

Impact of acid mine drainage on haematological, histopathological and genotoxic effects in golden mahaseer, *Tor putitora*

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Abstract

The present study was carried out to evaluate sub-lethal mechanism of acid mine drainage toxicity in fingerlings (9.5 ± 2.4 cm) of golden mahseer, *Tor putitora*. Exposed fingerlings showed significant reduction ($P < 0.01$) in blood erythrocytes, neutrophils, thrombocytes, lymphocytes and leukocytes in contrast to increase in number of immature circulating cells. Hyperplasia, degeneration of glomeruli, presence of inflammatory cells and increased number of melanomacrophage aggregates, vacuolization of cell cytoplasm, hepatocyte swelling were marked in kidney and liver of fish. Ladder in, an increment of 180-200 bp of hepatic and kidney DNA, by electrophoresis were consistent with DNA damage. 10 day exposure to acid mine drainage resulted in reduction of double stranded DNA to 46.0 and 48.0 in hepatocytes and kidney cells respectively. Significant increase ($P < 0.01$) in tail length and percent tail DNA was evident by comet assay. The results suggest that exposure to acid mine drainage might cause irreversible damage to immune cells, tissue and DNA of fish, and this model of DNA damage may contribute in identifying novel molecular mechanism of interest for bioremediation application.

Key words

Acid mine drainage, Aquatic toxicology, DNA damage, Immune cells

Introduction

Acid mine drainage is coal mining's most severe threat to environment worldwide, and mine draining acid can destroy rivers, streams, and aquatic life for forever. Many acid mine drainage impacted water bodies have pH < 4, with high sulfur and metals such as aluminium and iron contamination. Contaminated water can be toxic to aquatic organisms, thus leaving streams devoid of any living creatures and so water bodies receiving acid mine drainage is poor in taxa richness and abundance and often dominated by few tolerant organisms (Baldigo *et al.*, 2009).

Sporadic studies have been carried out to investigate toxicity of acid mine drainage on aquatic organisms,

especially on fish (Kaeser and Sharpe, 2001). Toxicity is primarily due to low pH and increased Al concentrations. Very low pH has been reported to restrict fish distribution (Mallik *et al.*, 2015). Acid mine drainage is formed by series of complex geochemical and microbial reactions that occur when water and air come in contact with pyrite (FeS₂) present in coal and exposed rock to form sulphuric acid and dissolved iron. The process of pyrite oxidation further leads to formation of Fe³⁺ and some or all of this iron can precipitate to cause red, orange or yellowish color of water and sedimentation at bottom of the stream. This "acid flow" or acid mine drainage aggravates with rich in dissolved metals may alter the physico-chemical parameters of the environment, adversely affecting the health of rivers and streams (Husain and Barik, 2004).

Tor putitora is a cyprinid fish endemic to Asia with natural distribution encircling the trans-Himalayan region. This fish is now identified as critically endangered species as depletion of mahseer populations has been reported from various parts of India and other Asian countries (Hussain and Mazid, 2001). *Tor putitora* is not only attractive as a sport fish, but it also has tremendous aquaculture potential.

Since the data on the coalmine toxicity to fish is very limited in India, the present study was focused on haematological, histopathological and genotoxic responses in *T. putitora* following a short-term exposure to acid mine drainage. The toxic effects of acid mine drainage on fish at cellular and genetic level, when exposed for short duration at sub-lethal concentration is evaluated. Although, long-term impact is important, the short-term exposure is a direct evidence of the acid mine drainage stress on fishes.

Materials and Methods

Acid mine drainage samples (6nos) were collected along the Simsang River at Nongal Bibra (25° 27.2' N 90° 42.1'E), East Garo Hill, Meghalaya, India in 20 l polyethylene cans and brought in chilled condition to the laboratory of ICAR-Directorate of Coldwater Fisheries Research (ICAR-DCFR), Bhimtal for necessary analysis. Standard methods were employed for estimation of physico-chemical parameters of acid mine drainage samples (APHA, 2005). pH, dissolved oxygen and temperature were measured *in-situ* using a multi-parameter probe (HI 9828, Hanna Instruments Inc., Romania), whereas quantitative estimation of trace metals was carried out using atomic absorption spectrophotometer (ANALYST 800, Perkin-Elmer, USA).

