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# Cytotoxic and genotoxic affects of acid mine drainage on fish *Channa punctata* (Bloch)

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ABSTRACT

The investigation deals with the effects of Acid Mine Drainage (AMD) of coal mine on fish *Channa punctata* (Bloch) by examining the incidence of haematological, morphological, histological changes and DNA fragmentation in tissues of *C. punctata* in laboratory condition. For this study fishes were exposed to 10% of AMD for a period of 30 days. The fusion of the primary and secondary gill lamellae, distortion, loss of alignment, deposition of worn out tissues and mucous on the surface of the lamella in the gills; degeneration of morphological architecture, loss of alignment of tubules, mucous deposition in the kidney; cellular damage, cellular necrosis, extraneous deposition on the surface, pore formation in the liver are some important changes detected by scanning electron microscopy. Fishes of AMD treated group showed gradual significant decrease in TEC, Hb and, increase in TLC and DLC as compared to that of the control. DNA fragmentation observed in kidney of fishes from treated group indicates an intricate pollutant present in the AMD. The high incidence of morphological and histological alterations, haematological changes along with DNA breakage in *C. punctata* is an evidence of the cytotoxic and genotoxic potential of AMD of coal mines.

## 1. Introduction

The deposition of coal in Meghalaya, India occurs along the southern fringe of Shillong plateau and distributed in Khasi, Garo and Jaintia Hills (Swer and Singh, 2004). East Garo Hills region is a major producer of coal in the North Eastern region of India. In Garo Hills coal mining is done by a primitive mining method called 'rat hole' mining which is crude, extravagant, vulnerable and unscientific. The coal is taken out through rate hole and cast off in nearby non mined area from where it is carried by trucks to the larger dumping places near highways for its trade. Entire road sides in and around the mining areas are normally used for piling of extracted coal which is a major source of pollution of air, soil and water (Sarma, 2010). The entire process of mining is done manually employing small outfit. Most of the mining activities are small scale ventures guarded by individuals who own the land. The unsystematic and unscientific mining, absence of post-mining treatment and management of mined areas are making the fragile ecosystems more vulnerable to environmental degradation hence leading to large scale land cover/ land use changes (Tiwari, 1996).

The prime cause of degradation of water quality and the declining trend of biodiversity in the water bodies of the mining area is attributed mainly to the AMD originating from mines, leaching of heavy metals, organic enrichment and silting by coal and sand particles, which makes water highly acidic and rich in heavy metal concentration (Pentreath, 1994). Excessive accumulation of AMD in this region since 1980's has altered the some area of the river devoid of any aquatic organism seasonally (Talukdar et al., 2015). AMD streams generally have lower pH, diverse blend of toxic metals, (Al, Fe, Mn, Zn, Cu, Ar, Pb), higher conductivity and higher sulphate concentrations (Grippo and Dunson, 1996). Heavy metal concentration in the coal mining areas may lead to its bioaccumulation in fish tissue (Canli et al., 1998), which leads to fish mortality. AMD is severe threat to environment which can destroy whole aquatic fauna and floras of the area through which it is drains and also has direct effect on fish by causing various physiological disturbances. The direct flow of AMD into the aquatic system makes water acidic and rich in certain heavy metals such as iron. Because of direct and continuous contact with the water body, different organs of fishes involved in many physiological processes such as respiratory gas exchange, osmoregulation, excretion of nitrogenous waste products and acid-base regulation are directly affected by contaminants present in the water. Since reports of AMD toxicity to aquatic ecosystem is very limited, present study deals with the studies on toxicological effects of AMD on haematological, morphological and, histological alterations and DNA fragmentation in fish *C. punctata*.

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## 2. Materials and methods

### 2.1. Collection and maintenance of fish

Healthy and sexually matured live freshwater fish *Channa punctata* (total length: 17 ± 2.5 cm, weight: 20 ± 1.5 g) were procured from market and disinfected with 0.1% solution of potassium permanganate for 1 min to avoid dermal infection. The fishes were allowed to acclimatize in a glass aquarium (25 L capacity) in the laboratory for one month. The water of the aquarium was also changed daily. Fishes were fed daily basis with commercial fish food to avoid effects of starvation.

