Genotoxic and histopathological effects of the Ili River (Kazakhstan) water pollution on the grass carp Ctenopharyngodon idella

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ABSTRACT

In the present study, genotoxic, histological and hematological effects of water pollution were evaluated in Ctenopharyngodon idella from the Ili river. Water and fish were sampled at three sites. The concentrations of the following heavy metals were measured in the water: Pb, Co, Mg, Cd, Cu, Zn, Fe. Water pollution with metals gradually increased from P1 to P3: in P1, Cu and Fe levels exceeded the maximum permissible concentrations for fish culture, in P2 – Pb, Cu, Zn, Fe, and in P3 – Pb, Cd, Cu, Zn, Fe. In fish from the Ili river, the highest frequency and severity of DNA damage and liver damage were noted in P3, the lowest in P1. Gill lesions were more pronounced and frequent in fish from P3 compared to grass carp from P2 and P1. Fish from P1 showed a higher frequency of neutrophils and a lower percentage of lymphocytes compared to the control. The results also revealed: genotoxicity measured by comet analysis and liver histology were the most sensitive and showed the magnitude of lesions directly related to the level of water contamination. Gill histology also clearly showed pathological changes caused by pollution, while differential leukocyte count was the least useful indicator, as it showed only minor differences between fish from unpolluted and polluted water.

1. Introduction

The aquatic ecosystem is the ultimate sink for many chemicals used in industry and agriculture, and thus aquatic pollution has become a global environmental problem [1]. Some chemicals present in industrial, agricultural and domestic effluents are highly toxic, depending on the dose and duration of exposure [2], having the potential to cause serious damage to aquatic organisms [3]. Therefore, fisheries and aquaculture are among the industries most vulnerable to aquatic pollution.

Heavy metals are potentially genotoxic and carcinogenic [4]. They have a devastating effect on the ecology of the host–environment balance and the diversity of aquatic organisms [5]. Water and sediment contamination with heavy metals and metalloids poses a serious threat to aquatic life due to their toxicity, long-term persistence, as well as bioaccumulation and bioamplification in the food chain [6]. The treatment of toxic industrial effluents is crucial for the protection of receiving reservoirs [7]. Bioaccumulation of heavy metals can cause stress and diseases in aquatic biota, increased mortality and extinction of sensitive taxa, and disturbance of ecological balance [8]. Morphological, cytological, histological and histopathological changes in various organs of the body in response to freshwater pollution were reported in fish [9].

Exposure of fish to waterborne metals is associated with their absorption and accumulation by the body, which leads to metal-induced disorders in the structure and function of various tissues and organs [10]. Fish are excellent objects for evaluation of the absorption, metabolism and storage of metals. They also show histopathological, mutagenic or carcinogenic changes caused by metal intoxication [11,12]. Therefore, fish are often used as bioindicators of water quality and biomonitors for the presence of metals and other pollutants [13].

The toxic effects of pollutants can manifest themselves at the cellular or tissue level before visible symptoms of intoxication occur [14]. The complexity of contaminants requires sensitive and reliable quantitative tools to assess toxicity. A powerful approach to analysis that evaluates DNA damage, both quantitatively and qualitatively, is the comet assay [15]. Comet assay is a widely used reliable method for determining genotoxicity (DNA damage) in aquatic organisms [16] that can be applied to the blood cells. Comet analysis, which in various modifications can reflect various DNA damages, has been successfully applied in fish because measurements are made on individual cells [17–19].
Histological analysis is another method commonly used to assess the effects of exposures to toxic substances in fish. Histopathological biomarkers react to biotic factors and water quality, so they are reliable indicators of environmental stress [20]. Histopathology can serve as a biomarker for environmental monitoring and studies of specific vital organs [21].