Experimental design: *T. putitora* fingerlings with an average length and weight 9.5±2.4 cm and 7.5±1.8 g were acclimatized for 10 days (6-8 fish per tank; specific density < 1.5 g of fish per litre of water) under laboratory condition, before the experiment. Fifteen fish were randomly distributed in aquarium 60 x 35 x 30 cm size and 60 l capacity. The experiment was carried out in triplicate with at least one control group. Fish were fed twice daily with a supplementary feed having 35% crude protein. Except control, all fish were exposed to 25% diluted acid mine drainage for 8 and 10 days. Control fish were kept in pretreated ground water. Water quality parameters of treatment groups were monitored daily, and the mean values recorded were as follows: temperature 20.0 ± 1.81 °C, pH 5.4 ± 0.43, dissolved oxygen 7.9 ± 1.20 mg l⁻¹, nitrite 0.05 ± 0.01 mg l⁻¹, nitrate 2.91 ± 0.31 mg l⁻¹ and ammonia < 0.01 ± 0.006 mg l⁻¹. At the end of 8 and 10 days, fish were sacrificed for haematological, histopathological and genotoxicity analysis.

Haematological parameters: Blood was collected from

caudal vein of three randomly selected fish per tank using heparinised needles and syringe for hematology parameters. Smear was prepared immediately after blood collection, air dried, fixed with methanol and stained by May-Grunwald Giemsa method. Slides were subsequently examined under a microscope (LEICA DM2000). Three slides per fish were taken for counting erythrocytes, leukocytes and other blood cell using a hemocytometer. Hemoglobin was determined within 2 hr of sampling using Drabkin's fluid (Qualigens, India).

Histopathological assay : Three fish from each tank were taken after 10 days of exposure for histopathological observation. Fish were sacrificed by overdose of tricaine methane sulfonate (200 mg l⁻¹ MS-222). Kidney and liver tissues were collected and placed in 10% neutral buffered formalin, followed by processing for paraffin wax embedding. Sections were cut at 4µm thickness with microtome (Leica RM 2245), and stained with haematoxylin and eosin (HandE). The stained sections were examined under a light microscope (LEICA, DMLS, TK-C1380E).

Genotoxicity assay : Three fish from each tank were collected and sacrificed as previously mentioned for genotoxicity analysis.

DNA ladder assay : Liver and kidney of *T. putitora* was rinsed with ice-cold phosphate buffer saline (PBS, pH 7.2), and lysed in buffer containing 0.1M Tris-Cl (pH 8.0), 1 M NaCl, 0.01 M EDTA (pH 8.0) for 30 min at 60 °C. RNase A was added, and sample was incubated at 68 °C for 10 min followed by centrifugation. DNA in supernatant was extracted using phenol/chloroform/ isoamyl alcohol and then precipitated using chilled isopropanol followed by centrifugation. DNA pellet was washed in 70% ethanol, dissolved in nuclease free water, and quantified spectrophotometrically (Qubit 2.0 Fluorometer, Invitrogen, Life Technologies). Three microgram of liver and kidney DNA was loaded in each well of 1.5% agarose gel containing 0.1 mg ml⁻¹ ethidium bromide (EtBr). The gel was photographed under ultraviolet light in gel documentation system (Gel doc XR⁺, Biorad).

Comet assay: Comet assay was carried out by the method of Singh *et al.*, (1988).

Fluorometric analysis of DNA unwinding (FADU): FADU was carried out by the method described by Bimboim and Jevcak (1981). Briefly, liver and kidney cells were lysed and chromatin was disrupted with a urea solution. DNA strand unwinding was allowed to occur at 15 °C for 75 min after adding an alkaline solution. The extent of DNA unwinding was measured at 15 °C for different durations: 0, 15, 30, 45, 60 and 75 min.

Statistical analysis : Statistical analysis was carried out using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Differences among treatment groups were tested by one-way analysis of variance (ANOVA) at significant level of $P < 0.01$.