### 2.2. Water analysis

Crude AMD samples were collected with five replicates near Nangalbibra site (longitude 90°44′39″E & latitude 25°28′22″N) and brought in chilled condition to the laboratory for further analysis. Dissolved oxygen (DO), conductivity and pH of AMD samples were determined immediately after collection using Systronics make digital water analyzing kit. Free $CO_2$, sulphate, nitrate and some of the heavy metals (Fe, Ni, Cd, Pb, Cu, Mn and Zn) were analyzed followed after APHA (2005).

### 2.3. Experimental design

$LC_{50}$ value of AMD for 96 h was determined (28% of the AMD) through probit analysis method (Finney, 1971) and 10% of the AMD was considered for experiment. Fishes were exposed to AMD for a period of 30 days to study the impact of short-term exposure of AMD. Along with that one control group of fishes were maintained in tape water. Each experiment was repeated for 3 times and for each experiment 5 fishes were used.

### 2.4. Light microscopic preparation

The tissues (gills, kidney and liver) were fixed in 10% formosaline fluid for 12 h and embedded in paraffin wax. Sections of 4 μm were cut with microtome and stained with hematoxylene and eosin (HE), for examination under the light microscope. Ten sections were analyzed per specimen and representative structural changes were photographed (Leica DM3000).

The presence of histological alterations for each organ was evaluated semi-quantitatively by the Degree of Tissue Change (DTC), which is based on the severity of the lesions. For DTC calculation (Poleksic and Mitrovic-Tutundzic, 1994), the alterations in each organ were classified in progressive stages of damage to the tissue: stage I alterations, which do not alter the normal functioning of the tissue; stage II, which are more severe and impair the normal functioning of the tissue; and stage III, which are very severe and cause irreparable damage. A value of DTC was calculated for each animal by the formula: DTC = (1 X *SI*) + (10 X *SII*) + (100 X *SIII*) where *I, II* and *III* correspond to the number of alterations of stages I, II and III, respectively. DTC values between 0 and 10 indicate normal functioning of the organ; values between 11 and 20 indicate slight damage to the organ; values between 21 and 50 indicate moderate changes in the organ; values between 50 and 100 indicate severe lesions and values above 100 indicate irreversible damage to the organ (Poleksic and Mitrovic-Tutundzic, 1994).

### 2.5. Scanning electron microscopic preparation

The tissues (gills, kidney and liver) were washed in distilled water and immersed in 2.5% glutaraldehyde (4 °C for 4 h), post fixation was done on 0.1 M sodium cacodylate buffer (4 °C for 15 min twice), dehydrated in acetone. The tissues were then dried with tetra methyl silane following the method of (Dey et al., 1989) and coated with gold plating.

### 2.6. Haematological studies

For haematological studies, blood was collected by severing off the caudal region of the fish. Clotting of blood was prevented by using EDTA (Drugs and Pharmaceuticals Ltd. Hyderabad). Hemoglobin content (Hb) was estimated by Sahli's Haemoglobinometer. Total erythrocyte count (TEC) and, total leukocyte count (TLC) were done by Neubaurs' Haemocytometer and differential WBC count (DLC) was done after staining the blood smear with Wright stain. The slides were subsequently examined under microscope (Leica DM3000).

### 2.7. DNA fragmentation assay

For DNA fragmentation assay DNA isolation was done as per method of Sambrook and Russel (Sambrook and Russel, 2001). Fresh kidney samples were lysed with the help of DNA lysis buffer and incubated at 37 °C for 24 h, followed by a 2 h incubation at 55 °C in the presence of 100 μg/ml of proteinase K, followed by addition of RNase A (10 μg/ml, 1 h at 37 °C). DNA was then extracted from fragmented samples, separated on 1.8% (w/v) agarose gels and visualised with ethidium bromide (6 μg/ml). Gels were illuminated with 300 nm UV light and photography was made in order to detect the qualitative damage to genomic DNA.

### 2.8. Statistical analysis

Mean values and standard deviation (SD) and standard error (SE) were calculated using MS Excel computer program. For all the experiments, *t*-test was performed and significance was assigned.

