26'49.36" E; river km 161.8), approximately 10 km upstream of the town of Ozalj. This area was chosen as the reference site since it has no recorded industrial inputs and it is known to receive minimal anthropogenic input and municipal wastes.

Water chemistry parameters (physico-chemical properties) at both sampling sites were assessed using standard methods.

After capture, fish were rapidly transferred (field animals in river water) to the laboratory prior to processing. Adult males and females varied in mass (2.4–13.5 g) and length (85–140 mm). Ten fish per site were used for the analyses. Blood samples (30 μl of each analyzed individual) were collected from the caudal vein with a heparinised syringe.

The comet assay was conducted with whole blood under yellow light to prevent UV induced DNA damage and performed as a three-layer procedure described by Singh et al. (1988). All chemicals were of analytical grade and purchased from Sigma Chemical Co. unless otherwise noted. Two fully frosted microscopic slides per specimen were prepared. Each slide was covered with a sandwich gel: 1% and 0.6% normal melting point (NMP) agarose. The blood sample (10 μl) was mixed with 0.5% low melting point (LMP) agarose.

The suspension was spread on slides previously coated with normal agarose and then covered by a single layer of 0.5% LMP agarose. Slides were immersed overnight in ice-cold freshly prepared lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Na-sarcosinate, pH 10) with 1% Triton X-100 and 10% dimethyl sulfoxide (Kemika) added fresh to lysed cells to allow for DNA unfolding. The slides were then randomly placed side by side in a horizontal gel-electrophoresis tank, facing the anode. The unit was filled with a freshly prepared electrophoretic buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13.0) and slides were left in this alkaline buffer for 20 min to allow for DNA unwinding and expression of alkali-labile sites. Denaturation and electrophoresis were performed at 4°C under dim light. Electrophoresis was carried out for the next 20 min at 25 V (300 mA). After electrophoresis, slides were washed gently three times at 5-min intervals with a neutralisation buffer (0.4 M Tris-HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20 μg/ml) and covered with a coverslip. Slides were stored at 4°C in humidified sealed containers until analysis.

Calibration of the comet assay and the optimization of the assay conditions were done with hydrogen peroxide (H_2O_2), as described in previous studies (Avishai et al. 2002, Pandey et al. 2006). This strong oxidising agent is frequently used as a "positive control" since it is able to produce an excess of single- and induce double strand breaks even at millimolar concentrations (Olive & Banáth 2006). Therefore, blood cells of fish collected at the reference site were treated *ex vivo* with 0.1 mM H_2O_2 in phosphate buffered saline (PBS; pH 7.4) for 5 minutes at 4°C. The slides were further processed in the same manner as described above.

All slides were evaluated blind by a single observer. 100 cells per specimen (50 cells/slide) were scored. Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells, cells near or in a trapped air bubble and superimposed comets. Slides were scored using an image analysis system (Comet Assay II, Perceptive Instruments Ltd., U.K.) attached to a fluorescence microscope (Zeiss, Germany), equipped with appropriate filters. The parameters selected for the quantification of DNA damage were: mean comet tail length, tail intensity (% DNA) and tail moment as calculated by the software. The extent of DNA damage, as recorded by the alkaline comet assay, was analyzed considering the mean (± standard error of the mean), median and range of the comet parameters measured. Statistical analysis was carried out using one-way ANOVA, complemented by the Duncan post-hoc test (Statistica Software 5.0, StatSoft, Tulsa, USA). Raw data were previously logarithmically transformed to normalize distribution and to equalize variances of the measured parameters. The minimal significance level in both statistical analyses was $p < 0.05$.

Results

The physico-chemical characteristics of the water are presented in Table 1. The pH of the water did not show significant variation (7.67–8.17). The water temperature also did not differ significantly compared to the typical values for that time of the year. Chemical analyses of water reveal differing concentrations of certain genotoxic metals (Sr, Hg, As, Cu, Cr, Mn) between the Sava and Kupa, with the highest pollution in municipal waters at Site 1 (location Ivanja Reka).

Morphometric (length and weight) parameters of collected fish are listed in Table 2. The loaches collected at Site 1 had an average weight of 6.2 ± 0.82 g and an average length of

Table 1. Physico-chemical properties of the water at the sampling locations.