The Ili river rises in Xinjiang Province of Western China and flows into the Western Balkhash, Kazakhstan [22]. The river is interrupted by several small dams in China and one large dam at Kapchagay in eastern Kazakhstan. This dam and Kapchagay Reservoir adversely affected the downstream Ili River by altering the hydrological cycle and increasing pollution load which resulted in reduced fish yield. Commercial catch of *Ctenopharyngodon idella* in the Ili river declined from 14.9 tons in 2010 to 0.7 ton in 2017 [23].

The present study was designed to evaluate genotoxic, histopathological and hematological effects of Ili river water pollution on grass carp, *Ctenopharyngodon idella*, a freshwater cyprinid herbivorous fish of commercial value.

2. Materials and methods

2.1. Study area

The length of the Ili river is 1,439 km, of which 815 km (56.6%) is on the territory of the Almaty Region of Kazakhstan. The objects of study were water samples taken at three sites (P1, P2 and P3) located along the river: P1 and P2 upstream of the Kapchagay Reservoir and P3 downstream. The location of water sampling points is shown in Figure 1.

2.2. Chemical analysis of water

Water samples were collected in June 2021, at a depth of 25 cm from the surface and near the bottom of each point (P1, P2 and P3). For sampling, polyethylene bottles (500 ml) were used and the surface and bottom samples were pooled. Immediately after collecting the samples, the samples were cooled to 4°C, transported to the laboratory of Al-Farabi Kazakh National University, Faculty of Biology and Biotechnology, and analyzed within 24 hours. The analysis included measurements of concentrations of Pb, Co, Mg, Cd, Cu, Zn and Fe using atomic absorption spectrophotometry [24]. The samples of water were acidified and then subjected to acid leaching and concentration for analysis by flame atomic absorption spectrophotometry (using the Varian-AA50B model). The water temperature was 22.06–24.5°C, the pH was 7.80–8.14, the concentration of dissolved oxygen in the water was 9.0–12.2 mg/dm³.

2.3. Fish sampling

Thirty-seven specimens of *Ctenopharyngodon idella* juveniles were used in the study, nine fish from each sampling point and 10 control specimens from the fishery pond Togan-4, Almaty Region, Enbekshikazakh District. The fish had an average total length of 16.6 cm.

Figure 1. Schematic map of the Ili river research area.
± 2.07 cm and a wet body weight of 157.05 g ± 13.70 g. The fish were harvested in September 2021 and transported alive in plastic bags with water to the research laboratory. Blood samples were collected by heart puncture. The blood was immediately transferred to 5 ml heparinized tubes to prevent blood clotting. After blood sampling, the fish were sacrificed and liver and gill tissues were collected, fixed in 4% formaldehyde and processed using standard histological methods [25].

2.4. Comet assay

For the analysis, 20 ml of blood cell suspension was mixed with 100 ml of 0.5% solution of low-melting agarose (type IV) in a phosphate-salt buffer with a pH of 7.4 at a temperature of 37°C and applied to slides pre-coated with a 1% layer of norm-melting agarose. Cell lysis was carried out by moving the slides into a cold lysing buffer (2.5 mol/l NaCl, 100 ml Na₂EDTA, 20 mol/l tris-HCL with pH10,1%, triton X-100 and 10% dimethyl sulfoxide (DMSO)) for 2 h. After cell lysis, the slides were placed in cold (40°C) tris borate TVE buffer with pH 8.2 (Sigma Aldrich) and kept for 20 min. Electrophoresis was performed in a TVE buffer at a voltage of 1.5 V/cm for 20 min. SYBR Green I (Sigma Aldrich) was used for DNA staining. Visualization of DNA comets was carried out under a microscope with an estimate of the magnitude of the ‘tail moment’ of the Olive (MHO). Microscopic analysis of the obtained preparations was carried out under a fluorescence microscope with an ×200 – ×400 magnification. Percentage of DNA in the ‘tail’ was evaluated and used as an estimate of the level of DNA damage (Figure 2).

