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Toxicological assessments of basic blue 3 dye in fresh water bivalve *Lamellidens marginalis*

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Abstract
The Basic blue 3 dye is a cationic dye gives colour shading from blue to green by addition with different chemicals. The acute exposure of this dye for 96 hours to bivalves showed altered physiological parameters. Increased level of antioxidant enzymes in gill, hepatopancreas, gonad and mantle of bivalve was recorded. At 70 ppm concentration of Basic blue 3 dye showed significant increase in gill and hepatopancreas while non-significant increase in gonad and mantle. At both the concentration 40 ppm and 70 ppm of the dye gives structural changes in gill, hepatopancreas and gonad assessed by histology. In gill, the structural changes were Swollen gill lamellae, ruptured epithelial cells, disorganization of gill filament and cell necrosis noticed; the hepatopancreas showed the abnormal shape of digestive tubules, destruction of digestive cells, mixing of cellular content of tubules, damages at basement membrane and degeneration of connective tissue while in gonad Stages of oocytes showed degenerative changes, the vacuolization in oocytes, degeneration of oolemma and ooplasm was observed. By comet assay tail DNA% and tail moment was recorded. At 70 ppm concentration, the gill cells showed significant tail DNA% and tail moment.

Keywords: Basic blue 3, *Lamellidens marginalis*, antioxidant enzymes, histology and comet assay.

1. Introduction
When dyes released in water, the soluble form was absorbed by aquatic organisms. Some dyes will change their form when entered in cell and the converted form will accumulate in cell cytoplasm, while some may bind to genetic material results in mutation. Basic blue 3 dye is colourful green light blue bronze powder. It gives colour shading from green to blue by addition of different chemicals, metals and also at varied temperature. It is used in dyeing of wool, nitriles, stick/acrylic blended fabric graft copolymerisation dyeing, and also can be used for direct printing of acrylic carpet. The acute toxicity was studied in fat head minnows (4 mg/lit) by Linda Little [36]. The cytotoxicity was studied in rat L6 cell [42].
From the past few decades, many species have been used as biomonitoring organisms as per their potential. Many species of molluscs from marine, fresh water and terrestrial ecosystems are the key species to determine the impact of pollutant, which will affect the molluscan population and results in negative impact on entire ecosystem [38]. The molluscs are somewhat sedentary, long lived, filter feeder, regionally abundant, having adequate tissue mass for analysis, accumulate contaminants from food, water and also from the ingestion of particulate material, hence fulfilling the criteria as a good bioindicator [44, 6, 27]. *Lamellidens marginalis* is used as bioindicator in fresh water system [33]. It has potential to accumulate heavy metals like copper, cadmium, chromium, nickel, and even pesticides and insecticides [55]. In variety of organisms, including mussels and fish, there were changes in the levels of antioxidant enzymes i.e. CAT, SOD and GPx [40]. Many workers have reported the pollutant induced oxidative stress. Pesticides are responsible for producing ROS and the natural immune system i.e antioxidant enzymes scavenge oxygen free radicals [19] Cadmium induced oxidative stress was observed in *Modiolus modiolus*. The rise in temperature also induced reactive oxygen species in the bivalve *Lateral elliptical* [26].
Histological observations are helpful tool for understanding the pathological condition or abnormalities and damages of tissues under toxic stress and also for assessing effects of toxicants at individual level.

Histopathological changes are mostly confined to organs directly involved in their metabolism and detoxification.
To evaluate DNA damage, comet assay is better test than other tests, because it requires only a very small number of cells and sensitive for detecting low levels of DNA [18]. The DNA damage in clam *Tapes semi decussatus* due to sediments was detected by comet assay [8]. Rigonato [46] observed the genotoxicity in heamolymph cells of *Corbicula fluminea* by comet assay exposed to methyl methane sulphonate.
In present investigation, the toxicity effect of Basic blue 3 at LC_0 and LC_{50} concentration for 96 hours was determined by using antioxidant enzyme assay, histology and comet assay in different tissues like gill, hepatopancrease, mantle and gonad of fresh water bivalve *Lamellidens marginalis*.

2. Materials and Methods
Fresh water bivalve *Lamellidens marginalis* about 7 to 8 cm in size were collected from Rajaram tank, Kolhapur (16^0 40' 43.4'' N 74^0 15' 53.0'' E) was as experimental animal. For experimental work, dyes were purchased from Sigma Aldirich. (USA). Basic blue 3 dye ("N-(7-(diethylamino-3H-phenoxazin-3- ylidene]-N-ethyl-, chloride) were purchased from sigma Aldrich (USA). The LC_0values (40 ppm) and LC_{50} values (70 ppm) of Basic blue 3 were determined by probit analysis for *Lamellidens marginalis*.

2.1 Antioxidant Enzymes
Three antioxidant enzymes were estimated from the tissues like gill, mantle, hepatopancreas and gonad of experimental and control group of bivalves.

2.1.1 Superoxide Dismutase (SOD) by Beauchamp and Fridovic method [4]
Preparation of sample: The sample was prepared from the fresh tissues of bivalve. The homogenate (1 mg/ml) was made in 0.25 M sucrose and 1 mM EDTA, centrifuged at 3000 rpm for 15 minutes. Supernatant was centrifuged at 10,000 rpm for 10 minutes at 4 ^{0}C. The pellet was suspended in 1 ml phosphate buffer.