Parameter	Unit	Sampling locations		
		Sava 1 45°46‘36.93“ N, 16°08‘42.90“ E	Sava 2 45°49‘42.58“ N, 15°49‘06.42“ E	Kupa 45°38‘37.20“ N, 15°26‘49.36“ E
Water temperature	°C	20.9	20.0	17.0
pH	-	7.67	8.07	7.98
Conductivity	μS/cm	485	425	335
$KMnO_4$	mg/L	4.7	2.0	1.6
Cl^-	mg/L	11.89	7.10	2.70
o-PO_4^{3-}	mg/L	0.11	0.02	0
SO_4^{2-}	mg/L	17.23	12.80	4.50
NO_3^-	mg/L	6.12	5.90	2.00
NO_2^-	mg/L	0.17	0.01	0
NH_4^+	mg/L	1.154	0.206	0.189
Fe	μg/ L	21.0	53.6	37.8
Pb	μg/ L	7.5	14.5	0
Cd	μg/ L	0.2	0	0
Hg	μg/ L	0.23	0	0
As	μg/ L	0.69	0	0
Cr	μg/ L	0.5	0	0
Zn	μg/ L	16.0	5.6	2.3
Cu	μg/ L	4.5	4.4	4.7
Mn	μg/ L	9.0	3.5	2.1
Sr	μg/ L	137.5	118.1	68.9
Al	μg/ L	43.8	107.7	35.8

103.5 ± 4.00 mm. Loaches collected at Site 2 had an average weight of 5.9 ± 0.81 g and an average length of 115.0 ± 4.92 mm. The loaches collected at non-polluted site in Kupa River were larger ($p<0.05$; with an average weight of 7.1 ± 0.87 g and an average length of 120.5 ± 4.54 mm) compared with those captured at both polluted sites in Sava River. However, weight did not significantly influence the statistical analysis.

The comet assay data for Balkan loaches captured from the Sava and Kupa Rivers are summarized in Table 2. Typical photomicrographs of comets due to DNA strand breaks are illustrated in Fig. 1.

The level of DNA damage in erythrocytes of fish was estimated based on three main comet parameters: tail length, tail intensity (% DNA) and tail moment. The main observation is that Balkan loaches from the Sava River show a lower degree of DNA integrity compared with control animals from reference site and that the comet assay has sufficient sensitivity to detect the genotoxicity. There was a pattern of increased DNA damage in fish erythrocytes from low levels at the clean Kupa site, through intermediate levels in the Sava 2, to higher levels in the relatively polluted site Sava 1. Inter-individual differences in erythrocyte DNA damage were also observed (Table 2), however, these were more pronounced in the group of loaches collected from the Sava River.

Analysis of variance with Duncan’s multiple range tests revealed that genotoxic levels differed significantly between sites sampled, and indicated the effect of site in modulating DNA integrity of fish erythrocytes (Fig. 2). A higher ($p<0.001$) level of DNA damage was measured by the comet assay in the polluted sites 1 and 2 in the Sava River compared to

the reference site in the Kupa River. The erythrocytes of specimens from sites 1 and 2 also showed a different degree of DNA integrity. Significant results were also recorded in all sampling sites in contrast with the positive control.

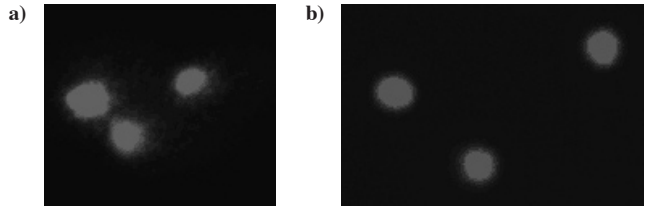

Fig. 1. Appearances of comets in erythrocytes of Balkan loaches. (a) Nuclei from erythrocytes of fish collected in the Sava River (Site 1) consist of a head with DNA migrating into the tail region as a result of extensive single strand breaks; (b) Nuclei from erythrocytes of fish collected in the Kupa River consist of a head with no or minimal DNA migration into the tail region.

Discussion

Although DNA integrity relies on complex intrinsic processes in the organism/cell, it is also significantly affected by many external factors, particularly the exposure to pollutants. Exposure to genotoxic chemicals in lower animals seldom results with neoplasia, but frequently with a variety of symptoms known as a genotoxic disease syndrome. These manifestations include impairments in enzyme function, altered protein turnover, impairments in general metabolism, production of initiators of cytotoxic injuries, inhibition of growth, degenerative processes and atrophy in tissues and organs, decreased scope for growth in organisms, faster ageing, decreased fitness and well-being, impairments in immunoresponse and reproduction, increased frequency of diseases and neoplasia, impairments in adaptation, survival and succession, and ultimately, the extinction of species (Kurelec 1993). This preliminary study is focused only to one aspect included in the genotoxic disease syndrome, i.e. the reduction of DNA integrity in erythrocytes of fish affected with varying water quality in their habitats.