Results and Discussion

Physico-chemical parameter of AMD, collected from Nongalbibra, Meghalaya is shown in Table 1. Water was light yellow-to-yellow reddish in color with $\text{pH} < 3.0$ with high sulfate, Fe and Al contamination. Water quality deteriorated due to acid mine drainage. Water seeping out from coalmines are very hazardous to the environment and any living creature due to low pH, and presence of Al and Fe due above permissible limit. At low pH, fish died to acidemia and toxicity of metals, especially Al as primary toxicant. The pH of water was below tolerance limit of fish fauna, and at Nongal Bibra fish fauna was completely absent. Similar condition of restricted biota inhabiting acid mine drainage has been reported worldwide (Cherry *et al.*, 2001; Schorr and Backer, 2006). Recruitment failure is commonly reported cause of fish population decline associated with acidification. Fish generally do not inhabit water severely polluted by coalmine drainage as CO_2 in water with pH values < 4.2 is free of CO_2 . Without buffering capacity from carbonates and bicarbonates, many aquatic animals die due to acute acidemia. Additional sources of toxicity of this water was sulfate and salts of iron and aluminium. The average concentration of acid mine drainage water was 390.00 mg l^{-1} and ranged as high as 480.63 mg l^{-1} while average Al concentration was 46.21 mg l^{-1} and ranged as high as 82 mg l^{-1} . Average sulfate concentration was 1230 mg l^{-1} and ranged as high as 1870 mg l^{-1} . At this site, pH was low ($\text{pH} < 3.0$), which explains the reason for such high level of trace metals in acid mine drainage water. At low pH, heavy metals are more soluble and enter water bodies from limestones, clays, rocks and organic substances present in mine drainage. Clay has ion exchange property and can absorb heavy metals, which only works at pH between 4-10 and below this range, the ion exchange process may not occur. In acid mine drainage water from Nongal Bibra, metal concentrations were equal to or greater than the level usually considered toxic to most fish species. Similar results of high concentration of metals and low pH were reported from acid mine drainage throughout the world (Druschel *et al.*, 2004; Kampe *et al.*, 2010). The apparent low species diversity provided evidence that conditions in Simsang river were stressful and toxic.

Total erythrocytes, thrombocytes, neutrophils, lymphocytes and leukocytes counts (Table 2) and number of immature cells were significantly higher ($p < 0.01$) in blood of exposed fish, relative to similar blood cells type counted from non-exposed fish. The hematocrit and monocytes cell

count didn't vary significantly between exposed and control groups. Adverse environmental conditions and water pollutants affect blood cells of fish. Therefore, evaluating fish blood parameters might be a useful tool for understanding the impact of extreme water conditions on fish health. Assessing the effects of acid mine drainage on blood cells related to immune functions, an idea could be drawn to understand the possible immuno-suppressive effect of this heavily polluted water. Following short-term exposure of acid mine drainage to golden mahseer, major changes in blood parameters related to immune functions could be noticed. In the exposed group, number of neutrophils were lower than control fish, and significant differences were observed in the number of circulating leukocytes and lymphocytes. In contrast, the number of immature cells was higher in acid mine drainage exposed group (Table 2). The presence of significantly higher number of circulating immature cells, as observed in the present work suggested that acid mine drainage might be directly toxic to leukocytes, and it might mitigate immune function by reducing leukocyte population. Therefore, in acid mine drainage receiving streams, river and lakes, fish may be more prone to infectious diseases due to immune suppression. The deleterious effect of acid mine drainage on immune cells should be a matter of further investigation.

