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Malachite green induced histopathology and genotoxicity in *Lamellidens marginalis*

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Abstract
Malachite Green (Basic Green 4) is basic, water soluble dye, used as biological stain and mostly used in aquaculture practices as antiparasite, antihelmenthes and antifungal but excess dose is cytotoxic and mutagenic. In present study, the first report of histopathological and genotoxic effects of Malachite green in bivalves for acute exposure were studied. Exposure to acute dose of Malachite green for 96 hours showed harmful action in the bivalve *Lamellidens marginalis*. Histopathological investigations were conducted in gill, hepatopancrease and gonad in 0.3ppm and 0.9ppm concentration compared to control bivalve. In gill, changes were observed as rupture of epithelial cells, vacuolisation, damaged cilia, fused gill lamelle and formation of granuloma. Like gill, in hepatopancrease the structural changes i.e breakdown of basement membrane, digestive cells changed their normal shape get distorted, vaculisation, karyolysis and necrosis were noticed. The female gonad of bivalve showed degeneration of follicle wall with less number of developing oocyte, severely damaged vitellogenic and previtellogenic oocyte and mixing of cellular content of oocyte. The comet assay was recorded in gill cells at both the exposure. The tail DNA % and tail moment was more significant in 0.9 ppm concentration. The results indicate that the histopathological alterations and genotoxic assessment in the organs of *Lamelliden marginalis* as reliable biomarker in biomonitoring of Malachite green toxicity in aquatic ecosystem.

Keywords: genotoxicity, gill, gonad, hepatopancrease, *Lamellidens maginalis*, malachite green

Introduction
Malachite Green or Basic Green 4 or dimethylammonium chloride is basic dye mostly used as biological stain in cell and tissue culture and also used as antiparasite and anti fungal in aquatic fishes. This dye can readily soluble in water. Due to this nature, in aquaculture industries it was used in fish breeding from last few decades. The enormous and frequent use of this dye showed therapeutic as well as toxicolical effects in aquatic animals. Therefore it is necessary to taking into account it's toxicological effects prior to use in bath treatments of aquatic animals. MG present in waste water which was discharged from aquaculture industries showed toxicological effects in fishes at less than 1 mg/lit concentration (Hidayah *et al*., 2013) [8]. This dye also possess carcinogenic and genotoxic effects which have potential risk to humans therefore the several Europian countries have banned MG (Srivasthav *et al*., 2004). Due to low cost, easily availability and highly effective; it is still being used in many countries (Srivastava *et al*., 2004) [29]. The exact chemical composition of malachite green was not known. From different scientific studies it was showed that malachite green and its reduced form that is leucomalachite green might be persist in edible fishes (Mitrowska, Posyniak, 2004) [16] and slowly excreted. Like LMG different derivatives of MG i.e N-oxide and MG N- demethylated were detected in edible fishes. The accumulation MG and LMG residues in organisms depend upon exposure, size and species. The persistance of MG mostly depends upon Exposure period, concentration and environmental conditions including pH and temperature. However the removal of MG from waste water received less attention compared to other toxicants.
From past few decades, molluscs are used as biomonitaring organisms for several reasons (Hung *et al*.,2001) [11]. Molluscs are good bioindicators due to sedentary mode of habitat, long lived, have suffiecient tissue mass for analysis, filter feeder (Huang *et al*., 2007) [9]. Comparison with fishes and crustacean, the bivalves are having low level of activity of enzymes which metabolize the persistent organic pollutants. So that bivalves accurately showed the amount of environmental pollutants (Phillips *et al*., 1990) [20]. Since last few decades, histopathology has been used to investigate the effects of disease, toxicant stress and parasite infestations in wild mussel population. The toxicity by histological assessment is due to metals, insecticides, pesticides and chemicals have been reported by many investigators (Mane and Muley, 1984; Muley *et al*, 1990; Huang *et al*., 2007; Bhosale *et al*., 2017) [13, 9, 2]. The comet assay is simple and sensitive technique to evaluate the genetic damage at single cell level (Speit and Hartmann, 1999;Tice *et al*.,2000; Lee and Steinert *et al*., 2003) [27, 31]. The comet like shape is due to migration of broken DNA during electrophoresis. The size of comet is equal to the extent of DNA damage. Due to sediments accumulation the DNA damage in clam *Tapes semidecussatus* was recorded (Coughlan *et al*., 2002) [3]. After exposure to methyl methane sulphonate the genotoxicity in heamolymph cells

of *Corbicula flumineao* was observed by comet assay (Rigonato *et al*., 2005) [23] and also due to gasoline (Fedato *et al*., 2010) [4].
In present study, histopathological and genotoxic effects were studied after acute exposure for 96 hours to malachite green in tissues like gill, hepatopancrease and gonad of fresh water bivalve *Lamellidens marginalis*.

Material and Methods
The fresh water bivalve (*Lamellidens marginalis*) about 7 to 8 cm were obtained from Rajaram tank, Kolhapur, India and transported to aquarium. The physico chemical parameter of fresh water (pH - 7.77 ± 0.057; Temperature 0C-29.4 ± 1.14; DO(mg\l) - 3.79 ± 0.208; Free CO2(mg\l) - 07.23 ± 1.12; Hardness(mg\l) - 184. 67 ± 0.578; Phosphate (mg\l) - 0.43± 0.125; Nitrate(mg\l) - 1.51± 0.76). Three groups of bivalves (10 in each) were used. One control group, two were exposed groups as LC0 for 0.3 ppm and LC50 for 0.9 ppm for 96 hours. At the end of experiment, gill, hepatopancease and gonad was removed and used for histology. While gill cells were selected to study genotoxicity by comet assay. Malachite green dye (*N*,*N*,*N′*,*N′*-Tetramethyl-4,4′-diaminotriphenylcarbenium oxalate) were purchased from Sigma aldirich.