Assay: The addition was done as follows:

Sr. No.	Reagents	Blank	Control	Sample
1.	Phosphate buffer	3.5	3.4	3.4
2.	EDTA (10 mM)	0.3	0.3	0.3
3.	Methionine	1.2	1.2	1.2
4.	NBT	0.6	0.6	0.6
5.	Sample	-	0.1	0.1

After addition, the tubes were exposed to 18 W fluorescent bulbs with addition of 0.4 ml riboflavin. The reaction was for 15 minutes and faint blue colour appears. The absorbance was measured at 560 nm with UV spectrophotometer.

2.1.2 Catalase (CAT) estimation by Luck method [37]
Preparation of sample
The sample was prepared from thawed fresh tissues of bivalves. The homogenate was made as 15 mg/ml concentration in phosphate buffer (pH 6.8) in prechilled Morter pestle. The homogenate was centrifuged at 10,000 rpm for 15 minutes at 4 ^{0}C. The supernatant was used as enzyme source. All procedure was carried out in cold condition.

Assay: For assay 3 ml H2O2 phosphate buffer was added in the cuvette, 0.05 ml enzyme source was added, mix it well. Immediately read at zero second at 240 nm on UV spectrophotometer. The readings were taken till it remains constant. i.e. maximum and minimum absorption.

2.1.3 Glutathion Peroxidase (GPx) estimation by Beers and Sizer [5]
Preparation of sample: The sample was prepared from thawed fresh tissues of bivalves. The homogenate was made as 15 mg/ml concentration in phosphate buffer (pH 6.8) in Pre-Chilled Morter pestle. The homogenate was centrifuged at 10,000 rpm for 15 minutes at 40C. The supernatant was used as enzyme source. All procedure was carried out in cold condition.

Assay: For assay 3 ml H2O2 phosphate buffer was added in the cuvette, 0.05 ml enzyme source and 0.01 ml 1mM sodium azide was added, mix it well. Immediately read at zero second at 240 nm on UV spectrophotometer. The readings were taken till it remains constant. i.e. maximum absorption and minimum absorption.

2.1.4 Statistical analysis
The data on antioxidant enzymes were analyzed for statistical significance between the control and experimental bivalves by Student 't' test. A significant difference was established at 0.05,0.01 and 0.001 significance level.

2.2 Histology
After acute and chronic exposure, the tissues like gill, hepatopancreas and gonad were fixed in bouins fixative along with control group for 48 hours. The tissues were dehydrated in ethyl alcohol grades (30% to 100%) and cleared in xylene. The tissues were embedded in paraffin wax (melting point 56 °C to 59 °C). Then they were sectioned at 5-6 µ.

2.2.1 Hematoxylene and eosin staining technique
The sections were deparaffinized in xylene and hydrated in alcohol grades. The hydrated slides were stained with hematoxylene then dehydrated in alcohol grades; stained with eosin; mount in DPX and observed under light microscope.

2.3 Comet assay
For comet assay procedure was adapted from Woods [55] with some modification.
For comet assay gill cells were used. Gills were separately macerated in Ca-Mg free PBS to obtain cell suspension and centrifuged for 10 minutes for 1000 rpm. The supernatant was discarded and pellet was resuspended in PBS. 10 µl of suspension was mixed with 60 µl low melting agar and incubated at 37 °C. Coat the slides with 1% normal melting agarose from one side. Keep it in refrigerator for 5 minutes to solidify. Take the sample embedded in low melting agar on normal melting agar coated slides. Put the coverslip on slide and keep it in refrigerator. Take out the slide, remove the coverslip and apply 100 µl low melting agar and put the coverslip on it and again keep the slide in refrigerator. Take out the slides and remove the coverslip. The low melting agar forms a protective layer to sample. Put the slides in lysis buffer in coupling jar covered with aluminium foil. Put the coupling jar in refrigerator overnight. Carry out electrophoresis at 25 V and 300 mA for 45 minutes. Neutralize the slide with neutralization buffer for 5 minutes keep in dark Figure. Ethanol drying was carried out for 5

minutes. Stain with DAPI for 10 minutes. The DNA was visualized using epifluorescence microcope. Measure the tail moment and tail DNA%, the results were statistically analyzed by one way ANOVA.

3. Results

To study the toxicity effect, the bivalves were divided into three groups i.e. control, LC_0 and LC_{50}. The alterations in antioxidant enzyme were noticed.

3.1.1 Superoxide Dismutase activity

In control group, the SOD response was maximum in gill (0.638 unit/mg protein/hour) while minimum in gonad (0.3564 unit/mg protein/hour). The SOD response was less significant in tissues for both the concentrations i.e. 70 ppm (LC_{50}) and 40 ppm (LC_0). The SOD response was non-significant in gonad and mantle at 40 ppm (LC_0) concentration, while significant in gill (17.03) and hepatopancreas (15.09). At 70 ppm (LC_{50}) concentration it was in the order of hepatopancreas (23.89) > gill (22) > gonad (21.92) > mantle (20.14) (Table No. 1)

3.1.2 Catalase (CAT) activity

In control group, the CAT response was maximum in gill (22.24 mmolH2O2/min/mg protein), while minimum in gonad (12.44 mmolH2O2/min/mg protein).

