



Chapter 13

Toxicity induced alterations as biomarker of environmental pollution

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Chapter contents

Introduction	174
Materials and methods	175
Experimental model	175
Exposure period and observations	176
Results and discussion	176
Conclusion	181
References	181

Abstract

Over the past few decades dye contamination of aquatic systems has attracted the attention of several investigators both in the developed and developing countries of the world. A large quantity of these dyes enters aquatic bodies from time to time because a substantial amount of a dye (10-15% unbound dyes) is lost in the effluent during dyeing processes. In return the aquatic bodies end up being the final destination of a large quantity of wastes from these sectors. Waste waters from dye manufacturing, paper, leather and textile industries bring tons of dyes into the aquifers, most of which are highly toxic to the flora and fauna of the receiving water bodies. Scanning electron microscopic observations were made for the changes in the surface ultra morphology of gills of *Cirrhinus mrigala* on exposure to lethal (0.1, 0.2, 0.4, 0.6 and 0.8 mg/L dye) doses of Basic Violet-1 (an important textile and hair colorant; CI: 42535, Trade name- Methyl Violet-2B). Present study was taken up as insufficient data exist regarding safety of this dye. The dye was observed to be cytotoxic in nature during the acute (96h) exposure to lethal doses. The dye caused reduction or complete loss of microridges, increase in mucous openings and

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degeneration of gill lamellae and rakers. Therefore, time to time monitoring of ultra morphology of tissues will provide us early indicators for the stress of very low levels of pollutants which may later cause mortality of the fish. The study holds importance because fishes are an important link in the food chain of man, respond to toxicants in a manner similar to higher vertebrates causing serious diseases.

Keywords: Acute exposure, *Cirrhinus mrigala*, Gills, Scanning electron microscopy, Ultra morphology

Introduction

Aquatic ecosystems are of extreme importance for the world population, as these are used for domestic, agricultural, industrial as well as recreational activities. In return the aquatic bodies end up being the final destination of a large quantity of wastes from these sectors. Waste waters from dye manufacturing, paper, leather and textile industries bring tons of dyes into the aquifers, most of which are highly toxic to the flora and fauna of the receiving water bodies (McCarthy, 1997). Uncontrolled discharge of dyes in water bodies causes serious problems due to change in colour of the water and production of even more toxic by-products after reduction in light (Chung, 1983). As a result, various dyes are banned and maximum residue levels exist in Europe and USA, however, in several other countries of the world, these dyes are openly sold in the market without any information regarding their chemical nature, purity, toxicity and possible mutagenicity (Mathur *et al.*, 2005). Unregulated use of dyes will therefore have grave consequences for human health and aquatic ecosystems in these countries. The aquatic environment is of primary concern because many a times these various toxic chemicals not only have significant implications for long-term survival of natural populations of the organisms living therein but cause heritable mutations that may lead to loss of the total genetic diversity of an ecosystem.

Many industries releasing industrial wastes and effluents contain various levels of organic and inorganic pollutants including acids, alkalis, inorganic ions, heavy metals etc. are one of the major sources of environmental pollutants in India. They discharge their effluents directly or indirectly into rivers and agricultural land used to irrigate agricultural fields, make the water and soil polluted, which is not good for agricultural purposes (Samanta *et al.*, 2018). The industries utilize many poisonous substances, which are very harmful to the plants and soil microorganism. The river water polluted with industrial effluent contains various toxic chemicals, dyes and heavy metals such as mercury (Hg), Cadmium (Cd), Chromium (Cr) and Zinc (Zn). When the effluent is used for irrigation, these metals are strongly bound to polypeptide and proteins of aquatic plants and animals. Of all the aquatic organisms fish have become vulnerable indicators for evaluation of the effects of such noxious compounds (Khidr and Mekkawy, 2008; Kaur and Dua, 2015) as these are the ultimate sufferers of pollution and form an important link in the food chain of humans.

By virtue of their high reactivity, dyes and other genotoxins contribute to structural modifications in the DNA of fish which then become the underlying cause of metabolic dysfunction and death. These disturbances being irreversible are transmitted to the future generations, have long lasting effects and appear even at those levels of toxins which are otherwise safe for survival.

