CYTOGENOTOXICITY OF AZO DYE ACID BLUE-113 (AB-113) TO Channa punctatus (BLOCH)
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ABSTRACT
A growing interest in environmental genotoxicity has led to the development of several tests for detecting genotoxins in the aquatic environment. One of the most popular and promising test is analysis of micronuclei (MN) in the stressed fish. This test has served as an index of cytogenetic damage over 30 years due to its sensitivity and simplicity. Acute (96h) exposure of azo dye AB-113 was given to Channa punctatus under laboratory conditions in semistatic system (with daily renewal of test water) and 96h LC50 was calculated to be 47 mg/l dye. Micronuclei, nuclear anomalies (binuclei, blebbed, notched, lobed, vacuolated and irregular nuclei), cellular anomalies (sphärocytic, echinocytic, blebbed, notched and vacuolated cells) and necrotic/apoptotic cells were recorded as a measure of cytogenotoxicity of the lethal doses of AB-113 in blood, gill, kidney, liver and spleen of fish. All the abnormalities showed a dose dependent increase and were maximum in the gill. It was observed that analysis of other abnormalities in nuclei and cells in several tissues along with MN yielded better results so their inclusion would help to decide permissible limits of dyes for aquifers in a more accurate way. The findings of this work will also throw light on the possible effects of small doses of the AB-113 to human beings on direct (occupational) or indirect exposures (by eating contaminated food) as the results obtained in fish are directly applicable to humans.

Key Words: Genotoxicity, Toxicity, Acid Blue-113, Channa punctatus, Micronucleus assay

INTRODUCTION
Development of modern technology and the use of complex artificial chemicals to improve the products has made it a serious problem with respect to the discharge of waste from industries into aquatic ecosystems. Wastewater effluents from the textile and other dye-stuff industries contain significant amounts of synthetic dyes in addition to harmful chemicals. Depending on the class of dyes, their loss in waste waters can range from 2% of the original concentration for basic dyes to as high as 50% for reactive dyes. Dyes persist in natural water bodies for quite a long time and render the water unfit for its intended use, at the same time the aquatic organisms also remain in contact with them for a very long time.

At present, there are about 3000 different dyes available on the commercial market and more than half of these are azo compounds. The annual world production of azo dyes is estimated to be around one million tons and these are being currently used in textile, food, leather, pharmaceutical and paper industries. Under natural conditions, the azo dyes before complete degradation are first broken down by microbes to aromatic amines, which are even more toxic than the parent dye. Azo dyes and their breakdown products not only cause mortality of organisms but are reported to be potent carcinogens and mutagens.

A growing interest in environmental genotoxicity has led to the development of several tests for detecting genotoxins in aquatic media. One of the most popular and promising test is analysis of micronuclei (MN) in the stressed fish. This test has served as an index of cytogenetic damage over 30 years due to its sensitivity. Presence of MN in cells is actually a reflection of stress induced chromosomal aberrations during mitosis and therefore they are potential indicators of fish health and water quality. In addition to MN which indicate only unrepairable damage to DNA, current trend is to use other nuclear
anomalies like notches, vacuolation and binucleation in cells for estimation of aneuploidy and abnormalities in tubulin polymerization and mitotic spindle that increases the sensitivity of a model organism for a genotoxin.6

Present investigation deals with the study of toxicity and cytogenotoxicity of lethal doses of an azo dye Acid Blue-113 (CI 26360) in Channa punctatus. Mortality was taken as toxicity index and frequencies of micronuclei (MN), Nuclear Anomalies (NA), Necrotic Cells/Apoptotic Cells (NC/AC) and Cellular Anomalies (CA) were observed as indices of cytogenotoxicity. These anomalies were observed in peripheral blood, gill, kidney, liver and spleen after short term (96h) exposures to the dye. Multi tissue analysis was done in the present study because there are tissue specific variations in susceptibility and response to pollutants.

AIMS AND OBJECTIVES
The findings of this work will throw light on the possible effects of small doses of AB-113 to human beings on direct (occupational) or indirect exposures (by eating contaminated food).