## 3. Results

### 3.1. Water analysis

Potential value of certain physico-chemical properties and heavy metal contains of AMD were analyzed as follows; low pH(2.7 ± 0.9), low dissolved oxygen, (4.5 ± 1.76 mg/l); high value of free $CO_2$ (26.3 ± 2.02 mg/l), nitrate (3.9 ± 1.76), high value of sulphate (758 ± 1.76), Fe (77 ± 9.23 mg/l), Ni,(9 ± 1.83 mg/l), Cd(2.7 ± 0.97 mg/l), Pb (5.21- ± 0.73 mg/l), Cu (3.83 ± 1.06 mg/l), Mn (2 ± 0.18 mg/l), Zn (15.6- ± 2.83 mg/l) and Cr(1.33 ± 1.54 mg/l) in comparison to prescribed standard (WHO, 2008).

### 3.2. Histopathological observations

The histological alterations found in the gills of the fishes exposed to AMD are depicted in Table 1. The severe changes were observed in stage I; includes dilation of the marginal channel, hyperplasia of the epithelial cells and lifting of the lamellar epithelium. In some cases where the hyperplasia was more prominent, fusion of some secondary lamellae has also been observed (Fig. 1b). Severe lesions were also observed in the gills (stage II) with lamellar aneurysms (Fig. 1c), hemorrhages with rupture of the lamellar epithelium and blood congestion (Fig. 1b). Stage III lesions were not observed in the gills of the studied fish. The degree of tissue change (DTC) for the gills of fish varies from 7.6 to 16.2 with a mean value of 10.6, indicates slight anomalies in the functioning of the organ (Table 2). Significant differences were detected in fishes after exposure and showed higher values of DTC than control. The alterations found in the kidney are shown in Table 1. The most important alternations found in the kidney were glomerular enlargement, resulting in enlargement of Bowman's space (Fig. 1f) and dilation of glomerulus (Fig. 1f). Tubular degeneration (Fig. 1e), melanomacrophage aggregation and hyaline droplet deposition were also observed. Very severe stage III lesions were not observed. In kidney DTC values ranged from 20.7 to 56.1, indicates moderate changes in the organ (Table 2). The main anomalies found in the liver

**Table 1**
Histopathological changes in the gills, kidney and liver of *C.punctata* indicating their respective stages of damage to the tissue. Stage I: do not alter the normal functioning of the tissue; Stage II: more severe and impair the normal functioning of the tissue; Stage III: very severe and cause irreparable damage.

| Stage | Gills | Kidney | Liver |
|---|---|---|---|
| I | Hyperplasia of gill epithelium | Dilation of glomerular capillaries | Cytoplasmic vacuolation |
| | Hypertrophy of gill epithelium | Glomerular enlargement | Irregular shaped cells |
| | Blood congestion | Cellular hypertrophy | Eosinophilic granules in the cytoplasm |
| | Epithelial Lifting of lamellae | Cytoplasmic vacuolation | Melanomacrophage aggregation |
| | Lamellar fusion | Dilation of tubular lumen | Haemaocyanin pigment accumulation |
| | | Melanomacrophage aggregation | Fatty infiltration |
| | Lamellar disorganization | | Increase of kupfer cells |
| | Lamellar shortening | | Widening of sinusoidal spaces |
| II | Rupture of epithelial lining | Bowman's space decrease | Cytoplasmic degeneration |
| | Haemorrhage | Blood in Bowman's space | Blood congestion |
| | | Hyaline droplet degeneration | Picnotic nucleus |
| | | Tubular degeneration | Blood vessel rupture |
| | | Blood vessel rupture | |
| III | | | Coagulative necrosis |

(Table 1) were; cytoplasmic vacuolation, fatty infiltration (Fig. 1h), presence of eosinophilic granules in the cytoplasm. Infiltration of lymphocytes (Fig. 1h) was identified. Cytoplasmic and nuclear degeneration was also very common; melanomacrophages were also detected. The majority of the alterations found in stage I and II, i.e. the tissue was moderately damaged, and recuperation was still possible as and when water quality improved. Only one stage III lesion was found (necrosis; Fig. 1i). This alteration caused damages to the tissues which was irreversible. The mean DTC for liver was 54.37 (values ranged from 30.3 to 165), indicates that in most cases, the hepatic lesions causes moderate to severe damage to the tissue (Table 2).