The alkaline comet assay was previously confirmed as an initial indicator of general, non-specific DNA damage/genotoxicity and an effective biomarker for environmental monitoring (Mitchelmore & Chipman 1998a,b, Frenzilli et al. 2004). It has great potential to estimate DNA damage in fish because neither metaphases nor knowledge of the chromosome numbers are required (Belpaeme et al. 1998). As it involves the analysis of single cells, inter-cell variability in responses may be also studied. Interaction of genotoxic agents with DNA forms strand breaks but also alkali labile adducts and other modifications, which due to enzymatic removal of damaged nucleotides can contribute to an increased level of DNA strand breaks. All the above-mentioned types of primary DNA damage could be sensitively detected by the alkaline comet assay, as used in our study. Overall comparison of main comet parameters measured in the erythrocytes of Balkan loach revealed the presence of significantly higher levels of DNA damage in fish captured from the Sava River at the polluted Site 1 (Ivanja Reka), suggesting the genotoxicity of this aquatic environment.

It is known that complex mixtures such as wastewaters and surface waters are composed of a multitude of chemical substances (Reifferschied & Grummt 2000). Sediments also are a sink for anthropogenic contaminants and may act as a pollution source for bottom-dwelling organisms. Many pollutants, including potential genotoxic polyaromatic and chlorinated substances, have been detected in water and sediment samples (Kamman

Table 2. Fish morphometric data and average DNA damage in erythrocytes of Balkan loaches collected in rivers Sava and Kupa and in positive control.

No.	Fish morphometric data			Comet tail length (μm)		Tail intensity (DNA %)		Tail moment	
	TL	SL	W	Mean ± SE	min-max	Mean ± SE	min-max	Mean ± SE	min-max
Sava River – Site 1									
1	121	106	8.2	18.47 ± 0.44	9.62 – 29.49	6.33 ± 0.56	0 – 20.50	0.86 ± 0.08	0 – 3.08
2	116	104	6.8	16.22 ± 0.65	8.97 – 39.74	2.54 ± 0.55	0 – 33.61	0.40 ± 0.10	0 – 6.25
3	85	76	2.4	13.72 ± 0.43	8.33 – 34.61	1.73 ± 0.38	0 – 26.26	0.23 ± 0.05	0 – 3.54
4	96	86	3.6	13.18 ± 0.41	8.33 – 28.85	1.87 ± 0.33	0 – 16.02	0.22 ± 0.04	0 – 2.16
5	92	82	3.0	13.24 ± 0.41	8.33 – 32.69	1.53 ± 0.33	0 – 22.23	0.20 ± 0.05	0 – 3.56
6	94	84	5.6	14.30 ± 0.32	8.33 – 21.15	0.85 ± 0.16	0 – 7.61	0.11 ± 0.02	0 – 0.98
7	122	106	10.7	17.99 ± 0.79	7.69 – 46.79	4.68 ± 0.62	0 – 36.67	0.67 ± 0.10	0 – 6.11
8	101	90	7.3	15.04 ± 0.47	8.33 – 32.05	3.35 ± 0.48	0 – 32.18	0.44 ± 0.08	0 – 6.19
9	102	91	6.0	19.11 ± 0.76	8.97 – 41.67	7.37 ± 0.99	0 – 40.21	1.19 ± 0.17	0 – 7.56
10	106	97	8.4	13.44 ± 0.36	8.33 – 25.64	1.73 ± 0.43	0 – 30.10	0.22 ± 0.06	0 – 4.25
Group means	103.5 ± 4.00	92.2 ± 3.37	6.2 ± 0.83	15.47 ± 0.18	7.69 – 46.79	3.20 ± 0.18	0 – 40.21	0.45 ± 0.03	0 – 7.56
Sava River – Site 2									
1	115	102	5.0	11.27 ± 0.37	8.33 – 29.49	1.06 ± 0.18	0 – 13.39	0.11 ± 0.02	0 – 1.63
2	120	107	5.9	13.14 ± 0.29	8.97 – 21.79	2.25 ± 0.24	0 – 9.98	0.24 ± 0.03	0 – 1.04
3	135	118	9.2	12.82 ± 0.45	7.69 – 27.56	0.95 ± 0.16	0 – 8.77	0.11 ± 0.02	0 – 1.08
4	110	97	5.3	11.03 ± 0.29	8.33 – 23.08	1.36 ± 0.18	0 – 11.06	0.13 ± 0.02	0 – 1.13
5	113	98	4.5	13.26 ± 0.43	8.33 – 27.56	1.96 ± 0.31	0 – 20.60	0.23 ± 0.04	0 – 3.17
6	98	86	3.1	11.42 ± 0.36	7.05 – 24.36	0.79 ± 0.15	0 – 9.75	0.09 ± 0.02	0 – 1.19
7	134	118	9.5	11.64 ± 0.35	7.69 – 25.00	0.99 ± 0.18	0 – 10.30	0.11 ± 0.02	0 – 1.12
8	137	122	9.6	12.69 ± 0.34	7.69 – 23.72	1.93 ± 0.34	0 – 25.57	0.21 ± 0.04	0 – 2.46
9	95	84	3.6	12.20 ± 0.36	7.69 – 29.49	1.38 ± 0.25	0 – 12.71	0.16 ± 0.03	0 – 1.71
10	101	88	3.5	11.44 ± 0.29	7.69 – 21.15	1.19 ± 0.17	0 – 8.40	0.13 ± 0.02	0 – 0.97
Group means	115.0 ± 4.92	102.0 ± 4.41	5.9 ± 0.81	12.09 ± 0.12	7.05 – 29.49	1.38 ± 0.07	0 – 25.57	0.15 ± 0.01	0 – 3.17