Although light microscopic studies provide good information about stress induced alterations in the cell morphology under stress but for obtaining finer and early details of the underlying causes of the death, electron microscopic technique-Scanning electron microscopic (SEM) studies for detailed information about the surface morphological variations in gills is preferred. Use of electron microscopy in relation to pollution and environmental conditions is a recent approach and is considered a very useful tool as it adds a valuable third dimension for understanding the structural deformations as well as for obtaining information much before fish exhibit many other visible symptoms of toxicity (Hidayati *et al.*, 2013). Therefore, in the present study electron microscopy (SEM) has been used collectively for evaluating toxicity of Basic Violet-1 (a triphenylmethane dye) because toxicity data for this dye is not available (Diamante *et al.*, 2009). Dyes with triphenylmethane pharmacophore (Basic Violet-1, Gentian Violet, Crystal Violet) have a long history of human use (Souza Pietra *et al.*, 2013) but these interact with lipid bilayers of the cellular membrane and perturb membrane structure as well as ionic balance of the cell (Dell Antoneet *et al.*, 1972; Aljofan *et al.*, 2009). In living systems these are biotransformed to demethylated derivatives which react with DNA and lead to tumour development (Culp *et al.*, 2006). Present work envisages evaluation of toxic potential of Basic Violet-1 (BV-1, CI:42535: Trade name- Methyl Violet-2B) which is commonly used as a direct synthetic (non oxidative dye) fibre/textile dye and a hair colorant (Diamante *et al.*, 2009). *Cirrhinus mrigala*, an Indian Major Carp, abundantly present in fresh waters of India has been selected as a test model for this study to detect most prominent changes in the epithelial cells and morphology of the gill that can act as a biomarker for the stress of very low doses of such dyes. Predominantly, the gills constitute a multifunctional organ (respiration, acid base regulation, ion regulation, nitrogenous waste excretion) accounting for well over fifty per cent of the total surface area of the animal. They are the major site of uptake for most water borne toxicants and site of toxic impact for many of them. (Ale *et al.*, 2018).

The main aim was to assess the suitability of ultramorphological changes in the pavement cells, chloride cells, and mucous openings in the gill as early indicators of the dose dependent stress of Basic Violet-1. This research work has been envisaged as insufficient data exist to support the safety for this dye (Diamante *et al.* 2009).

Materials and methods

Experimental model

For the present study *C. mrigala* was selected as a model for evaluating acute toxicity of a dye, BV-1.

Procurement and acclimation of fish: Fish weighing 22 ± 1.25 g and measuring 10.50 ± 1.22 cm were procured from the Government Fish Farm, Rajasansi, Amritsar, Punjab, India. The fish were given a bath in 0.1% KMnO_4 for 2-3 minutes for disinfection, diseased fish were sorted and not subjected to experimentation. Fish were acclimated for 21 days under laboratory conditions and fed with a pelleted commercial Toya feed on alternate days @ 2% of the biomass.

Toxicant used: BV-1 (CI: 42535), purchased from the local market, Amritsar, India was used for the present study. The commercial grade preparation was a green coloured powder soluble in water.

Test containers: Toxicity tests were conducted in plastic pools of 200 L capacity.

Dilution water and control: Dechlorinated tap water was used as control and for making various concentrations of the dye.

Exposure period and observations

Acute exposure: Ten fish were exposed in triplicate to 0, 0.10, 0.20, 0.40, 0.60, 0.80 and 1.0 mg/L dye. Semi static daily renewal 96h bioassay was conducted according to OECD guidelines (1992) and APHA (1998). No food was given 24h prior to the exposure during the bioassay. Mortality was recorded at 24h intervals and dead fish were removed immediately to avoid asphyxiation of other fish. A fish was considered dead when no opercula movement was there and it did not move on prodding with a glass rod.

Observations: Observations were recorded at the beginning and at the end of the acute exposure for ultra morphological changes (scanning electron microscopic studies on gill) in the fish during the study.

Gill: Second and third gill arch from the right side were immediately excised and fixed in the Karnovsky's fixative for 6-12h. Post fixation was done in 1% Osmium tetroxide for 2h at 4°C. Three washings were given in 0.1M phosphate buffer (pH 7.4), and dehydration of the sample was carried for 15 min each in 30, 50, 70, 90 and 100% anhydrous alcohol. The dehydrated tissue was critical-point dried and placed over the silver tape attached to the aluminium stub.

Coating and viewing: Gold coating of the samples was done with a sputter coater [Model: Q150RES (QUORUM)] for viewing under Carl Zeiss EVO | LS 10, Scanning electron microscope at The Central Instrumentation Facility, Guru Nanak Dev University, Amritsar and Carl Zeiss EVO | MA 10 at The Indian Agricultural Research Institute, New Delhi at low acceleration voltage of 10-20 kV. Till the time of viewing, the stubs were kept in a desiccator.