After 96 hours exposure, in 40 ppm conc. of basic blue 3 showed less significant activity of CAT in all selected tissues, while in gonad the activity of CAT was non-significant. At 70 ppm conc. of basic blue 3, highly significant ($P< 0.001$) increased activity of CAT was observed in gill (26.7%), hepatopancreas (21.96%) as compared to other tissues. In 40 ppm concentrations, the increased CAT activity was found less significant ($P< 0.05$) in hepatopancreas (15.49%), gill (12.81%) and mantle (11.45%); while non-significant in Gonad. (Table No. 2)

3.1.3 Glutathion Peroxidase Activity

In control group, the GPx activity was maximum in hepatopancreas (1.90 mmol NADPH/min/mg protein) and minimum in gonad (0.78 mmol NADPH/min/mg protein).After 96 hours exposure to 70 ppm (LC_{50}) and 40 ppm (LC_0) concentration, the gonad showed no significant change in activity of GPx at both the concentrations. At 40 ppm (LC_0) concentration, the significant ($p<0.05$ increased) activity was observed in gill (23.75%), hepatopancreas (22.44%) and mantle (15.21%). At 70 ppm (LC_{50}) concentration, highly significant ($p< 0.001$) increase in activity was observed in gill (29.62%) and hepatopancreas (28.1%). (Table No. 3)

3.2 Histology

After acute exposure to Basic blue 3 dye, the drastical histological changes were noticed. The control group of bivalves showed normal histology with no changes, while the LC_0 and LC_{50} group showed structural changes in gill, hepatopancreas and gonad.

3.2.1 Gill

Compared to control group, at 40 ppm concentration of Basic blue 3 (LC_0) group for 96 hours, showed the lamellae get fused to interlamellar junction at proximal end and degenerative changes were observed at distal end. Hypertrophy of gill lamellae, vacuolization of epithelial cells of gill lamellae, disorganization of epithelial cells and formation of ciliary tufts were observed. The lamellae get fused at which tissue get ruptured. The connective tissues also get damaged. At 70 ppm concentration (LC_{50}) after 96 hours, more damages were observed as compared to LC_0group. The formation of clavate-globate structure of gill lamellae with maximum number of vacuolization in epithelial cells was observed. At distal region, the gill lamellae get fused. Connective tissue, ciliary damages are common. Large irregular spaces were observed in connective tissue core. Swollen gill lamellae, ruptured epithelial cells, disorganization of gill filament and cell necrosis were found (Figure No. 1).

3.2.2 Hepatopancreas

After acute exposure for 96 hours to 40 ppm concentration (LC_0 group), the digestive tubules showed elongated structure with disruption of digestive cells. The normal structure of digestive cell get damaged. The cells discharged from basement membrane and fall in lumen. Vacuolization and necrosis was commonly observed. The connective tissue and muscle fiber got degenerated. The mixing of cellular content of hepatic tubule was found. At 70 ppm concentration (LC_{50} group) of Basic blue 3 showed less damages and these damages were similar as LC_0group. The abnormal shape of digestive tubules, destruction of digestive cells, mixing of cellular content of tubules, damages at basement membrane, degeneration of connective tissue and muscle fiber, infiltration of hemocytes were seen (Figure No. 2)

3.2.3 Gonad

Exposure to 40 ppm concentration of Basic blue 3 for 96 hours showed not much damages as compared to other exposed groups. The damages were commonly observed in the nuclei of oocytes. There was fragmentation of nuclei. The slight degeneration in previtellogenic and vitellogenic oocytes was observed. After 96 hours exposure to 70 ppm concentration of Basic blue 3 showed more damages as compared to LC_0 group. The follicle wall was with less number of oocytes. The developing oocytes were spread in ovules. Stages of oocytes showed degenerative changes. The vacuolization in oocytes, degeneration of oolemma and ooplasm was observed. The nuclear fragmentation was also noticed in some oocytes. The central space of ovule was occupied by fluid material (Figure No.3).

3.3 Comet Assay

The acute exposure of Basic blue 3 showed less significant damages. The LC_0 group (40 ppm) showed non-significant tail DNA% and tail moment as compared to control group. The LC_{50} group (70 ppm) showed significant ($P< 0.05$) damages in tail DNA% (32.01±1.98) and tail moment (28.9±1.73). The comet from LC_{50} group was included in class I DNA damage (Table No. 4; Figure No. 4)

4. Discussions

Application of pollutant exerts the xenobiotic effects which influences the redox cycle. The excessive formation of reactive oxygen species (ROS) is toxic to the biological component like Protein, lipid and glycogen [45]. SOD, CAT and GPx are the antioxidant defence system which inhibit the formation of oxyradicals [12, 45]. Study of antioxidant response was used as biomarker in fresh water bivalve *Unio tumidus* in different contamination profile [7]. Kesavan [30] observed the

increased antioxidant response in SOD, CAT and GPx in *Oreochromis mossambicus* after exposure to Malachite green. The induction of SOD activity is due to the activity of stress response in defense mechanism. The production of superoxide anion radical is due to stress and neutralized by SOD and converted into hydrogen peroxide and oxygen [2]. The acute and chronic exposure to Basic blue 3 dye showed highly significant activity in hepatopancreas. The obtained results were similar to the other toxicants induced toxicity in mussels with increase in SOD and CAT response [11, 14]. Pandey [40] observed that, the heavy metal exposure to *L. marginalis* induced the SOD activity in mantle, gill and hepatopancreas. Jing [28] noticed increased SOD response in the mantle of pearl oyster, *Pinctada fucata*. The pesticide cypermethrin after acute exposure induced higher activity of SOD in gills compared to control in fresh water mussel *Unio gibbus* [32]. Li [34] observed the increased activity of SOD and CAT in fish *Carrasius auratus* after exposure to three dyes, Direct blending rebbin, Direct blending scarlet and Direct blending yellow. In present study, the acute and chronic exposure to Basic blue 3 dyes showed significant change in CAT response. The gill showed highly significant response after acute exposure to Basic blue 3 dyes.