In each group, blood samples from two to three fish were analyzed and 36–91 DNA comets without overlapping tails were evaluated in each preparation. Total comet score (TCS) was calculated as a percentage of cells showing DNA damage.

2.5. Histopathological analysis

The tissues were fixed in a 4% solution of paraformaldehyde phosphate and embedded in paraffin in accordance with the standard protocol [25] (Humason, 1979). Sections of 5 μm thickness were obtained using a microtome (Accu-Cut® SRM™ 200 Sakura, Japan). The sections were placed in a water bath (M-1450 Sakura, Japan) at a temperature of 56°C, so that they floated above the surface of the water and the folds were removed. For proper attachment of the sections, the egg white was applied to clean slides. The sections were installed above the slide and placed on the slide for hot drying in a plate (mod. 1452, Sakura, Japan) for 30–40 minutes, followed by placing in an oven with hot air for 2–3 hours for drying and removing excessive paraffin. After final drying, the slides with

![G₁ - practically intact cells (5% of DNA in the "tail")](image1)

![G₂ - low level of damage (5-20%)](image2)

![G₃ - medium level of damage (20-40%)](image3)

![G₄ - high level of damage (40-95%)](image4)

![G₅ - completely damaged cells (more than 95%)](image5)

Figure 2. Various levels of DNA damage as a percentage of DNA in the ‘tail’.
tissue sections were stained with hematoxylin and eosin (H&E) in accordance with the methods provided by Bio-Optica (Milan, Italy) staining kits. In each fish, 150 hepatocytes, 12 primary lamellae and 494 secondary lamellae were randomly selected from each image and analyzed individually for quantitative histopathological evaluation using a Leica microscope with a Leica DC 300 camera connected to the BioVision 4.0 computer image analysis system.

2.6. Differential leukocyte count

Thin smears of freshly collected blood were made, dried, preserved with methanol and stained with May-Grunwald and Giemsa solutions. The slides were fixed with Histokitt (Karl Hecht GmbH & Co KG, Sondheim/Rhon, Germany) and cover glasses. Differential leukocyte counts were performed at ×1000 magnification using Nikon Eclipse 300 (Japan) microscope and the

**Figure 3.** Histology of liver of grass carp from Ili river and control group. Normal hepatocytes (HP) with one nucleus (n) each, sinusoid (S) and hepatocytes with pyknotic nuclei (PN), pancreatic vein (P), blood vessel (BV), FC (fatty change), hydropic degeneration (HD), N (necrosis) and degeneration of pancreatic tissue (DP).
results were shown as a percentage of neutrophils, lymphocytes and monocytes (100 leukocytes in each smear were viewed).

2.7. Statistical analysis
Statistical analysis was performed using IBM SPSS 25 statistics (IBM Inc., Chicago, USA). The means and standard deviations of means of the data were calculated. Homogeneity of variance was tested using Levene’s test, one-way analysis of variance and Tukey’s HSD test were used to evaluate the significance of differences in multiple comparisons. The significance level was set at p < 0.05.

3. Results

3.1. Heavy metal concentrations in water
In the samples of water, the content of the following heavy metals was determined: Pb, Co, Mg, Cd, Cu, Zn and Fe and average values from three samplings were compared with the maximum permitted concentrations for fishery reservoirs (Table 1) [26]. Concentrations of Pb at P3 and P2 were 8.7 and 5.3 times higher than MPC, respectively. Co and Mg levels at all three sites were below the MPC, while Cd at P3 exceeded MPV 5.7 times. Cu, Zn and Fe were higher compared to MPC at all three sites from 1.7 (Fe at P1) to 5.3 times (Cu at P2).

3.2. Genotoxicity
The results of comet assay revealed that fish from the control group showed only 17.5% of erythrocyte nuclei at the lowest stage of DNA damage, while 82.5% of cells remained intact (Table 2). In grass carp from the Ili River most cells showed DNA damage (from 70.3% at P1 to 100% at P3). Frequency and severity of DNA damage increased with the river course and pollution level (from P1 to P3).