### 3.3. Scanning electron microscopy

Primary and secondary gill lamellae exhibited fusion, distortion, loss of alignment. Deposition of worn out tissues and mucous on the surface of the lamella and rackers were prominent. Rupturing and uplifting of the gill epithelium was observed in the exposed fishes. A complete disarrangement of primary and secondary lamellae was clearly visible in gills. Damaging of tissue especially at their tips and secondary lamellae (Fig. 2b) and epithelial membrane breakdown (Fig. 2c) on gills were also observed frequently. Scanning electron Microscopy of the kidney of fishes from control group showed the presence of well aligned tubules (Fig. 2d) while the kidney of fishes from exposed group exhibits abnormal structural features such as outgrowth observed at the surface of the tissue. Blood cell deposition (Fig. 2e), disturbance in alignment of the tubules (Fig. 2f) and some extraneous depositions on the surface were also seen. In contrast to the control group of fishes which have showed normal architecture of the parenchyma (Fig. 2g), liver from fishes of exposed group showed unusual outgrowths or tissue masses at certain locations, pore formation (Fig. 2h) and damage of the tissue at some portions, rough surface and distortion of different magnitude, Moreover, extraneous depositions are also seen on the surface (Fig. 2i).

### 3.4. Haematological observations

Fishes from AMD treated group showed gradual significant decrease in TEC, Hb and increase in TLC as compared to that of the control (Table 3). It has been observed from the DLC that there was an increase in the number of lymphocytes and basophills and decrease in the other cells i.e, eosinophils, monocytes and neutrophils.

### 3.5. Analysis of DNA fragmentation

The genomic DNA extracted from kidney samples of control group of fishes showed a very weakly stained smear like pattern, without any fragmentation (Fig. 3, Lane A). In contrast, a characteristic laddering mixed with a smear-like pattern observed in AMD treated group of fishes, revealed DNA fragmentation (Fig. 3, Lane B).

## 4. Discussion

The physical and chemical characteristics of water play a very important role in evaluation of whole scenario of the aquatic biota. Deterioration of water quality due to AMD is a major problem associated with the mining areas. The water from coalmine seeping out to the environment and cause hazardous environmental affects and living organisms present in that area. It is because of presence of high amount of sulphate and other heavy metals in the AMD with low pH. The pH of a water body is one of the most critical parameter necessary for survival of fishes. Fishes may die due to acidemia in water body having low pH because of loss of buffering capacity from carbonates and bicarbonates, this turn it increases the level of water free $CO_2$. Fish cannot survive in the AMD polluted water because, in addition to low pH, AMD polluted water has also high sulphate and heavy metals. Heavy metals may originate either from natural processes (such as weathering and erosion of bedrocks) or human activities. Natural erosion and weathering of crustal materials take place over long periods of time and the amount of heavy metals released is small (John et al., 1995). When compared to the natural exposure through erosion, the exposure rate through mining is over ten times faster (Massachusetts Institute of Technology, 1970). Leaching of mine tailings and drainage from mined areas can introduce substantial amounts of metals into the aquatic body (John et al., 1995).

Water parameters are one of the major factors responsible for individual variation in fish haematology. It has been reported that the blood parameters remarkably vary in different fishes and this is considered to reflect adaptations to the varied environment conditions (Ramaswamy and Reddy, 1978; Moyle and Cech, 1982). In the present study, *Channa punctata* after exposure to AMD showed decrease in the RBC count and Hb content. It has also been observed that the decrease in total erythrocyte count may be attributed to the toxic pollutant present in the AMD. The toxicants in the form of high amount of sulphate and some of the heavy metals coming from AMD influences the malfunctioning of the haemopoetic system of fishes. Therefore, the haemopoetic tissues fail to release the blood cells which subsequently released into the blood stream. The observed depiction in the hemoglobin content could be attributed in the lysis of erythrocytes and significant destruction in hemoglobin is an indication of anaemia (Soivio and Oikari, 1976). A significant increase of lymphocytes and decreased in neutrophil and eosinophil observed in present investigation might be due to immunological reaction to produce more antibodies to cope up with the stress induced by the AMD.