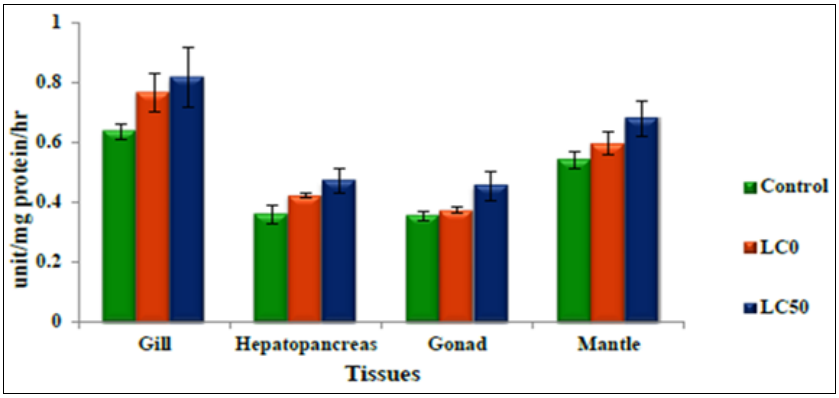
The SOD catalyze the dismutation of superoxide radical which was catalyzed by CAT into H2O and O2 to prevent oxidative damage and cell homeostasis [57]. The CAT was less as compared to SOD activity. Mundhe [39] observed the SOD and CAT response in different tissues of *Lamellidens marginalis* after MCP exposure. The heavy metal as Cd and Pb [1] and cypermethrin [32] induced increased CAT response in gill after acute exposure. Damiens [10] observed significantly increased CAT activity in larvae of *Crassostrea gigas* after exposure to pesticides. The role of glutathion peroxidase (GPx) having role in reduction of H2O2 and lipid hydroperoxidase with glutathione oxidation (GSH) [25], [49]. This cycle lowers the NADPH/NADP+ ratio to prevent oxidative damage. The acute exposure of Basic blue 3 showed highly significant GPx activity in hepatopancreas, gill and mantle. Acute exposure of lead and paraquat caused increased activity of antioxidant enzymes CAT and GPx in digestive gland of brown mussel [9]. Iron, copper and cadmium induced stress in mussels with increased activity of GSH [3]. Franco [15] studied the GPx activity after exposure to zinc, which showed increased activity of GPx in gill of green mussel. Khandekar [31] observed similar findings as increased level of antioxidant enzymes SOD, CAT and GPx in *Lamellidens marginalis* when exposed to malachite green dye for 96 hours. From present findings, the histopathological effects on gill of *L. marginalis* due to Basic blue 3 were depend upon similar to the toxicant induced. Dye induced histopathology were observed in some animals. The gill of *Heteropneustes fossilis* showed necrosis, karyolysis, desquamation of epithelial lining cells and vacuolisation after exposure to MG [50]. Sukumaran [52] noticed enlargement of gill fillament, loss of interlamellar junction and vacuolisation after heavy metal exposure in gill of *Meretrix casta*. The exposure of Malachite green to Nile Tilapia showed damages in gill lamellae, hyperplasia which considered as defense mechanism which reduces the branchial superficial area. Stalin [51] observed the hyperplasia, clavate globate shape, tissue necrosis, fusion of gill lamellae in *L marginalis* after exposure to chlorpyrifos.

The hepatopancreas in mollusc is the largest digestive gland formed by vast number of tubules, with different size and shape. After treatment to dyes for different concentration and exposure period showed the degeneration of basement membrane, swelling and degeneration of digestive tubules, fragmentation of digestive cells was observed. These results are similar to the work done by [21, 43, 23, 22]. The digestive gland of bivalve *Crenomytilusgrayanus* showed vacuolization of digestive cells, lipofuscin, necrosis and lysis of cells in connective tissue due to polluted water [53]. The hepatopancreas of *Helix aspera* showed the damages to the digestive tubules, breakdown of the basement membrane leading to severe damages like deterioration of the tissue after introduction of Thiamethoxam [22]. The gonad showed different developmental stages *viz.* gonad development, spawning and fertilization and development and growth is controlled by seasonal and size specific variation. In present study, the gonad showed spawning and fertilization stage. The reduced size and generation in the gonadal cells of *Helix aspera* due to cadmium exposure was observed [29]. The snail *Indoplanorbis exustus* showed vacuolisation in ooplasm, increased number of nutritive phagocytes in follicles and nuclear picnosis after mercury exposure. Werner [54] reported the accumulation of metals, PAH and organochlorine pesticides which results into histopathological lesions in gonads of *Macoma nasuta*. The stress produced by tributyltin reduces progesteron levels, which delays gametogenesis and vitellogenesis in *M. arenaria* [16, 17]. The calcium chloride and lead nitrate induced histopatological alterations in testes of fresh water bivalve, *Lamellidenscorrianus* [39]. In present study, the histopathological effects of Basic blue 3 dyes on female gonad of *Lamellidens marginalis* was observed, which were similar to the observations of other gonadal changes due to toxicity stress. The integrity of DNA assessment is important in determining genotoxic pollutants related to stress in organisms. The comet assay is the technique used to measure the integrity of DNA. Genotoxicity is one of the most important parameter in testing chemical toxicity and risk assessment [24]. The results of the comet assay presented here have demonstrated the concentration and the time dependent response to dyes exposure in *L. marginalis*. There was positive correlation found between oxidative stress and DNA damage in gill and digestive cells of *Uniotumidus* [35]. Grazeffe [20] classifiedthe comet in four categories (0-3) as per tail DNA damage in gill cells of *Biomphalaria glabrata* after γ irradiation. The similar results were observed in *Nerita chamaeleon* after chronic exposure to cadmium [48]. Santos [47] observed that, the atrazine and roundup induced the biochemical changes as increased response of SOD, CAT and GST which increases the genotoxic effect in gill and digestive cells of *Corbicula fluminea*.