3.3. Histopathology
The liver, due to its function as a detoxification site, is considered a target organ of xenobiotics. Histopathological analysis revealed that in fish from all three sites of the Ili river, the frequency of abnormal hepatocytes was significantly higher compared to the control. Hydropic dystrophy and progressive fat deposition, degenerative changes, pyknotic nuclei, and especially an increase in vacuolation of hepatocytes, as well as hepatocyte necrosis were the most common pathological changes (Fig. 3, 4). Similarly, as genotoxic effects, the highest frequency and severity of hepatic histopathological lesions were observed in fish from the P3 in which hepatocyte necrosis predominated. In fish from the P2, the percentage of damaged hepatocytes was also high, but they showed mainly hydropic degeneration. The lowest frequency of pathological hepatocytes occurred in fish from the P1 in which necrosis and hydropic degeneration were equally frequent. Frequency of all types of hepatocyte lesions significantly differ among groups, except for hepatocytes with pyknotic nuclei, the frequency of which did not significantly differ between P1 and P3.

The gills showed a normal arrangement of primary and secondary lamellae in the control group in which no histopathological lesions were observed. Primary lamellae or gill filaments were arranged perpendicularly and along the gill arch, while secondary lamellae were thin and slightly curved, arranged in bilateral symmetry with respect to the first (Fig. 5, 6). Histopathological analysis revealed the pronounced changes in the gills of grass carp from all three sampling sites of the Ili river. The most frequent gill changes were lamellar degeneration
and epithelial necrosis, sometimes leading to fusion of the lamellae and elevation and exfoliation of the epithelium. The highest frequencies of all gill lesions occurred in the P3 group and it was the only group in which fusion of the primary lamellae was observed. The frequency of respiratory epithelium exfoliation and necrosis of secondary lamellae was significantly higher in fish from the P3 site compared to the P2 and P1.
Figure 6. Frequency [\%] of histopathological gill lesions in grass carp from three points of the Ili river and the control (mean ± S.D., n = 4, ANOVA, Tukey HSD test, different letter superscripts indicate significant differences at p < 0.05).

Table 1. Metal concentrations (mean ± S.D.) in water samples taken at three different points of the Ili River.

| Sampling points | Pb µg/ml  | Co µg/ml  | Mg µg/ml  | Cd µg/ml  | Cu µg/ml  | Zn µg/ml  | Fe µg/ml  |
|-----------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| P1              | 0.2 ± 0.5 | 0.6 ± 0.5 | 6.0 ± 2.0 | 1.7 ± 1.7 | 3.9 ± 0.8*| 46.7 ± 0.3| 166.7 ± 0.3*|
| P2              | 53.3 ± 5.0*| 1.7 ± 1.0 | 5.3 ± 3.0 | 1.7 ± 1.0 | 5.3 ± 4.0*| 33.3 ± 0.2*| 266.2 ± 0.0*|
| P3              | 87.0 ± 7.0*| 3.3 ± 2.0 | 3.9 ± 3.0 | 28.7 ± 6.0*| 3.7 ± 0.5*| 43.3 ± 0.1*| 266.7 ± 0.9*|
| MPV             | 10.0      | 10.0      | 100.0     | 5.0       | 1.0       | 10.0      | 100       |

*The values exceeding the maximum permitted value MPV

Table 2. Percentage of blood cells showing various degrees of DNA damage (detected using comet assay) in Ctenopharyngodon idella from three points of the Ili river (±S.D.). The mean values differ significantly (ANOVA, Tukey HSD, different letter superscripts indicate significantly different values, p < 0.05).