Fish physiology can reflect degraded environmental conditions through altered immune response or the nonspecific defenses. Fish gills play a very important role in gaseous exchange, osmoregulation, acid base balance and excretion of nitrogenous compound (Olson, 1991) and particularly sensitive to changes in the quality of the water are considered the primary target of the contaminants (Mazon et al., 2002), Fernandes and Mazon (2003)]. Water quality changes can cause distortion, epidermal detachment, fusion of gills and necrosis of rackers etc, causing tremendous stress to the fish in its respiratory and other functions (Acharya et al., 2005). The detachment of the lamellar epithelium is the first sign of pathology in fish (Thophon et al., 2003). In

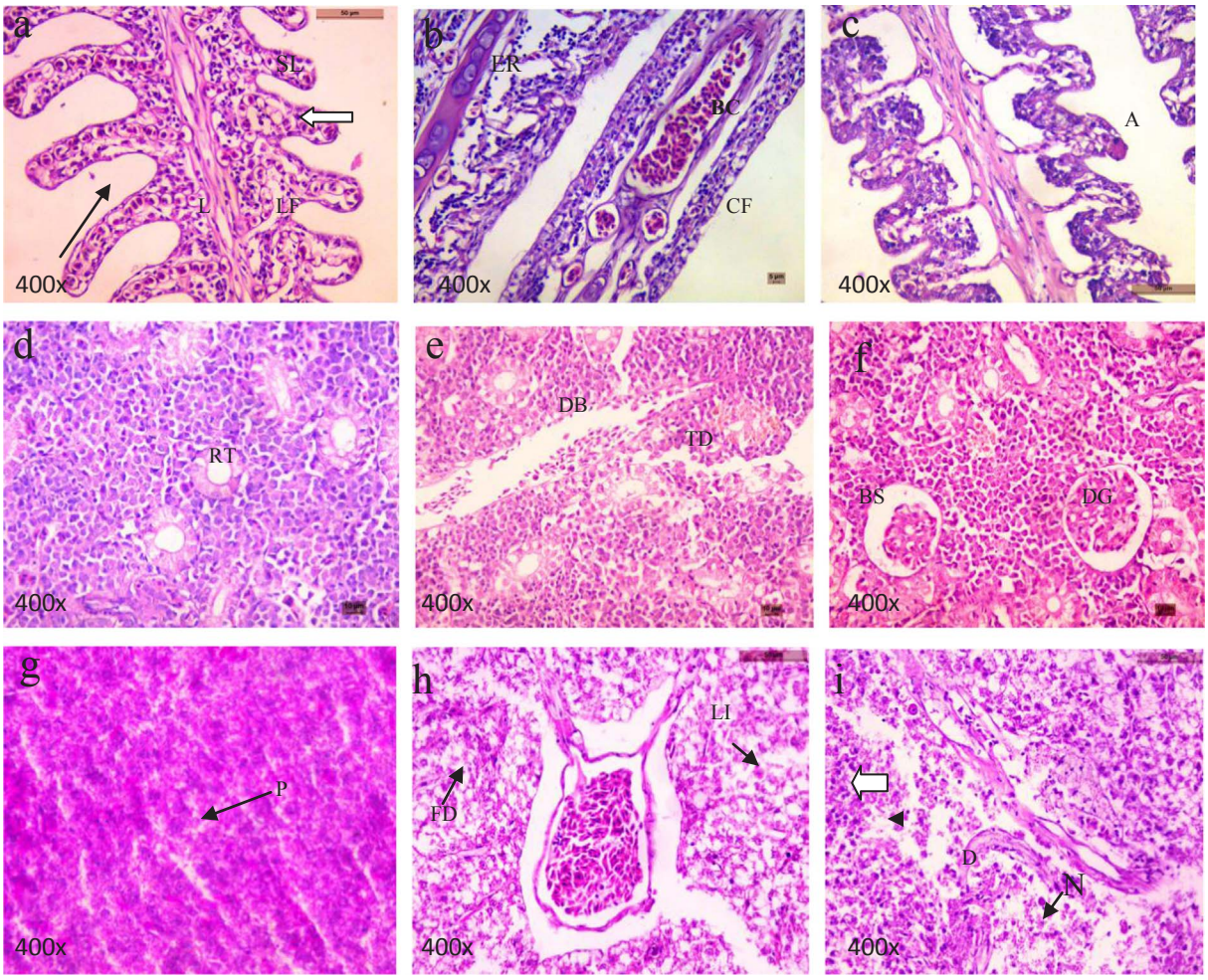


**Fig. 1.** Photomicrographs of Gills, kidney and liver of *C. punctata.* (a) gill showing normal primary lamellae (L), secondary lamellae (SL), lamellae filament (LF), pillar cell (white arrow), and water channel (black arrow) of control group of fish; (b-c) gills of fish treated with AMD showing; blood congestion (BC), complete fusion of some lamellae (CF), epithelium rupture (ER), lamellar aneurysm (A).(d) kidney of fish showing normal renal features i.e., many renal tubules (RT) and hematopoietic tissue in between the tubules in control group of fishes; (e-f) kidney of fishes treated with sub-lethal concentration of AMD showing dilated blood vessel (DB), tubular degeneration (TD), dilation of glomeruli (DG), enlargement of Bowman's space (BS); (g) showing normal liver architecture, liver parenchyma (P), hepatocytes (arrow) with round nucleus and prominent nucleolus in control group; (h-i) liver of fishes treated with sub-lethal concentration of AMD showing fatty infiltrations (FD), infiltration of lymphocytes (LI), individualization of the hepatocyte (white arrow head), absence of plasma membrane in the hepatocytes (black arrow head), degeneration of liver parenchyma (D), necrosis (N).