On microscopic examination, liver and kidney of golden mahseer revealed severe damage to these two organs in acid mine drainage exposed group. Kidney cells were degenerated with increase in melanomacrophage aggregates (Fig. 1a, b). Necrosis, profusion of inflammatory cells,

Table 1 : Physico-chemical parameters of acid mine drainage collected from Nongal Bibra, Simsang River, Meghalaya, India.

| Parameters | Values |
|---|--------------------|
| Temperature ($^{\circ}\text{C}$) | 25.03 \pm 3.20 |
| pH | 2.40 \pm 0.32 |
| DO (mg l^{-1}) | 4.26 \pm 0.93 |
| Conductivity ($\mu\text{m s}^{-1}$) | 136.0 \pm 9.50 |
| TDS (ppm) | 68.00 \pm 11.64 |
| NH_4^+ (mg l^{-1}) | 0.36 \pm 0.04 |
| NO_2^- (mg l^{-1}) | 0.16 \pm 0.01 |
| NO_3^- (mg l^{-1}) | 4.18 \pm 0.94 |
| PO_4^{3-} (mg l^{-1}) | 0.83 \pm 0.08 |
| Alkalinity (mg l^{-1}) | 26.40 \pm 3.61 |
| Hardness (mg l^{-1}) | 96.00 \pm 4.82 |
| Sulfate (mg l^{-1}) | 1230 \pm 98.32 |
| Potassium (mg l^{-1}) | 12.58 \pm 0.59 |
| Fe (mg l^{-1}) | 390.16 \pm 27.90 |
| Al (mg l^{-1}) | 46.21 \pm 3.94 |
| BOD ₅ (mg l^{-1}) | 9.45 \pm 2.80 |

DO, Dissolved oxygen; TDS, Total dissolved solid; BOD, Biological oxygen demand

Table 2 : Haematological parameters of control and exposed *T. putitora* fingerlings for 8 and 10 days to 25 % of acid mine drainage water

| Blood parameters* | Control | Exposed | |
|--|------------------------|------------------------|------------------------|
| | | 8 days | 10 days |
| Hematocrit (%) | 37.4±3.1 ^a | 34.9±2.0 ^a | 35.9±2.0 ^a |
| Erythrocytes (x 10 ⁶ μl ⁻¹) | 1.59±0.06 ^a | 1.26±0.04 ^b | 1.03±0.04 ^b |
| Immature cells (%) | 0.20±0.03 ^a | 0.71±0.06 ^b | 0.86±0.06 ^b |
| Thrombocytes (x 10 ³ μl ⁻¹) | 5.43±0.8 ^a | 4.21±0.72 ^b | 3.31±0.72 ^b |
| Total leukocytes (x 10 ³ μl ⁻¹) | 5.31±0.6 ^a | 3.06±0.14 ^b | 2.61±0.14 ^b |
| Neutrophils (x 10 ³ μl ⁻¹) | 0.29±0.09 ^a | 0.09±0.03 ^b | 0.07±0.03 ^b |
| Monocytes (x 10 ³ μl ⁻¹) | 0.40±0.16 ^a | 0.31±0.04 ^a | 0.29±0.04 ^a |
| Lymphocytes (x 10 ³ μl ⁻¹) | 4.69±0.18 ^a | 2.09±0.31 ^b | 1.91±0.31 ^b |

The results represent mean ± SE for each category. Different superscripts (a, b) in same row indicate significant statistical difference ($P < 0.01$)