MATERIAL AND METHODS
Live specimens of C. punctatus, with an average length 13.50±1.38 cm and an average weight 25±1.41 g were procured from the local fish market in Amritsar, Punjab, India. These were transported to laboratory in oxygenated water-filled polythene bags. The fish were disinfected with 0.1% KMnO4 solution for 2-3 minutes and were kept for two weeks in plastic pools of 200 litre capacity with dechlorinated water at room temperature (23.0±1.95℃), for acclimation to laboratory conditions. The fish were fed on boiled egg white ad libitum during acclimation except the bioassay and 24h prior to the commencement of bioassay.

Azo dye, Acid Blue-113 (CI- 26360) used for the present study was purchased from Punjab Rang Udyog, a dye manufacturing unit in Amritsar, Punjab, India. This dye has been selected for the present study because it is used at a large scale in textile industries and effluents with unbound dye and dye products are released into the aquatic environment at high rates.

Toxicity studies
For assessment of toxic potential of AB-113, semi static bioassays were conducted according to standard methods for the examination of water and wastewater7 in plastic tanks of 200L capacity. Ten fish of almost same weight and same length were exposed in triplicate to various concentrations of the dye. Tap water was used as diluent and control after dechlorination. Test water of each tank was changed daily to remove the waste metabolites of fish and to maintain required concentration of the dye. Dead fish were removed immediately to avoid asphyxiation of the live fish. A fish was considered dead when it did not move on prodding with a glass rod. Mortality was recorded at 24h interval for 96h and behaviour of the fish was also recorded during the exposure period.

Cytogenotoxicity studies
Cytogenotoxic potential of the lethal doses of azo dye AB-113 was observed with respect to incidence of abnormalities were recorded after 96h exposure to 0, 25, 50, 65 and 75 mg/l were scored at 100X under compound microscope.8 MN, NA (blebbed nuclei, notched nuclei, irregular nuclei, binucleation and multinucleation), NC/AC (Necrotic/Apoptotic cells) and CA (blebbed, irregular, echinocytic, notched, vacuolated and spherocytic cells in erythrocytes, head kidney, spleen, gill tips and liver cells of C. Punctatus.

Statistical analysis
Data for mortality were subjected to probit analysis for calculation of 96h LC50 of AB-113, for C. punctatus9, Safe Application Rate (SAR) was calculated by using formula given by Basak and Konar10 data for cytogenotoxicity were subjected to one-way ANOVA.

RESULTS AND DISCUSSION
In the present study observations were recorded for toxicity and cytogenotoxicity of AB-113 to C. punctatus in a semistatic system.

Toxicity of AB-113
Table 1 shows the data for 96h LC50, fiducial limits at 5% level, X2, standard error, variance mean, SAR and regression equation of AB-113 for C. punctatus. 96h LC50 came to be 47mg/l, 100% mortality was observed in 85 mg/l
(LC\textsubscript{100}) and 0\% mortality was observed in 10 mg/l of dye (LC\textsubscript{0}). The value of Safe Application Rate (SAR) of AB-113 was calculated to be 5.5 mg/l. There was no mortality in control during the experimental period.

Table 1: LC\textsubscript{50}, SAR and statistical values of AB-113 for \textit{C. punctatus}

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<table>
<thead>
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<tbody>
<tr>
<td>96h LC\textsubscript{50}</td>
<td>47 mg/l</td>
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<tr>
<td>Variance (Vm)</td>
<td>1.38</td>
</tr>
<tr>
<td>Standard Error (S.E.)</td>
<td>1.91</td>
</tr>
<tr>
<td>Fiducial limits</td>
<td></td>
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<tr>
<td>5% Upper</td>
<td>61.46</td>
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<tr>
<td>5% Lower</td>
<td>27.41</td>
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<tr>
<td>$\chi^2$</td>
<td>3.14</td>
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<tr>
<td>Safe application rate</td>
<td>5.5 mg/l</td>
</tr>
<tr>
<td>Regression equation</td>
<td>$Y=1.124X-10.89$</td>
</tr>
<tr>
<td>Coefficient of correlation</td>
<td>r = 0.98</td>
</tr>
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</table>

When the fish were exposed to various doses of AB-113 for 96h, body and gills of the exposed fish turned bluish black in color in a dose-dependent manner, indicating that dye had high affinity for the tissues of fish, it could enter through the plasma membrane and accumulate in the cells. Periodical change in body color of \textit{Colisa chuna} has been reported on exposure to chrome black T.\textsuperscript{11} He correlated it with destruction of melanophores and their nerve supplies by the dye. Accumulation of present dye in the gill cells might have resulted in respiratory distress and mortality of the experimental fish as the stress symptoms like surfacing and jumping were more frequent in the higher concentrations of the dye.