| DNA damage/Points | \(G_0\) | \(G_1\) | \(G_2\) | \(G_3\) | \(G_4\) | TCS |
|-------------------|---------|---------|---------|---------|---------|-----|
| Control           | 82.5 ± 0.7*| 17.5 ± 0.7*| 0.0 ± 0.0*| 0.0 ± 0.0*| 0.0 ± 0.00*| 17.5 ± 0.7*|
| P1                | 29.7 ± 2.1 | 27.7 ± 3.1 | 24.3 ± 6.8 | 12.7 ± 4.7 | 5.7 ± 3.1 | 70.3 ± 2.1 |
| P2                | 18.0 ± 5.0 | 30.7 ± 3.1 | 21.0 ± 4.4 | 24.7 ± 1.5 | 5.7 ± 1.2 | 82.0 ± 5.0 |
| P3                | 0.0 ± 0.0 | 27.0 ± 1.4 | 26.5 ± 3.5 | 35.5 ± 2.1 | 11.0 ± 0.0 | 100 ± 0.0 |

TCS – total comet score.

Figure 7. Differential leukocyte count in grass carp from the control group and three sites of the Ili river (means ± S.E., n = 3, ANOVA, Tukey HSD test, different letter superscripts indicate significant differences at p < 0.05).
3.4. Differential leukocyte count

Differential leukocyte count showed lesser differences among the fish groups compared to the genotoxic and histopathological parameters (Figure 7). In all groups, neutrophils predominated and their frequency was significantly higher in P1 compared to the control, while the percentage of lymphocytes was significantly lower. The percentage of monocytes was the highest in P3 and the lowest in the control but no significant differences occurred.

4. Discussion

The Ili river is widely used for agricultural purposes, and numerous settlements are located along its valley. Measurements of the content of Pb, Co, Mg, Cd, Cu, Zn and Fe showed that Pb level exceeded the maximum permissible concentration (MPC) for fishery water bodies at two points (P3 and P2), Cd at one point (P3), while the levels of Cu, Zn and Fe were higher than MPC at all three sites. Out of these metals, Pb and Cd are xenobiotics, while Cu, Zn and Fe are essential elements but toxic at high concentrations [27]. Toxicity of these metals to cyprinid fish is Cu > Cd > Zn > Pb > Fe and it is in accordance with the ranking developed by Shuhaimi-Othman et al. [28].

Heavy metals are known to cause DNA damage [29]. High concentrations of metals can bioaccumulate which leads to oxidative damage and mutagenicity in exposed organisms [30,31]. Genotoxic changes caused by metals can be determined as the levels of DNA damage using comet assay performed on fish erythrocytes [32]. In the grass carp from the Ili River most of the cells showed DNA damage (from 70.3% at P1 to 100% at P3). DNA damage in erythrocytes of Labeo rohita, Cirrhina migratoria, Catla catla and Ctenopharyngodon idella exposed to As, Cu and Zn was also reported by Matsumoto et al. [33]. Genotoxicity of As, Cu, Zn, Cd and P in Clarias gariepinus was observed by Javed and Kousar [34]. According to Farombi et al. [35], Astyanax lacustris showed significant genotoxic damage after exposure to stream water polluted with a mixture of metals. According to Pereira da Silva et al. [36], urban reservoir water pollution with Cr, Ni and Pb caused genotoxic effects in Channa striatus and Heteropneustes fossilis.

Histopathological examination is important for assessing morphological changes in cells and tissues. The liver and gills are vital organs of detoxification and accumulation of toxic substances. Histopathological biomarkers are useful to evaluate the effects of pollutants on fish [37]. Heavy metals may adversely affect the structure and functions of various organs of aquatic animals, often causing necrosis of hepatocytes, fusion of gill lamellae and inducing genotoxicity [38,39]. In the present study, histopathological analysis revealed pronounced changes in hepatocytes of grass carp from all three sites of the Ili. Frequency and magnitude of lesions were proportional to the level of water pollution. The most frequently observed pathological changes were hydropic dystrophy and progressive fat deposition, degenerative changes and especially increased vacuolation of hepatocytes, as well as their necrosis. Similar results were obtained by Ahmed et al. [40] who reported degenerative changes, pyknotic nuclei, increase in hepatic hepatocyte vacuolation and hepatocyte necrosis in the liver of the grass carp. Degenerative changes of hepatocytes, including vacuolation and pyknosis, were detected by Khan et al. [41]. Shah et al. [42] reported hepatic melanomacrophages and hepatocyte necrosis in grass carp due to the bioaccumulating action of Cu, Cr and Pb. The abundance of melanomacrophages increased with increasing concentration of trace metals and exposure.