Table 2. continued

Kupa River – Site 3									
1	140	125	10.3	11.22 ± 0.18	7.69 – 17.95	0.46 ± 0.07	0 – 4.56	0.05 ± 0.01	0 – 0.58
2	128	113	7.8	11.35 ± 0.16	8.33 – 17.31	0.35 ± 0.10	0 – 8.96	0.04 ± 0.01	0 – 0.84
3	130	115	8.2	10.53 ± 0.21	7.05 – 17.31	0.36 ± 0.10	0 – 6.52	0.04 ± 0.01	0 – 0.79
4	117	105	6.5	11.21 ± 0.13	7.69 – 15.38	0.51 ± 0.06	0 – 2.34	0.05 ± 0.01	0 – 0.26
5	109	97	5.6	10.52 ± 0.16	7.69 – 16.03	0.36 ± 0.05	0 – 2.45	0.04 ± 0.01	0 – 0.31
6	110	98	4.9	11.90 ± 0.26	7.05 – 17.95	0.89 ± 0.17	0 – 9.41	0.10 ± 0.02	0 – 1.15
7	126	112	9.3	10.63 ± 0.13	8.33 – 17.95	0.36 ± 0.06	0 – 3.08	0.04 ± 0.01	0 – 0.32
8	105	92	3.8	11.20 ± 0.20	7.69 – 18.59	0.39 ± 0.08	0 – 3.20	0.04 ± 0.01	0 – 0.38
9	100	88	3.0	10.53 ± 0.16	8.33 – 17.31	0.34 ± 0.07	0 – 5.94	0.04 ± 0.01	0 – 0.88
10	140	125	11.2	11.01 ± 0.12	8.33 – 16.03	0.34 ± 0.05	0 – 2.91	0.04 ± 0.01	0 – 0.37
Group means	120.5 ± 4.92	107.0 ± 4.14	7.1 ± 0.87	11.01 ± 0.06	7.05 – 18.59	0.43 ± 0.03	0 – 9.41	0.05 ± 0.003	0 – 1.15
Positive control (0.1 mM H_2O_2 *ex vivo*)									
1	140	125	10.3	25.49 ± 0.62	12.82 – 41.02	17.41 ± 1.20	0 – 48.95	2.88 ± 0.21	0 – 9.22
2	128	113	7.8	23.99 ± 0.57	11.54 – 36.54	14.57 ± 1.14	0 – 44.36	2.38 ± 0.20	0 – 8.32
3	130	115	8.2	43.00 ± 1.05	12.18 – 66.02	32.36 ± 1.21	2.38 – 49.64	7.50 ± 0.38	0.49 – 15.58
4	117	105	6.5	41.27 ± 0.74	16.67 – 55.13	31.22 ± 1.02	5.04 – 48.52	6.62 ± 0.29	0.71 – 13.56
5	109	97	5.6	40.58 ± 0.59	16.03 – 56.41	35.74 ± 0.98	8.41 – 48.94	7.60 ± 0.26	1.34 – 12.64
6	110	98	4.9	38.34 ± 0.68	17.95 – 69.23	30.53 ± 0.95	5.66 – 48.99	5.89 ± 0.26	1.45 – 12.61
7	126	112	9.3	34.88 ± 0.74	11.54 – 54.49	31.83 ± 1.22	1.24 – 48.89	6.28 ± 0.30	0.18 – 11.91
8	105	92	3.8	33.04 ± 0.55	17.31 – 46.79	28.90 ± 1.00	3.10 – 47.92	5.50 ± 0.23	0.58 – 10.44
9	100	88	3.0	38.82 ± 0.74	18.59 – 49.36	35.18 ± 1.02	5.65 – 49.82	7.10 ± 0.28	0.83 – 13.53
10	140	125	11.2	29.22 ± 0.79	14.74 – 51.92	23.04 ± 1.18	1.24 – 46.72	3.82 ± 0.24	0.21 – 11.89
Group means	120.5 ± 4.92	107.0 ± 4.14	7.1 ± 0.87	34.86 ± 0.30	7.05 – 29.49	28.08 ± 0.41	0 – 49.82	5.56 ± 0.10	0 – 15.58