Table 1: Changes in SOD activity after acute exposureof L. *marginalis*to basic blue 3 (values are expressed in unit/mg protein/hour)

Tissues	Control	LC0 (40ppm)	% Change over control	LC50 (70 ppm)	% Change over control
Gill	0.638±0.023	0.769±0.063*	+17.03	0.818±0.09*	+22
Hepatopancreas	0.360±0.031	0.424±0.006*	+15.09	0.474±0.04*	+23.89
Gonad	0.3564±0.015	0.3758±0.009NS	+5.06	0.456±0.050*	+21.92
Mantle	0.543±0.029	0.598±0.038NS	+5.5	0.680±0.057*	+20.14

Values are Mean ± S.D., *, **, ***, NS indicates significance level $p< 0.05$, $p< 0.01$, $p<0.001$, Non-significant (n = 3)

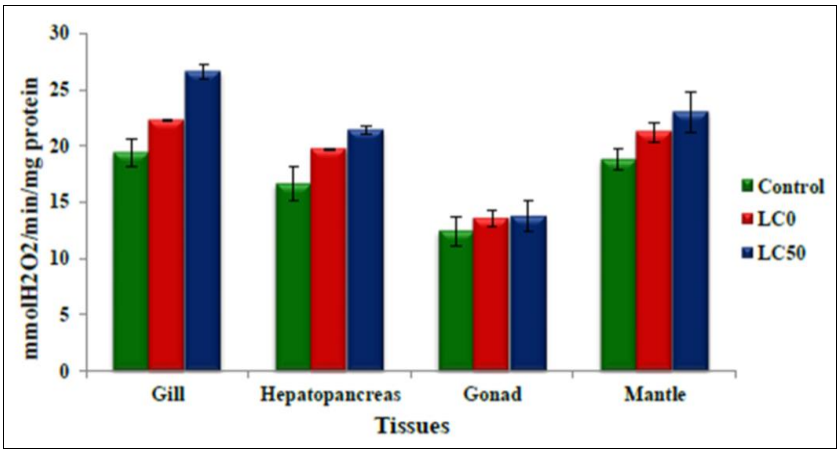


Graph 1: Changes in SODactivity in different tissues of fresh water bivalve, *Lamellidens marginalis* after acute exposure to basic blue 3(values are expressed in unit/mg protein/hour)

Table 2: Changes in CAT activity after acute exposureof *L. marginalis*to basic blue 3 (values are expressed in mmolH2O2/min/mg protein)

Tissues	Control	LC0 (40ppm)	% change over control	LC50 (70ppm)	% change over control
Gill	19.39±1.19	22.247±0.09*	+12.81	26.57±0.66***	+26.7
Hepatopancreas	16.63±1.50	19.687±0.12*	+15.49	21.37±0.38***	+21.96
Gonad	12.44±1.27	13.54±0.73NS	+8.12	13.71±1.37NS	+9.26
Mantle	18.797±0.88	21.22±0.88*	+11.45	22.97±1.78*	+18.19

Values are Mean ± S.D., *, **, ***, NS indicates significance level $p< 0.05$, $p< 0.01$, $p< 0.001$, Non-significant (n = 3)

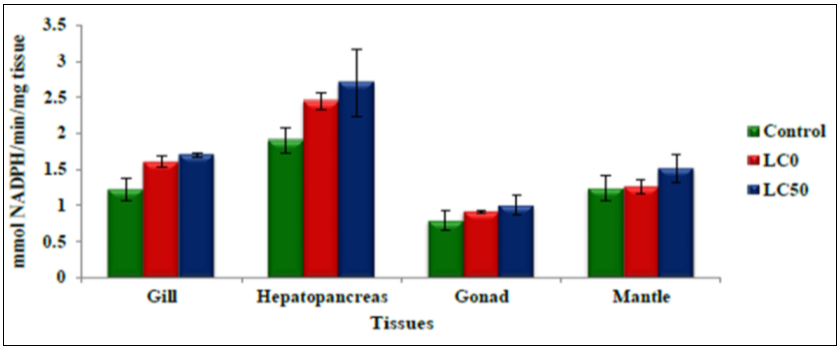


Graph 2: Changes in CAT activity in different tissues of fresh water bivalve, *Lamellidens marginalis* after acute exposure to basic blue 3 (values are inmmolH2O2/min/mg protein)