scanning electron microscopy, loss of alignment of primary and secondary lamella result in increased diffusion distance which is bound to cause difficulties in gas exchange (Nowak, 1992). Fishes expose to water with low pH causes significant damage to the gills which includes lifting, sloughing, necrosis of the branchial epithelium and shortening of tight junctions between cells of branchial epithelium (Rosseland and Staurnes, 1994). The profuse mucous secretion on the surface epithelium of the gill lamellae of *C. punctata* observed in the present study indicated a high mucous secreting character of the gills (Laurent et al., 1985; Saboia-Moraes et al., 1996) in toxic environmental condition. The secretion of large amount of mucous was the first response observed in the external tissues of fish to toxic substances present in the water.

**Table 2**
Degree of Tissue Change for the gills, kidney and liver of *C. punctata*.

| | Degree of Tissue Change | | |
|---|---|---|---|
| | **Gills** | **Kidney** | **Liver** |
| **Control** | 6.5 ± 1.81 | 24.8 ± 1.78 | 34.7 ± 2.26 |
| **Treated** | 13.8 ± 3.2* | 40.6 ± 2.64* | 52.6 ± 2.38* |

Results are mean ± SE.
* Indicates statistical difference in $P < 0.05$.

The teleostean kidney is one of the first organs to be affected by contaminants in the water. Most common alterations found in the kidney of fishes due to toxic contaminated water are tubule degeneration (cloudy swelling and hyaline droplets) and changes in the corpuscle, such as dilation of capillaries in the glomerulus and reduction of Bowman's space (Takashima and Hibiya, 1995). The presence of tubule degeneration, coupled with absence of necrosis in the kidney observed in the present investigation indicates that the kidney suffered cellular damage due to presence of toxicant in the AMD.

Fish liver is the organ which is associated with the detoxification and biotransformation process and due to its function, position and blood supply (Van der Oost et al., 2003) and also one of the organs most affected by contaminants in the water (Rodrigues and Fanta, 1998). The acid water and heavy metals caused alternations in liver parenchyma, necrosis destroying overall cellular architecture of liver. High amounts of ions (especially nitrogen and potassium) in mining wastewater have drastically changed the water chemistry, and affected species composition, the food chain structure and fish health. Similar histological findings of liver and gills of fish was observed (Tkatcheva et al., 2012) in lakes receiving mining effluents indicating unfavorable