Results and discussion

For evaluating toxicity of Basic Violet-1, fingerlings of *C. mrigala* were given acute (96h) exposures to BV-1. Data were recorded for ultra structural changes in the gills of fish during the exposure period. *C. mrigala* has four pairs of lateral gills which are reddish in colour and protected by an operculum. Each gill arch bears two rows of primary filaments upon which are situated two rows

of secondary lamellae. Gill filaments, lamellae and rakers were found to be lined by pavement cells (simple squamous epithelial cells) which were polygonal or hexagonal in shape. A single or double ridged border (Micro border) was seen between pavement cells or pavement cell and chloride cell junction. The dye induced changes in the morphology of filaments, lamellae as well as rakers of the gill. Two rows of stiff denticular gill rakers are present on the other side of gill arch. The microvilli or micro ridges overlying the pavement cells of the gill arch, primary filament and secondary lamella showed considerable disorganization with respect to their size and shape in the dye exposed fish.

On exposure to different concentrations of the dye, there was no change in gill morphology of control (after 96h) and 0.10 mg/L dye exposed fish (after the acute exposure) in the present study (Figure 13.1a-b). No change was observed in the gillrakers of control and 0.10 mg/L dye exposed fish as they were spaced equally (Figure 13.1c) while a dose dependent increase was observed during the acute exposure. Microridges of pavement cells of primary and secondary gill lamellae of control and 0.10 mg/L dye exposed fish were observed to be shorter, interwoven and random-

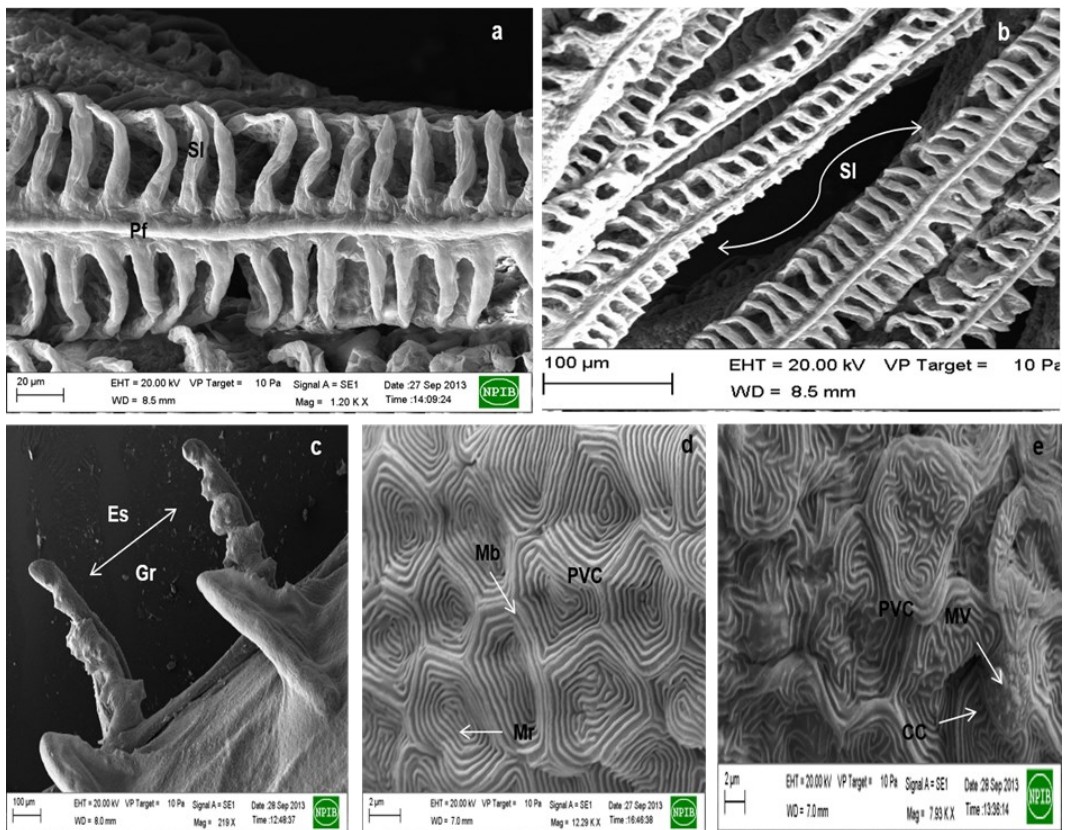


Figure 13.1. Gills of control *C. mrigala* after 96h exposure (a-e); Pf – Primary gill filament, Sl- Secondary lamellae, GR- Gill rakers, Es- Equally spaced, PVC- Pavement cells, Mr- Microridges, Mb- Microborder, CC- Chloride cells, MV- Microvilli.