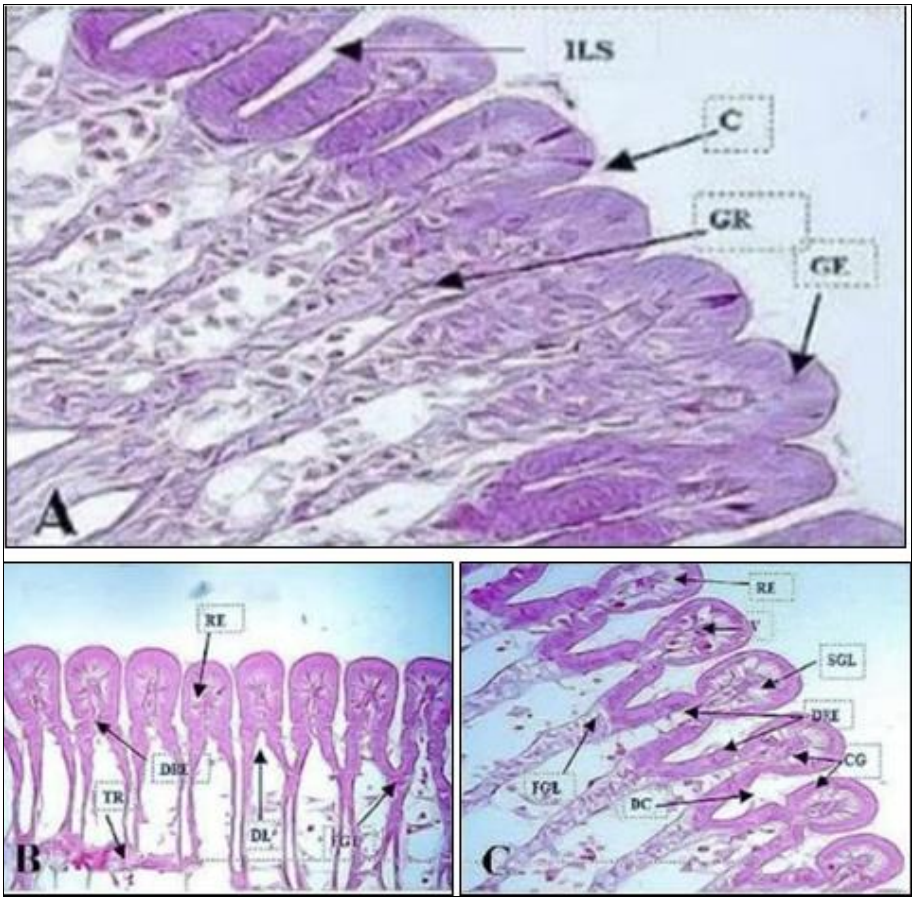
Table 3: Changes in GPx activity after acute exposure of *L. marginalis* to basic blue 3 (values are expressed in mmol NADPH/min/mg protein)

Tissues	Control	LC0 (40ppm)	% change over control	LC50 (70ppm)	% change over control
Gill	1.22±0.15	1.607±0.80*	+23.75	1.697±0.02***	+28.1
Hepatopancreas	1.90±0.17	2.45±0.121*	+22.44	2.707±0.446***	+29.62
Gonad	0.785±0.18	0.928±0.032NS	+15.21	0.997±0.13NS	+20.7
Mantle	1.20±0.31	1.362±0.36NS	+11.89	1.38±0.14*	+13.04

Values are mean± S.D., *, **, ***, NS indicates significance level $p< 0.05$, $p< 0.01$, $p<0.001$, Non-significant (n = 3)



Graph 3: Changes in GPx activityin different tissues of fresh water bivalve, *Lamellidens marginalis* after acute exposure to basic blue 3 (values are in mmol NADPH/min/mg protein)



GE – Gill Epithelium, GR – Gill Rod, C – Cilia, ILS – Interlamellar Space, V – Vaccuolation, RE – Ruptured Epithelia, DC - Damaged Cilia, TR- Tissue Rupture, D-EP - Damaged Epithelium, ROE=Reorganizing epithelia
SGL- Swollen Gill Lamellae, FGL- Fused Gill Lamellae, N – Necrosis, TGR - Thickened Gill rod, DE - Desquamated/disorganized nuclei, DL=Dilate sinus. DRE- Degenerated Respiratory Epithelium, CG – Cavate Goblet.

Fig 1A: Section passing through gill of fresh water bivalve *Lamellidensmarginalis*from control group (400X). B: Effect of 40 ppm (LC0) of Basic Blue 3 on gill of fresh water bivalve *Lamellidensmarginalis* after 96 hours exposure (400X) C: Effect of 70 ppm (LC50) of Basic Blue 3 on gill of fresh water bivalve *Lamellidenmarginalis* after 96 hours exposure (400X)