High affinity of azo dyes for the gills and its correlation with anoxemia and mortality of fish has been reported earlier also.\textsuperscript{12} There is a variation in the toxicity of various azo dyes, some being very toxic and others being moderately toxic to the fish.

96h LC50 of azo dye Methyl Red for \textit{Poecilia reticulata} was observed to be 24 ppm.\textsuperscript{13} On the other hand 48h LC\textsubscript{50} of C.I. Acid Violet 66 and C.I. Acid Red 217 was observed respectively to be 8.20 and 71.04 mg/l for \textit{Onchorhynchus mykiss}.\textsuperscript{14} 96h LC\textsubscript{50} value of aniline for \textit{O. mossambicus} was observed to be 69.4 mg/l. A reduction in appetite, growth rate and yield has been observed even at 0.02 mg/l of aniline.\textsuperscript{15} The fish became restless on exposure to the present dye and the response was dose-dependent. Immediately after exposure, surfacing increased and the fish banged in the walls of the aquarium and tried to jump out of water but gradually the fish became lethargic, stopped moving and gathered in a corner. Mucus secretion increased in the exposed fish and dead fish had a thick coat of mucus on the body and gills. Color of the fish and gills became blackish blue on exposure to AB-113. Coagulation of film anoxia might have been responsible for mortality of fish in the present study as a thick layer of mucus was present on the gills and body of dead fish. Hypoxia due to a reduced gill surface area has also been observed in \textit{C. punctatus} due to stress of malathion.\textsuperscript{16} Azo dye Malachite Green produced sinusoidal damage, congestion, focal necrosis in liver and also damaged mitochondria, lamellar cells and gill epithelium of \textit{H. fossilis}.\textsuperscript{17} In our study also the darkening of gills and body was more intense in higher doses of dye at the same time nuclear and cellular abnormalities increased dose-dependently in all the tissues which clearly indicates that degenerative changes in these organs may have hampered their normal functioning and caused mortality of fish on exposure to AB-113.

Erratic opercular movements and gasping increased immediately after the exposure but gradually these subsided. Aquatic pollutants generally affect fish directly by causing respiratory or circulatory distress through their interference with excretory function of gills. On absorption through gills, lining of mouth and gastro-intestinal tract these pollutants are concentrated and metabolized by fish.\textsuperscript{18} The present dye may have stressed the brain and latero-acoustic system of \textit{C. punctatus} as they
swam laterally for quite some time (dose-dependently) and turned upside down before death.\textsuperscript{19} Failure of latero-acoustic or neuromast system was responsible for loss of equilibrium before death in \textit{P. reticulata} due to stress of heavy metals was also suggested.

**Genotoxicity of AB-113**

Exposure to lethal doses (25, 50, 65 and 75 mg/l) for 96h brought a significant dose dependent increase (p<0.01) in the frequencies of MN, NA, CA and NC/AC in the selected tissues of fish. The increase in frequencies of all the parameters was dose-dependent (Fig. 1 to Fig. 4). There were significant differences within treatments (p<0.01) and with their respective controls (p<0.01). Many xenobiotic compounds are transformed by fish to carcinogenic and mutagenic metabolites and used in DNA or proteins.\textsuperscript{20} Genotoxicity bioassay using micronuclei induction therefore helps to evaluate toxicity of minute quantities of pollutants especially in the situations when there is no mortality.