ly oriented at the beginning as well as at the end of exposure as well as microvilli of chloride cells were observed to be normal (Figure 13.1d-e).

There was a dose dependent loss of secondary lamellae and collapse of structural integrity of the epithelial cells after 96h exposure. On exposure to 0.20 and 0.40 mg/L dye, fusion of secondary lamellae (Figure 13.2a) initiated while in 0.60 mg/L dye, mucous cell openings and sloughing of the epithelium of primary gill filaments at tips was also observed at some places (Figure 13.2b-c). In 0.80 mg/L dye, mucous cell openings, necrosis and degeneration of epithelium on the secondary gill lamellae were noticed all over the gill surface (Figure 13.2d).

Alteration in the shape of the microridges started appearing in 0.20 and 0.40 mg/L dye and became more pronounced with the increase in dose (Figure 13.3a). Mucous cell openings and gall like structures were noticed in some of the pavement cells on exposure to 0.60 mg/L dye which increased further in 0.80 mg/L dye and were accompanied with degeneration of microridges of the whole surface of gill (Figure 13.3a-b). Slight changes were observed in the morphology of gill rakers of fish exposed to 0.20-0.40 mg/L dye but there was a marked change in structural conformity like swelling, erosion and necrosis of the surface of rakers on exposure to 0.60 and 0.80 mg/L dye (Figure 13.3c). There was no change in the pavement cells of the rakers of 0.20 and 0.40 mg/L dye exposed fish. Fusion of pavement cells, loss of micro ridges was also noticed in 0.60 mg/L dye along with these changes, degeneration of pavement cells, microridges and microvilli of the rakers was common in 0.80 mg/L dye (Figure 13.3d).

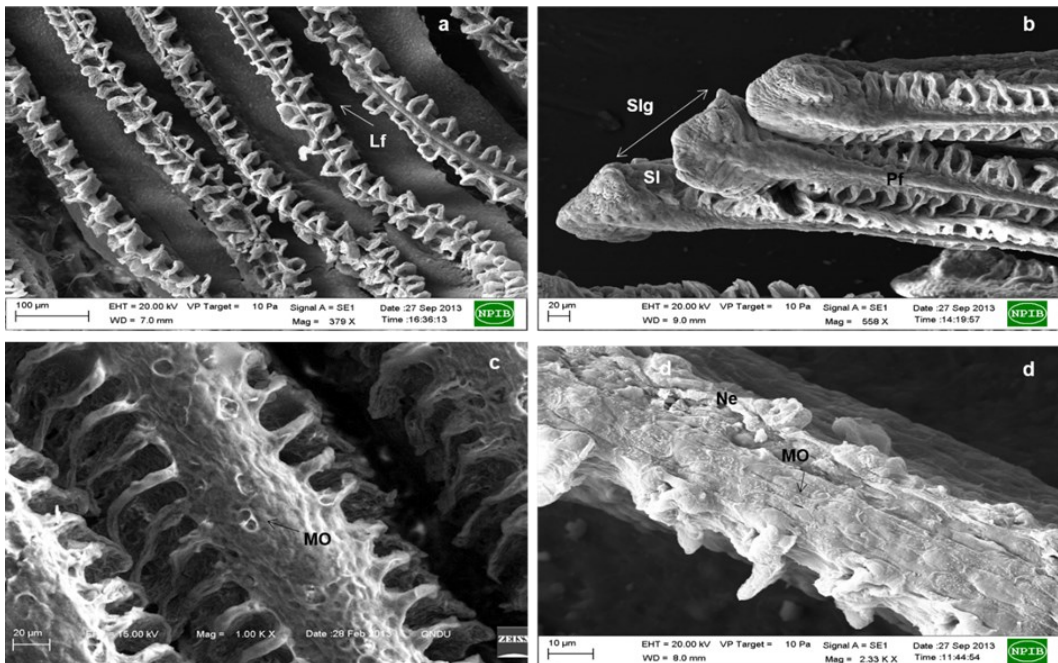


Figure 13.2. Gills of dye exposed (0.20-0.80 mg/L dye) *C. mrigala* (a-d); Pf- Primary gill filament, Sl- Secondary lamellae, Lf- Lamellar fusion, Slg- Sloughing, Mo- Mucous opening, Ne- Necrosis.