Legend: TL – total length (mm); SL – standard length (mm);W – weight (g).

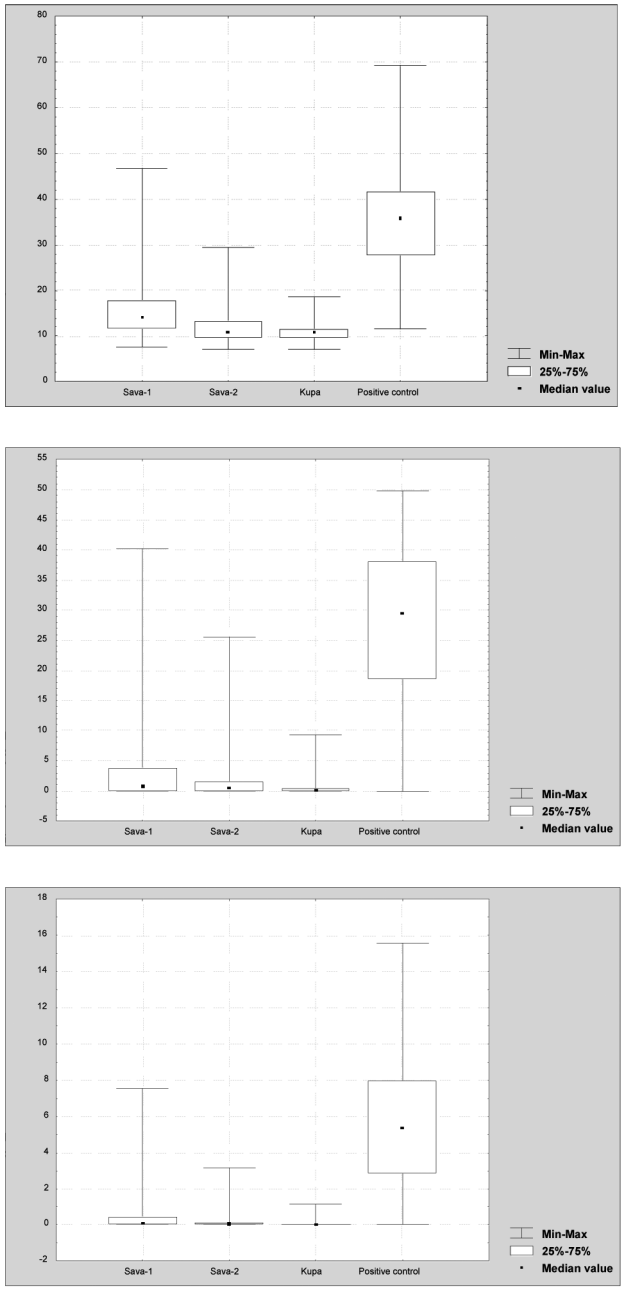

Fig. 2. Comparison between the values of three mean comet parameters recorded in erythrocytes of Balkan loaches collected at polluted sites in the Sava River, the reference site in the Kupa River, and positive control (0.1 mM H_2O_2, *ex vivo*).