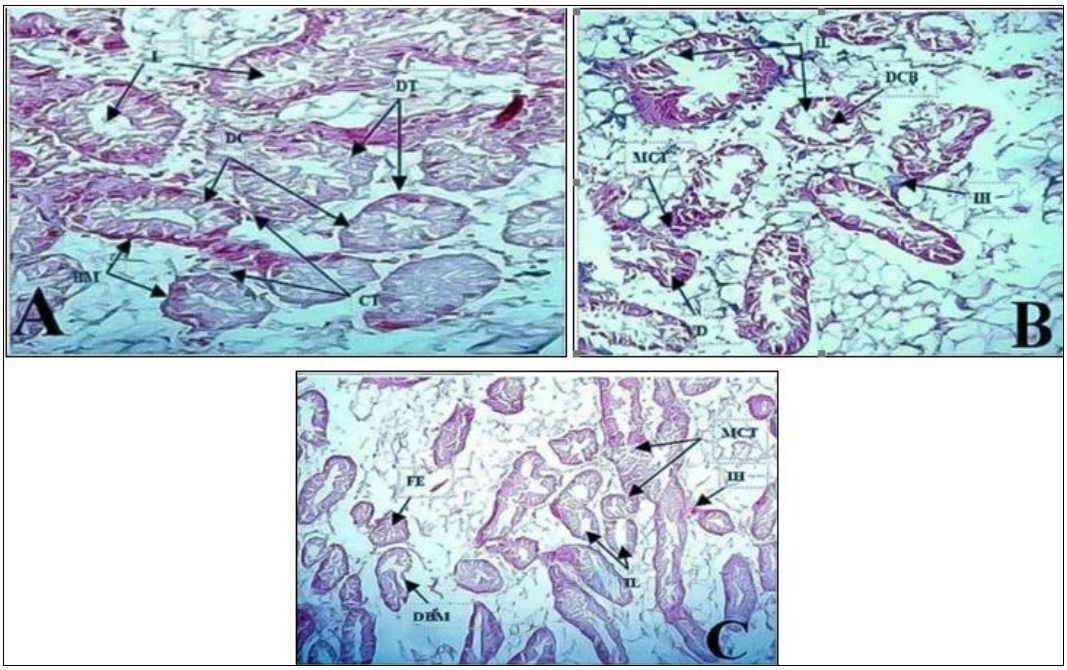
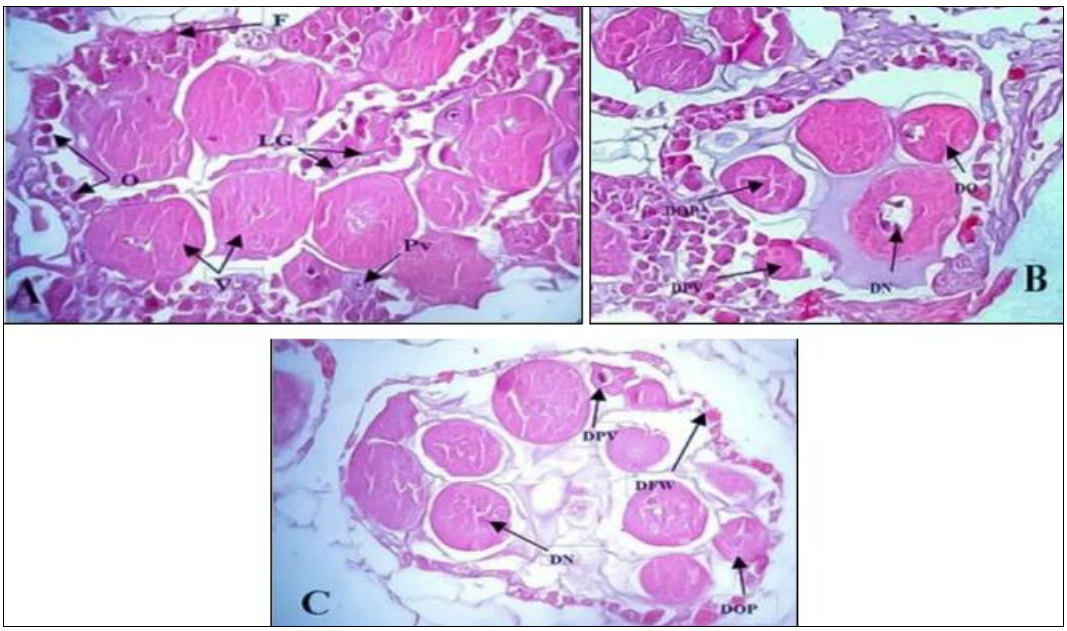


Fig 2A: Section passing through hepatopancreas of fresh water bivalve *Lamellidens marginalis* from control group (400X). B: Effect of 40 ppm (LC_0) of Basic Blue 3 on hepatopancreas of fresh water bivalve *Lamellidens marginalis* after 96 hours exposure (400X) C: Effect of 70 ppm (LC_{50}) of Basic Blue 3 on hepatopancreas of fresh water bivalve *Lamellidens marginalis* after 96 hours exposure (400X)

DC – Digestive Cells, DT – Digestive Tubule,
L – Lumen, BM – Basement Membrane,
CT – Connective Tissue, IL – Interruption of Lumen Lining
VD – Vacuolar Degeneration, SD – Swollen Digestive Tuules,
HI – Hemocyte Infilteration, FE - Fusion of Epithelial cells,
DCB - Detachment of cells from basement DBM - Disintegration of Basement Membrane
NT - Necrotic Tissue,
SN - formation of Syncytium layer of Nuclei.



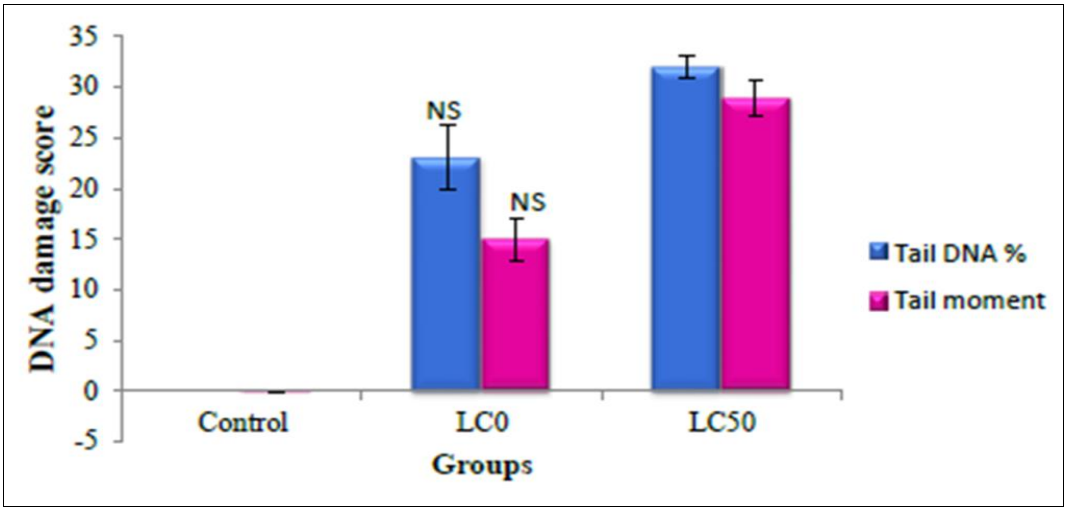
O – Oocytes, Pv – Previtellogenic Oocyte,
F – Follicle, LG – Lipid Globule,
V – Vitellogenic Oocyte, OP – Ooplasm,
DVO – Degeneration of Vitellogenic Oocyte, N – Necrosis,
DOP – Degeneration of Ooplasm, DFW - Degeneration of Follicle wall
DO - Degeneration of Oocyte, DN – Degeneration of nucleus,
DPVO - Degeneration of previtellogenic Oocyte.