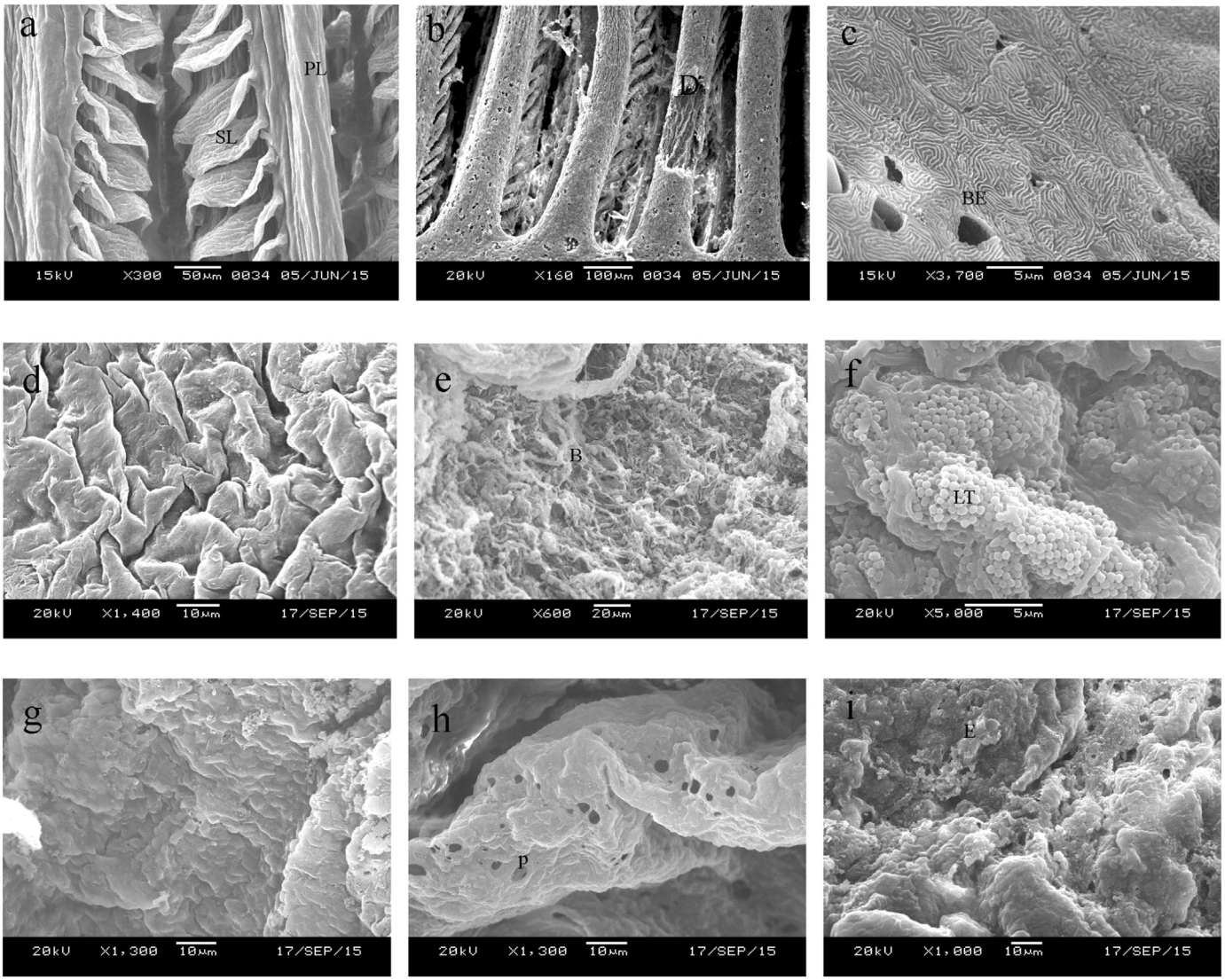


**Fig. 2.** Scanning Electron Micrographs of the Gills, kidney and liver of *C. punctata.* (a) gill of fishes showing normal primary lamellae (PL), secondary lamellae (SL) parallel to each other in control group of fish; (b-c) gill of fish treated with sub-lethal concentration of AMD showing damage (D) of secondary lamallae at places, breakdown of epithelial membrane (BE); (d) kidney of fish showing normal alignment of kidney tubules in control group; (e-f) kidney of fish treated with sub-lethal concentration of AMD showing blood cell deposition (B); loss of tubules arrangement (LT); (g) liver of normal fish showing morphological architecture in control group; (h-i) liver of fish treated with sub-lethal concentration of AMD showing pore formation (P), extraneous deposition on the surface (E).

environmental conditions for aquatic biota.

Different tissues are usually used for the determination of DNA damage, e.g., intestine, liver, kidneys, spleen and gills (Lee and Steinert, 2003). Kidney, the main excretory organ is exposed directly to the environmental pollutants. Therefore, kidney was considered to determine the genotoxicity potential of AMD. The environmental oxidative stress and, damages of tissues and macromolecules (DNA, proteins and lipids) are caused by heavy metals by generating reactive oxygen species such as hydrogen peroxide, superoxide anions and hydroxyl radicals (Pandey, and Farombi et al., 2003, 2007). A regulatory role of heavy metals such as iron, copper and zinc in endonuclease activity and apoptosis has been suggested by several authors (McCabe, and Shiokawa et al., 1993, 1994). At the molecular level, large DNA fragments of 50–200 kb and nucleosome size fragments of 180–200 bp (Higuchi, 2003) are recognized to indicate apoptosis in a single cell. Therefore, the composition of different types of metal present in the AMD lowers the pH of water and leads to fragmentation in DNA.