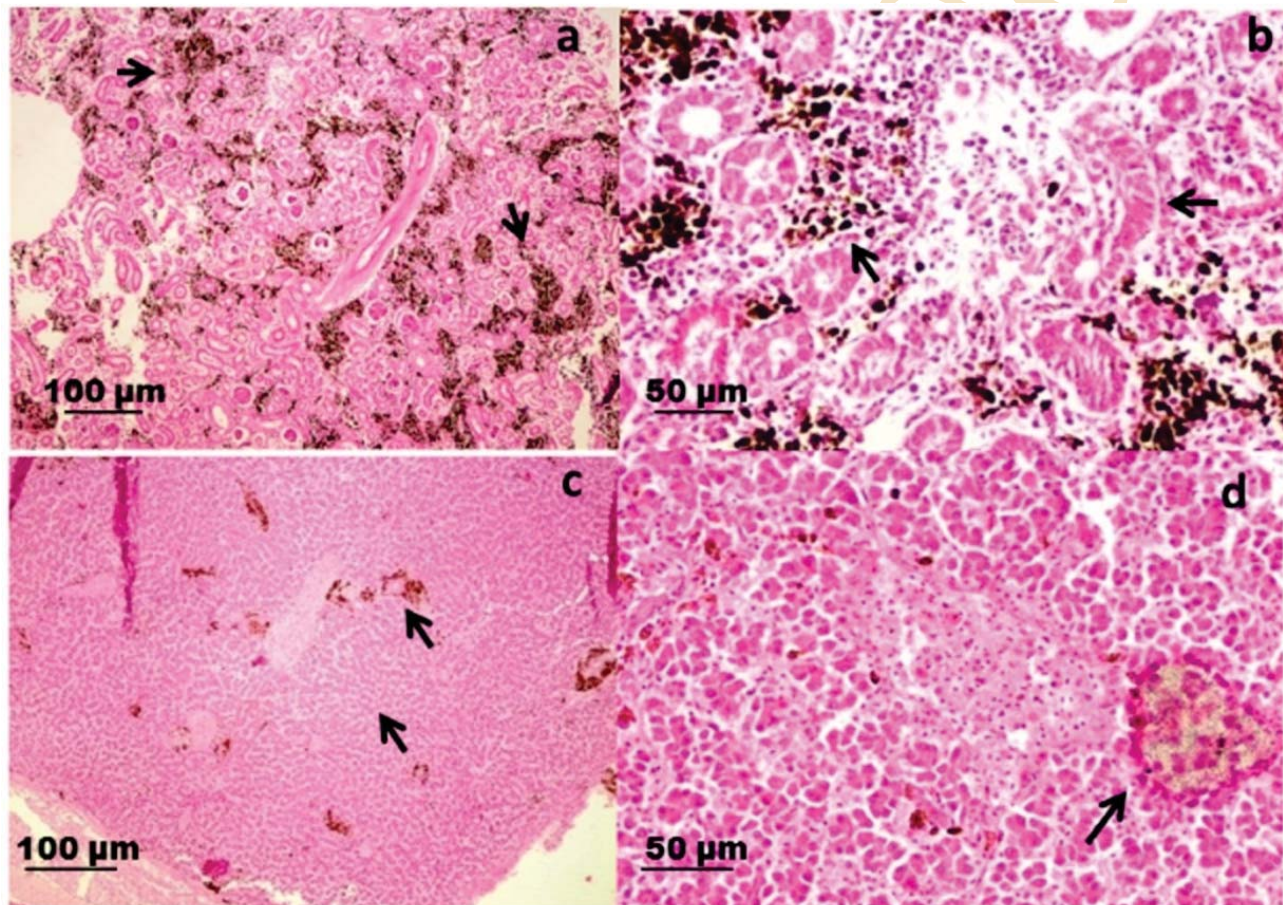


Fig. 1 : Histopathology of the kidney and liver tissue of *T. putitora* exposed to acid mine drainage water for 10 days. (a) Kidney of fish showing tubular and glomeruli degeneration (40X); (b) Kidney had congestion and hemorrhage with increased number of melanophages (200X); (c) hepatocytes had focal necrosis with infiltration of inflammatory cells (40X); (d) nuclear atrophy and increase in size of melano-macrophage centres (MMC) (200X)

nuclear atrophy, degeneration of hepatocytes, cytoplasm vacuolation and hypertrophy in hepatocytes of exposed fish were also recorded (Fig. 1c, d). Fish inhabiting polluted environment can reflect degraded environmental conditions through altered activity of immune system or nonspecific

defenses. Change in size of melano-macrophage centres (MMC) can occur in association with environmental pollutants. Wolke *et al.* (1985) persuasively advocated MMC as biomarker for assessing the effects of environmental exposure to pollutant chemicals. Although environmental