Fig 3A: Section passing through gonad of fresh water bivalve *Lamellidens marginalis* from control group (400X). B: Effect of 40 ppm (LC0) of Basic Blue 3 on gonad of fresh water bivalve *Lamellidens marginalis* after 96 hours exposure (400X) C: Effect of 70 ppm (LC50) of Basic Blue 3 on gonad of fresh water bivalve *Lamellidens marginalis* after 96 hours exposure (400X)

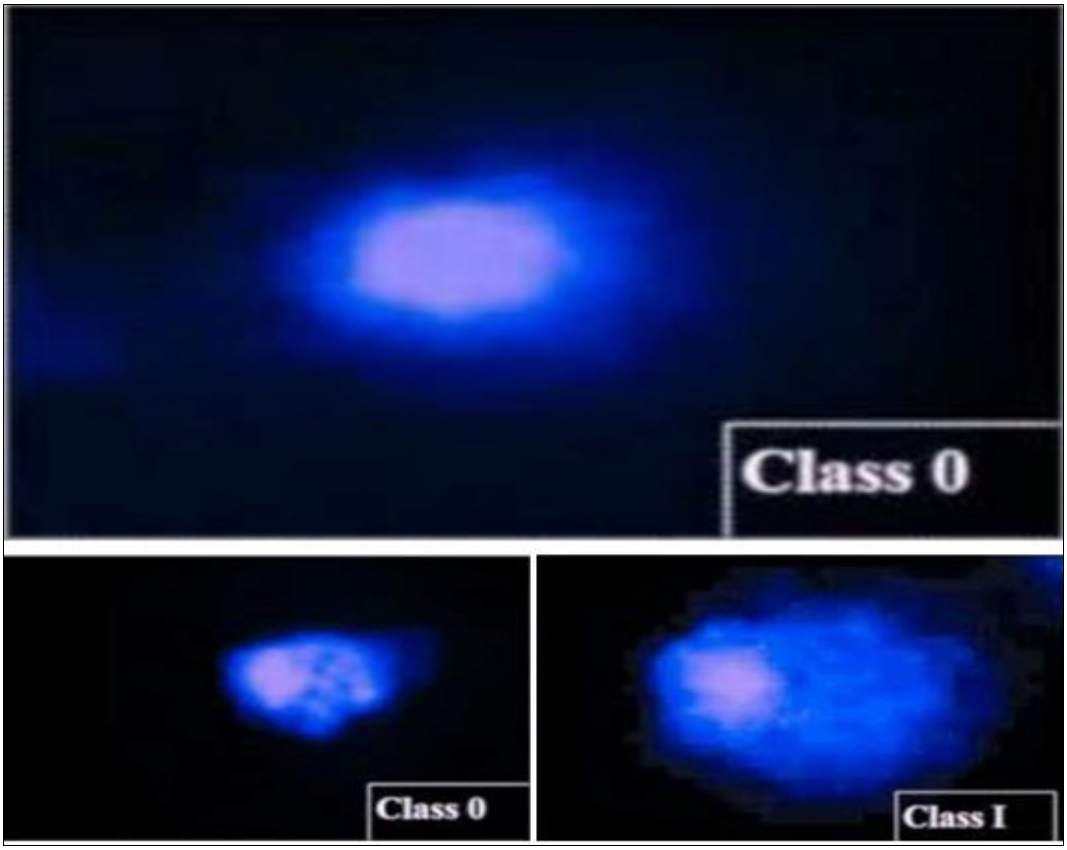
Table 4: Comet assay, tail DNA% and tail moment after acute exposure to Basic blue 3in *L. marginalis*.

Group	Tail DNA%	Tail moment
Control	1.1155±0.0084	0.0178±0.06
LC0	23.0729±3.21 NS	14.99±2.1 NS
LC50	32.0098±1.09 *	28.90±1.73 *

Values are Mean ± S.D., NS, *, indicates significance level Non significant, $p < 0.05$ (n =70)



Graph 4: DNA strand breaks in gill cells of *Lamellidens marginalis* after acute exposure to Basic blue 3.



According to Tail DNA% damage the three class were described-
Class 0 = 0 – 20%, Class I = 20 – 40%, Class II = 40 – 60%, Class III = over 60%

Fig 4A: Photomicrograph of gill cell of *Lamellidens marginalis* for 96 hours exposure; degree of damage class 0 B: Photomicrograph of gill cell of *Lamellidens marginalis* for 96 hours exposure to 40 ppm concentration of Basic blue 3; degree of damage class 0 C: Photomicrograph of gill cell of *Lamellidens marginalis* for 96 hours exposure to 70 ppm concentration of Basic blue 3; degree of damage class I

5. Conclusion

A small concentration of Basic Blue 3 affect physiology of bivalves. At acute exposure to this dye resulted into increased concentration of antioxidant enzymes which is defence mechanism of toxicological stress. The vital organ i.e. gill, hepatopancrease and gonad showed drastical structural changes which indicated that pathological effect of basic blue 3.Genotoxicity is the key tool which examine the depth of toxicity. The tail DNA% damages showed genotoxic effect of Basic blue 3 dye in fresh water bivalve, *Lamellidensmarginalis*.

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