et al. 2000, B u s c h i n i et al. 2004). Based on the results obtained, it is not possible to reveal a single chemical in the Sava River that causes the reduced DNA integrity observed in the present study. However, they imply that synergistic effects from a combination of

chemicals cause DNA damage and possibly also have an effect on immune response in loaches collected at the polluted sites. This assumption is also sustained by the results of the chemical analysis of water, showing increased concentrations of certain genotoxic metals (Sr, Hg, As, Cu, Cr, Mn) in municipal waters at the location Ivanja Reka (Site 1). In previous studies, cadmium and mercury are considered two of the most toxic metals which toxicity and genotoxicity for fish are reported (Ayllon & Garcia-Vazquez 2000, Risso-de Faverney et al. 2001). Genotoxic and carcinogenic effects of arsenic and copper have also been well documented (Reifferschied & Grummt 2000, Gabbianelli et al. 2003). Fish are recognized as ideal indicators of heavy metal contamination in aquatic systems because they occupy different trophic levels and are of varying size and age (Gabbianelli et al. 2003).

It is important to stress that the level of DNA damage as detected by the alkaline comet assay is not limited to influence by exogenous environmental pollutants only. Oxidative DNA damage alone contributes to background levels of DNA damage and is also relevant to the secondary effects of many pollutants. The metabolism of several pollutants, heavy metals among them, generates reactive oxygen species that can attack cellular macromolecules as DNA, fatty acids, carbohydrates and proteins, leading to serious damage (Livingstone 2001, Gabbianelli et al. 2003, Mamaca et al. 2005).

Our results indicate inter-individual differences in erythrocyte DNA damage, but these were more pronounced in the group of loaches collected from the Sava River. This variability was pronounced more when the values of tail intensity among animals were compared. It is well known that the comet tail length usually reaches a plateau after migrating a certain distance. After this maximum was reached, the tail would not extend in length, but grow in intensity (Bowden et al. 2003). The DNA damage variability could be explained by different genetic susceptibility, DNA repair activity, the number of alkali-labile sites, metabolic activity, antioxidant concentrations and other factors known to vary among individuals (Mitchelmore & Chipman 1998a,b, Akcha et al. 2003, Buschini et al. 2004). In this study, possible variability in DNA damage between the sexes was not investigated. However, previous comet assay studies on fish suggested controversial data on the involvement of sex in regulation of DNA primary damage (Devaux et al. 1998).

When using fish for biomonitoring purposes, it is also important to consider the possible influence of migratory movements (Akcha et al. 2003). Many aspects of the biology and ecology of Balkan loaches still remain poorly investigated. Loach movements in the Sava River and its tributaries seem to be limited in the study area. It is also supposed that they do not exhibit a clear spatial segregation of juveniles and adults, and no distinct migratory movements associated with the reproduction process have yet been described for the Balkan loach (Mrakovčić, personal comm.).

Although the majority of fish species avoid long-term exposure to pollutants dissolved in the water at a particular site by actively swimming, some territorial species have a more stationary behaviour and thus may be exposed for longer time. This was assumed to be the case for the Balkan loach, a benthic species that feeds on bottom-dwelling organisms. Consequently, in addition to direct accumulation of pollutants from water, it is also exposed indirectly by feeding. Considering our previous studies, populations of Balkan loach are surprisingly abundant in many polluted sites along the Sava River, especially as juveniles. Cotelle & Ferard (1999) suggested that sediment-feeders are especially suitable and recommended for biomonitoring studies.

The estimation of genotoxic effects is essential to any comprehensive study of pollutants in the aquatic environment. Although based on a relative small data set, our results confirmed high sensitivity of the comet assay for the detection of DNA damage in erythrocytes of Balkan loach. They are also promising for further standardization and the use of the comet assay on fish in environmental risk assessment.

The present study showed a good DNA damage pattern for Balkan loach affected by water pollution, thus proving this species is a potential bio-indicator of genotoxicity. However, due to its global (data deficient, Ref. 53964) and regional (vulnerable) conservation status, only the restricted use of a small number of specimens per sampling site could be permitted. Since the Balkan loach is considered particularly susceptible to pollutant exposure, results of the present study will be useful for future work involving the biomonitoring of regions inhabited by this fish. Moreover, they will help in the assessment of its genetic susceptibility necessary to be known to minimize threats to this vulnerable species and improve strategies for its protection.

A c k n o w l e d g e m e n t s

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