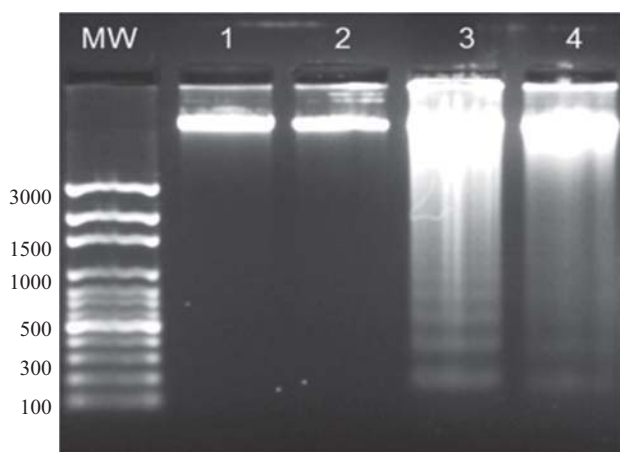


Fig. 2 : Agarose gel electrophoresis of DNA from liver and kidney of acid mine drainage water exposed and control fish. Lane assignment; MW corresponds to a 100 bp ladder DNA marker (Axygen Biosciences, USA), Lane 1 and 2 is DNA from the unexposed fish of organ liver and kidney, respectively. Lane 3 and 4 is DNA from liver and kidney of fish exposed to 25% acid mine drainage water. DNA was extracted 10 days after exposure. Numbers to the left of the figure indicates DNA size in base pairs (bp)

pollutants may directly cause fish mortalities, sub-lethal effects are more common (Kalff, 2002). A number of physiological and pathological responses have been suggested as indicators of environmental contamination. Liver of acid mine drainage exposed fish has apparent MMC, which clearly indicates that even after dilution; acid mine drainage water is highly toxic to fish. Prominent MMC in the hepatocytes of fish might be due to exposure to metals (Long *et al.*, 1995; Couillard and Hodson 1996; Meinelt *et al.*, 1997). Therefore, MMC can provide sensitive indicators of stressful conditions in aquatic environment of coalmine drainage area. The acid water and trace metals also caused in vacuolization and necrosis in liver parenchyma. These alterations are often associated with degenerative-necrotic condition. There are reports that chronic accumulation of some heavy metals in fish liver causes hepatocyte lysis, cirrhosis and eventually death (Varanka *et al.*, 2001). Similar condition was not observed in the present study as fish were exposed for shorter duration. As in higher vertebrates, kidneys of fish perform an important function in electrolyte and water balance. Following exposure of fish to toxic agents, histological alterations have been found at the level of tubular epithelium and glomeruli. Ortiz *et al.* (2003) found that renal lesions might be good indicator of environmental pollution.

DNA isolated from liver and kidney of exposed golden mahseer had DNA laddering (Fig. 2). However, DNA isolated from liver and kidney of control fish did not exhibit

laddering of low molecular weight DNA, indicating that the procedure did not produce artifactual breakdown of DNA during the isolation process. DNA laddering showed an increment of 180-200 bp in both the organs. Fluorometric analysis of DNA unwinding method is based on the rate of alkaline unwinding of DNA and the effect on DNA unwinding at 15 °C for different duration for liver and kidney (Fig. 3a and b), respectively. The percentage of double-stranded DNA (DSD) in liver and kidney of control fish varied from 86 to 84.4 and 81.0 to 74.0 respectively. DNA sample in both the organs of treated fish showed significant decrease in DSD percent. In liver and kidney of exposed fish, the percentage of DSD was 82.0 to 46.0 and 78.0 to 48.0, respectively. With increase in duration of treatment, there was linear decrease in percentage of DSD. This indicates that the effect was time dependent. Comet TL and % tail DNA in hepatocytes and kidney cells of fish exposed to AMD (Fig. 4a, b, c and d) for 8 and 10 days. Exposure to AMD led to significant increase in TL as compared to control, but no time-related effect of AMD on TL was detected. TL and % tail DNA in hepatocytes and kidney cells did not differ among fish treated for two different durations. DNA laddering assay, FADU and comet assay are simple and sensitive technique for analyzing and quantifying DNA strand breakage and is commonly applied to fish cells for detecting genotoxicity of a wide range of chemicals. The mechanism of genotoxicity induced by heavy metals is quite complex. The metal induced toxic effects mainly include DNA-protein cross-links, DNA strand breaks and oxidative DNA damage etc. Moreover, heavy metals are positively charged ions and easily bind with DNA or nucleophilic sites to cause mutagenesis. DNA strand breakage is a sensitive indicator of genetic damage, and a useful tool for monitoring the genotoxic effect of pollutants on aquatic organisms. Comet assay is a simple and sensitive technique for analyzing and quantifying DNA strand breakage and is widely applied to the cells of aquatic organisms, including fish, to detect genotoxicity of a wide range of chemical agents. In the present study TL and % tail DNA, the most frequent DNA damage indicators, were significantly increased when fish were exposed to 25 % acid mine drainage water for 8 and 10 days. The results suggested that acid mine drainage had potential to induce DNA damage in liver and kidney of fish at low concentration. Comet TL and % tail DNA did not show an increase trend with increasing durations. The acid mine drainage was heavily contaminated with metals, as exposure to Al and Fe is known to produce comets in organs of fish (Pulsford *et al.*, 1992). Gel electrophoresis analysis of DNA isolated from liver and kidney of exposed fish indicated that acid mine drainage induced random fragmentation and degradation typical of apoptosis, which might be due to activation of DNA endonuclease. However, further studies on the exact sequences of molecular events by which acid