The selected anomalies were observed to be maximum in gill, however, frequencies of MN and NA were minimum in the liver and that of NC/AC and CA in spleen. MN are the result of chromosome breaks (or mitotic anomalies) which are normally extruded along the main nucleus and their presence suggests their origin at a more recent cell cycle.\textsuperscript{24} Micronucleus assay provides an indirect measure of structural cellular anomalies\textsuperscript{22} and has been proven and used as bioassay in toxicology studies especially when no mortality is observed. Liver cells have low mitotic index as compared to other haemopoietic organs like kidney and spleen\textsuperscript{23} so it may be the reason for lowest number of micronuclei and NA in the liver of \textit{C. punctatus} in our study. In the gills, the number of the MN, NA, CA and NC/AC was 36.84, 98.50, 81.40 and 25.17 respectively in the highest dose (75 mg/l) of the dye. In the liver on the other hand, the numbers were 19.83, 36.0, 3.50 and 52.35 respectively for MN, NA, NC/AC and CA. Although MN in peripheral erythrocytes provide a feasible approach to monitor the effects of genotoxic agents in fish but recording other nuclear and cellular abnormalities along with it has been considered to be a more reliable approach for assessment of toxic effects of contaminants. At the same time studying these parameters in many tissues helps to increase sensitivity of model organisms in toxicity related studies.\textsuperscript{24} This corroborates findings of the present research also. A few NC/AC were present only in the blood (0.97) and gills (1.0) of control fish but these were observed in much higher numbers in all the tissues of the dye exposed fish. In spleen, liver and kidney there was only a slight increase in the number of NC/AC (2.35, 3.50 and 3.27 respectively) as compared to a marked increase in the NC/AC of blood (24.0) of the fish exposed to the highest dose (75mg/l) of AB-113. It is possible that repair system of spleen and kidney had acted and damaged cells had been removed as suggested.\textsuperscript{25} Metabolic rate/capacity, antioxidant enzyme machinery and DNA repair capabilities introduce a great variability in the frequencies of anomalies.\textsuperscript{26}

![Fig. 1](image_url): Frequency of MN (number/2500) in various tissues of \textit{C. punctatus} on exposure to lethal doses of AB-113 after 96h
A dose dependent significant increase in MN clearly hints towards clastogenic nature of the present dye as MN are believed to be derived from chromosome fragments or whole chromosomes that are not incorporated into daughter nuclei at the time of cell division and increase in their frequency is an indicator of the stress on cells. Induction of MN actually represents unreparable and higher damage to DNA and helps in distinction of clastogens and
aneugens.\textsuperscript{27} It was hypothesized that nucleus may have a capacity to sense excess DNA that does not fit well in the nuclear matrix. This extra DNA is eliminated via nuclear budding to form nuclei/MN selectively at the periphery of the nucleus.\textsuperscript{28} In the present study there was 2-4 fold increase in MN even due to 25 mg/l dye. The biotransformation of xenobiotics after absorption results in production of reactive intermediates such as Reactive Oxygen Species (ROS) which are highly toxic and can cause oxidative damage to DNA. Although all organisms are equipped with an antioxidant defense system but when ROS production exceeds the capacity of defense mechanisms, cellular and genetic lesions appear.\textsuperscript{29,30} Along with this increase in incidence of necrosis and frequency of abnormal shapes of cells and nuclei in all the tissues of \textit{C. punctatus} due to the selected doses of the present dye clearly hints towards superimposed pathology in the cells of exposed fish. Hypoxia was opined to depress ATP and to be responsible for transformation of cells on exposing \textit{C. punctatus} to malathion.\textsuperscript{31} Various types of nuclear lesions have been used as indicators of genotoxicity by many authors\textsuperscript{32-36} but origin of these has not yet been elucidated. The dose dependent increase in frequency of MN along with NA and CA in all the tissues of \textit{C. punctatus} clearly suggests that AB-113 has genotoxic as well as cytotoxic potential. In the present study gills showed maximum frequency of all the abnormalities whereas, liver and spleen were least affected by the lethal doses after 96 h exposure. Gills are actually in direct contact with the contaminants as compared to other tissues of the body, abnormalities in them therefore increase several folds as compared to other organs on exposure to pollutants in water. It was observed in the present study that observation of other abnormalities along with MN in more than one tissue yielded better and comprehensive results as the incidence was NA > CA > NC/AC > MN.

CONCLUSION

AB-113 is very toxic to \textit{C. punctatus} as 96h LC 50 was 47 mg/l and 96h LC 100 was 85 mg/l. Variable frequency of the abnormalities in different tissues hints towards a variation in susceptibility, metabolism, repair system and response of the tissues to AB-113 and also highlights that a comparative study in many tissues would yield better results as compared to recording abnormalities in erythrocytes alone. Study also points towards achieving better results with inclusion of NA, NC/AC and CA along with MN where fishes are used as bio-indicators of the health of aquatic ecosystems.

REFERENCES


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