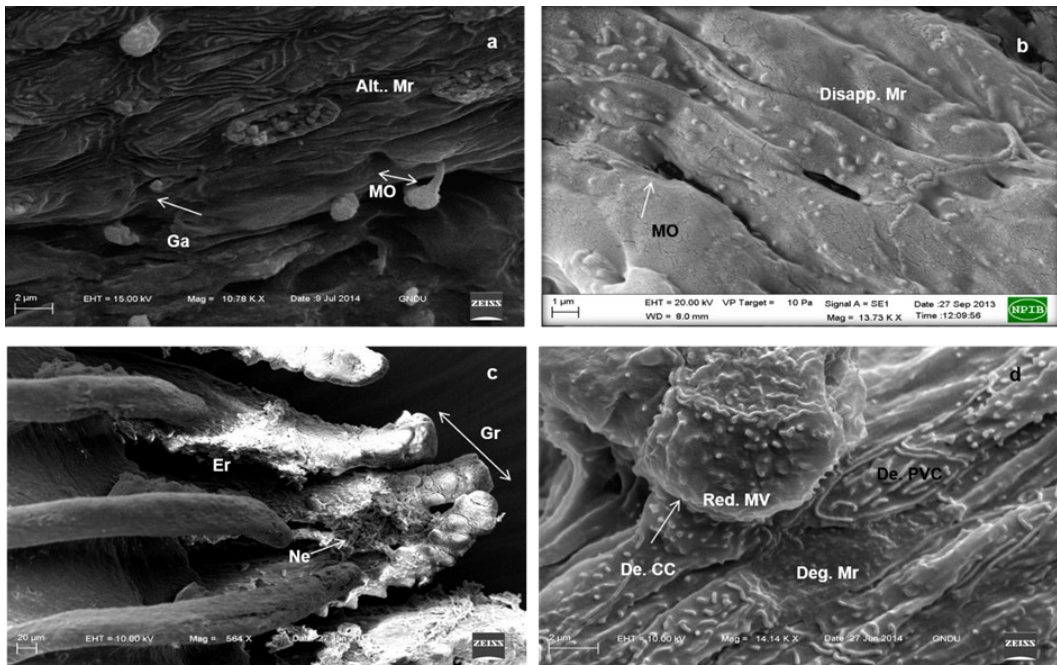


Figure 13.3. Gills of dye exposed (0.60-0.80 mg/L dye) *C. mrigala* showing degeneration of microridges (Pavement cells) and microvilli (Chloride cells) of lamellae and gill rakers (a-d); Alt. Mr- Alternating microridges, Ga- Gall like appearances, MO- Mucous opening, Disapp. Mr- Disappearance of microridges, Er- Erosion, Ne- Necrosis, Dr- Gill rakers, Red MV- Reduction in Microvilli, De PVC- Degenerating Pavement Cells, Deg. Mr- Degenerating microridges, De. CC- Degenerating Chloride Cells.

In the present study, ultra structural changes were observed in *C. mrigala* after acute (96h) exposure to BV-1. Toxic effects of the present dye on the gill of fish can be divided into two categories: lesions and reactions. Lesions, the direct and deleterious effects including necrosis and rupture of respiratory epithelium (Temminck *et al.*, 1983) were observed to be dose dependent during acute exposure. Lesions due to the present dye may have developed either due to the stress induced autolysis by the cell's own enzymes or due to the direct cytotoxic action of the dye (Mallatt, 1985). Perturbation of membrane structure due to direct binding of the cationic dyes to lipid bilayers has been reported by Zachowski and Durand (1988). The reactions like epithelial lifting, fusion, hypertrophy, hyperplasia, increase in mucous secretion, vascular stasis, mucous cell proliferation, loss of microvilli and chloride cell proliferation (Morgan and Tovell, 1973; Mallatt, 1985; Dutta *et al.*, 1996) are generally considered to be defensive mechanisms for reducing the surface area in contact with the toxins (Dutta *et al.*, 1997) but in the present study these were replaced with lesions with the dose of the dye.