Since the fishes are the most sensitive fauna and any little change that occurs in their habitat have immediately influenced their physiology. Hence, the present investigation confirms that stress due to AMD has created disturbances by affecting the immune system and making the fish vulnerable to diseases. This implies the need for greater seriousness in terms of AMD discharge point, and more efforts towards eliminating the constant sources of pollution.

**Table 3**
Haematological parameters of fish *Channa punctata* exposed to AMD (All values are expressed in mean ± standard deviation).

| Parameters | Control | After 30 days of exposure |
|---|---|---|
| TEC ($\times 10^6$/mm$^3$) | 4.29 ± 0.66 | 2.49 ± 0.21* |
| Hb (g/dL) | 13.11 ± 0.35 | 10.3 ± 0.53** |
| TLC ($\times 10^3$/mm$^3$) | 4.3 ± 0.26 | 7.6 ± 0.09** |
| DLC(%) (n = 300) | | |
| Neutrophil (%) | 19.65 ± 0.91 | 10.7 ± 0.46** |
| Monocyte (%) | 11.2 ± 0.85 | 8.9 ± 0.75** |
| Eosinophil (%) | 5.4 ± 0.88 | 3.7 ± 0.55* |
| Basophil (%) | 2.6 ± 0.69 | 4.4 ± 0.29* |
| Lymphocyte (%) | 61.15 ± 1.36 | 72.3 ± 0.94** |

* Show statistical significant ($P < 0.01$).
** Show statistical significant ($P < 0.05$).

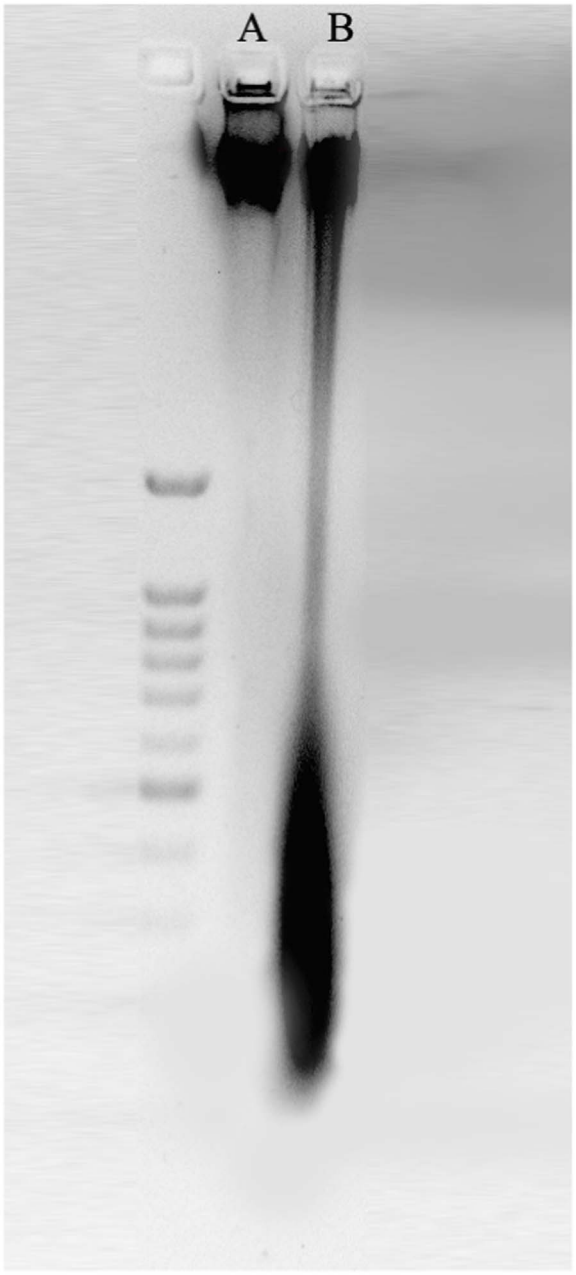


**Fig. 3.** Agarose gel electrophoresis of DNA from kidney of AMD exposed and control fish. First lane corresponds to a 100 bp ladder DNA marker (Axygen Biosciences, USA), Lane A is DNA from the unexposed fish, Lane B is DNA of fish exposed to sub-lethal concentration of AMD.

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