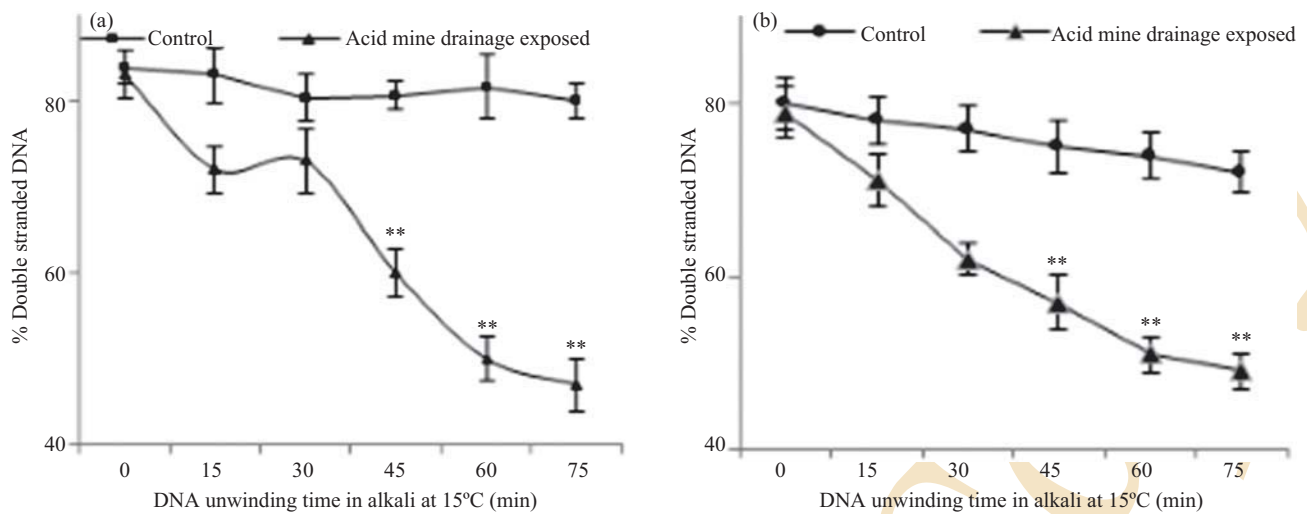


Fig. 3 : The kinetics of DNA unwinding of control and acid mine drainage (AMD) exposed (10 days exposure period) *T. putilora* in alkali at 15°C in (a) liver cell and (b) kidney cell suspension. Percentage of double-stranded DNA (DSD) remaining after unwinding at different durations. Control fish are not exposed to AMD. Significance at **P < 0.01 by student t-test