During the acute exposure, reactions were prominent in 0.20 and 0.40 mg/L dye but the incidence of lesions and reactions was equal in 0.60 and 0.80 mg/L dye. As a result, lesions appeared even due to very low doses of the dye with the increasing dose of exposure. Drastic changes like loss of

structural integrity and necrosis hint towards carcinogenic or mutagenic nature of this dye. Several dyes have been reported to be carcinogenic, mutagenic and teratogenic with a potential to cause chromosomal fracture (Khanna and Das, 1991). Being water soluble, the present dye might have been degraded by anaerobic intestinal microorganisms (Chung and Stevens, 1993) and this could have activated a procarcinogen to a mutagen (McCoy *et al.*, 1977). On the other hand, dose dependent increase in extrusion of mucous, epithelial lifting, lamellar fusion and reduction of micro ridges in lower doses of acute (0.20 and 0.40 mg/L dye) exposure can be considered to be defensive reactions of the dye stressed fish. Loss of microridges in fish gills under the stress of toxicants has been observed by many workers (Hart and Oglesby, 1979; Jacobs *et al.*, 1981; Jagoe and Haines, 1983; Roy *et al.*, 1986; Roy and Munshi, 1991). Lamellar swelling, epithelial lifting and reduction of micro ridges in the present fish may have been to reduce the surface area of the gills in contact with the dye so as to sustain the progressive loss of the basic function of the gill (Temmink *et al.*, 1983). This may also have been to increase the barrier distance for diffusion from outside to blood capillaries (Dutta *et al.*, 1992).

Present dye seems to be abrasive in nature as it promoted necrosis, mucous cell openings and copious mucous secretion in a dose dependent manner (Kaur and Jindal, 2016). The dose dependent increase in extrusion of mucus may have been to facilitate respiration in the dye exposed fish as mucous decreases the co-efficient of drag for water flow across the gills, reduces the resistance of gill and plays a role in ion exchange and water balance under stress (Shephard, 1994; Macirella and Brunelli, 2017). However, loss of microridge pattern and copious mucous seems to have reduced the effectiveness of exchange processes, especially gaseous exchange and further stressed the fish and caused mortality. Enormous damage to the epidermis and destruction of cell processes and nerve supply of melanophores due to absorption of Chrome Black T has been reported by Singh (2007) in *Colisa chuna*. Misreplication of dye induced DNA lesions after its N-hydroxylation (Culp *et al.*, 2006) may have resulted in mutations that resulted in appearance of gall like structures in 0.60 mg/L dye after acute exposure to BV-1.

The present dye seems to affect the ionic balance of the fish as it caused erosion of rakers in some doses. Dose dependent increase in the appearance of chloride cells but reduction in their microvilli (the short and stubby microridges) during the acute exposure could also be an effort of the stressed fish for maintaining ionic balance under the influence of this cationic dye as the chloride cells or ionocytes are associated with electrolyte balance of the body. An increase in intracellular concentration of calcium and a large decrease in sodium ion concentration due to loss of cellular membrane integrity under the stress of cationic dyes have been reported by Aljofan *et al.* (2009). Similar changes in chloride cells have been reported by Ghanbousi *et al.* (2012) in *Aphanius dispar* exposed to deltamethrin. However, Wong and Wong (2000) reported augmentation of micro ridges in pavement cells and an increase in the density and apical membrane area of chloride cells of *O. mossambicus* after a short term exposure to cadmium. This highlights the significance of ultrastructural changes in gills over mortality as early indicators of the stress of cationic dyes like BV-1.

Conclusion

The electron microscopic studies in gills will provide us early indicators of the stress of very minute doses of cationic dyes like BV-1. Therefore, time to time monitoring of ultramorphology of tissues will provide us early indicators for the stress of very low levels of pollutants which may later cause mortality of the fish. The results clearly show that BV-1 is mutagenic, carcinogenic as well as cytotoxic in nature and this effect becomes more prominent at 0.80 mg/L dye showing deleterious effects. The toxicants present in the industrial dyes or effluents when come in contact with biological environment may create serious long-term toxicity effect to the living organisms. However, the extent of toxicity depends upon their concentration and duration of exposure to the vulnerable site.

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Author contributions

The study was designed by A Kaur and provided overall supervision and management of the work. K. Kaur performed the experiment and wrote the first draft. Both the authors were involved in interpretation of results, critical evaluation, and approval of the final manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest.

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