Considerable changes in the gills of the grass carp from all three sampling sites of the Ili river were also observed. The most frequent changes in the gills were lamellar degeneration (interruption) and necrosis of the epithelium, sometimes leading to fusion of the lamellae, elevation, and a slight detachment of the epithelium. The gills are the important target organ for waterborne pollutants, and they are the main route of metal absorption [43]. Similar changes, lamellar degeneration and elevation of the respiratory epithelium, were described, and a histopathological study showed that higher concentrations of aflatoxin B1 (AFB1) in grass carp caused pathological changes and structural and functional changes in liver, kidney, gill tissues [44]. Kaur et al. [45] showed that gills of grass carp exposed to chlorpyrifos depicted necrosis of epithelial and columnar cells, distortion of secondary lamellae and increased vacuolation. Locally, the primary gill lamellae were detached from the base. Histological changes such as epithelial lifting, interlamellar spaces, swelling and fusion of lamellae, abnormal cells, destruction of epithelial cells, cellular necrosis and inflammatory cells were observed in grass carp exposed to various doses of heavy metals [46]. The damage may be a result of protective reaction of the gills to prevent the uptake of toxic substances [47].

Leukocytes are effector cells of immune response; thus, their number and percentage of particular cell types are indicators of the immune status of an organism. However, differential leukocyte count showed less pronounced effects of exposure to polluted water compared to genotoxic and histopathological endpoints. In all groups, including the control, neutrophils predominated over lymphocytes which is not a typical
phenomenon in fish including grass carp [48–50]. Significant differences in the percentage of lymphocytes and neutrophils occurred only between the control and the fish from P3 (the least polluted site). According to da Silva et al. [51], Oreochromis niloticus from polluted water showed more monocytes and eosinophils compared to the control. In contrary Shah et al. [46] reported increase in lymphocyte frequency and decrease in monocytes and neutrophils in grass carp intoxicated with Cu, Cr and Pb. According to Witeska [52], exposure of Cyprinus carpio to Pb, Cu, Cd or Zn caused a decrease in lymphocyte count, while Pb and Cd caused a considerable increase in the count of neutrophils.

Comparison of sensitivity of different toxicity endpoints used in the present study: genotoxicity, hepatic and gill histology and differential leukocyte counts showed that genotoxic effect measured using comet assay and quantitative histological examination of hepatocytes were the most responsive to aquatic pollution and the magnitude of anomalies was directly related to the level of water pollution. Gill histology is also a sensitive indicator of toxicity. Differential leukocyte count was the least useful endpoint.

5. Conclusion

This study demonstrated the genotoxic and histopathological effects of water pollution with metals in Ctenopharyngodon idella from the Ili river water of the urban area of Almaty. Chemical analysis showed the presence of anthropogenic contamination with high concentrations of Cu, Zn, Fe, Pb and Cd, most increasing with the river course. The water pollution level was the highest downstream the Kaphchay Reservoir. The observed significant genotoxic and hepatic histopathological alterations were directly related to the level of pollution. Considerable gill lesions were also observed in fish from the river compared to the control. Only slight effects of pollution on differential leukocyte count occurred which make this parameter little useful for evaluation of toxic effects in grass carp. However, further studies, particularly of the most sensitive developmental stages of grass carp, embryos and larvae, are necessary to elucidate the effects of the Ili river pollution on the population of this species.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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