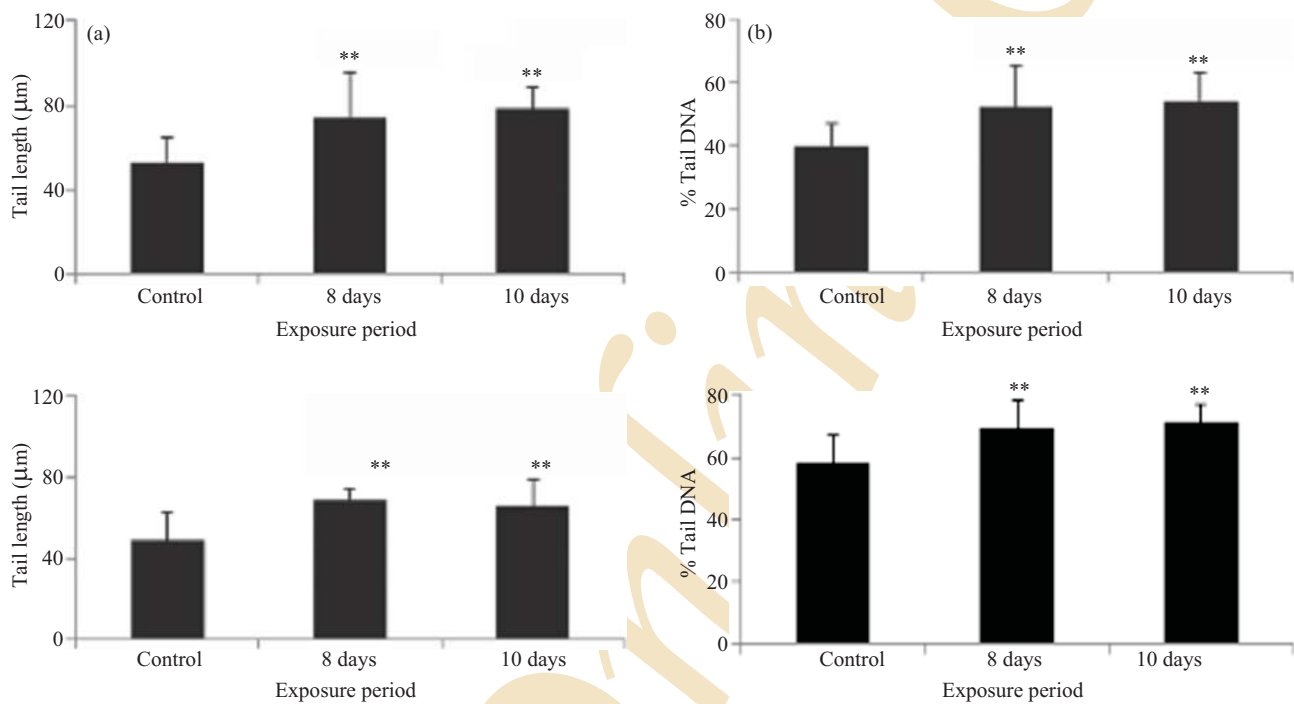


Fig. 4 : Comet tail length (TL) and percentage tail DNA in hepatocytes (a and b) and kidney (c and d) of *T. putilora* after 8 and 10 days of exposure to acid mine drainage water from Nongal Bibra, Simsang River, Meghalaya. Each bar represents the mean \pm standard error of mean (SEM). Three comet slides were prepared for each fish, and 40 comets were counted from each slide. Significant difference from the control is designated by **P < 0.01

mine drainage causes DNA fragmentation *in vivo* and *in vitro* are necessary. In FADU assay, reduced DNA integrity for fish was observed. The rate of DNA unwinding of DNA from liver and kidney of control and exposed fish were compared. The acid mine drainage produced readily observable effects unwinding rate. An apparent decrease in percentage of DSD

with time liver and kidney cells of treated fish was observed in the present investigation. A comparison between the exposed animals and their corresponding 'time controls' provided evidence that exposure to acid mine drainage did induce DNA strand separation. These strand separation at DNA level is probably one of the key mechanisms for

causing DNA damage.

Acid mine drainage generated from coal mine is very toxic in cellular and genetic level and the pollutant is capable of suppressing immune cells and inducing DNA damage in vivo. Findings of this study are of direct and importance relevance for local authorities to remediate environmental pollution by initiating some activities on habitat restoration.

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