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 embeded in paraffinwax (melting point 56^0C to 59^0C). Then they were sectioned at 5-6 μ. 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.

Comet Assay
For comet assay procedure was adapted from Woods (1999) 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. Pellet was resuspended in PBS. 10 μl of suspension was mixed with 60 μl low melting agar and incubated at 37^0C. The slide with 1 % normal melting agar refrigerated for 5 minutes Spread sample evenly on slide. 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 for 5 minutes. Fix with ethanol. Washed with PBS (pH 7.5). Stain with DAPI for 10 min. Wash the slides with PBS. Coverslip was over the gel and 25 randomly chosen nucleoids per sample was analysed, as only the DNA that is visualised using an epifluorescence microscope. DNA damage was expressed as tail moment, which was determined as the product of the tail length and the fraction of DNA in the tail (Olive *et al*., 1990; Pavlica *et al*., 2001) and measured using the imaging analysis software CASP. After measuring the tail moment and tail DNA %, the results were stastically analyzed by one way ANOVA.

Results
The bivalves were divided in three groups i.e. control, LC_0 and LC_{50}. The remarkable structural changes were observed in all target organs when compared to control after acute exposure for 96 hours to Malachite green.

Gill
The gills are the vital organs in bivalves.The Plate I showed histopathological changes in gill. Plate I Figure A represents the normal structure of gill consists of epithelial cells, interlamellar space, cilia and chitinous rod. Each gill lamellae is free at their distal end.Gill lamellae are free and supported by interbranchial septa. Each lamellae were covered by monolayer of epithelial cells. Plate I Figure B represents the structural changes in gill of *Lamellidensmarginalis* after acute exposure to 0.3 ppm concentration of Malachite green. There was loss of interlamellar junction, some cells showed irregular vacuolization, at the basement membrane. The damaged cilia were observed and the dye residues were observed in epithelial cells which changes the normal structure of gill. Plate I Figure C showed drastical changes in structure of gill of *Lamellidensmarginalis* after 96 hour exposure to 0.9 ppm concentration of malachite green. There was reduction in cell number and nuclear size with elongated gill fillaments was observed. The gill lamellae get swollen and reduced lumen size. Inter lamellar space and fused to each other. In connective tissue, there was formation of granuloma (appearance of cells with brown colour).

Hepatopancrease
Plate 2 Figure A represents the hepatopancrease of *L marginalis* is the digestive gland which consist of hepatic ducts and digestive tubules in the form of bundles: indistinctly separated and connected to each other by inter lobular connective tissue of collagenous fibers. The digestive tubule consists of digestive cells or columnar type of cell. These cells secrete different digestive enzymes. The secreted products are seenin the lumen. After acute exposure for 96 hours 0.3 ppm and 0.9 ppm concentration of Malachite green, the hepatopancrease of *L marginalis*showed remarkable changes. After exposure to 0.3 ppm concentration (Plate II Figure B) the tubules were swollen, the basement membranes get ruptured at many places, the digestive cells were abnormal in shape and nuclei moved at the basement membrane.Infilteration of haemocytes between the tubules were observed.

While after exposure to 0.9 ppm concentration of Malachite green the hepatopancrease showed more drastical changes compared to control and LC_0 group. The tubules were shrinked, the basement membrane were ruptured at many places resulted into mixing of cellular content. The digestive cells changed their original structures and get distorted. The cells showed vacuolization, necrosis and karyolysis (Plate III Figure C). Gonad – In control group, thefemale gonad of L marginalis showed developing oocytes in different stages (Plate III figure A). The female follicles were compactly arranged with dark staineooplasm. The developing oocytes were seen with prominent nucleus along with follicles. The oocytes are large in size with granular cytoplasm. At 0.3 ppm concentration of Malachite green for 96 hours showed severe damages in follicle wall. Degeneration of follicle wall with less number of developing oocytes was observed. The lumen was more as compared to control group. The granular cytoplasm was degenerated in some vitellogenic oocytes (Plate III, Figure B). At 0.9 ppm concentration of Malachite green for 96 hours, showed the ooplasmspreaded out of the follicle. The follicles from the follicular wall were detached. The previtellogenic and vitellogenic oocytes were severely degenerated. The content of all oocytes were mixed with each other. The nuclei of some oocytes were degenerated (Plate III Fifgure C).

Comet Assay

The exposure of malachite green for 96 hours showed stastistically significant ($p < 0.01$) increase in tail DNA % and tail moment. Compared to control group, the damages were more in LC50 group. The % tail DNA was more in LC50 group cell (39.32 ± 1.98) than that of LC0 group (31.84 ± 2.54). The tail moment showed clear dose dependant increase i.e at 0.3 ppm showed 25.92 ± 3.11 a.u while at 0.9 ppm showed 35.78 ± 2.32 a.u. The comet tail was included in class I for both LC0 and LC50 group while the control was included in class 0. The images are shown in Plate no IV, Figure no. A, B and C.

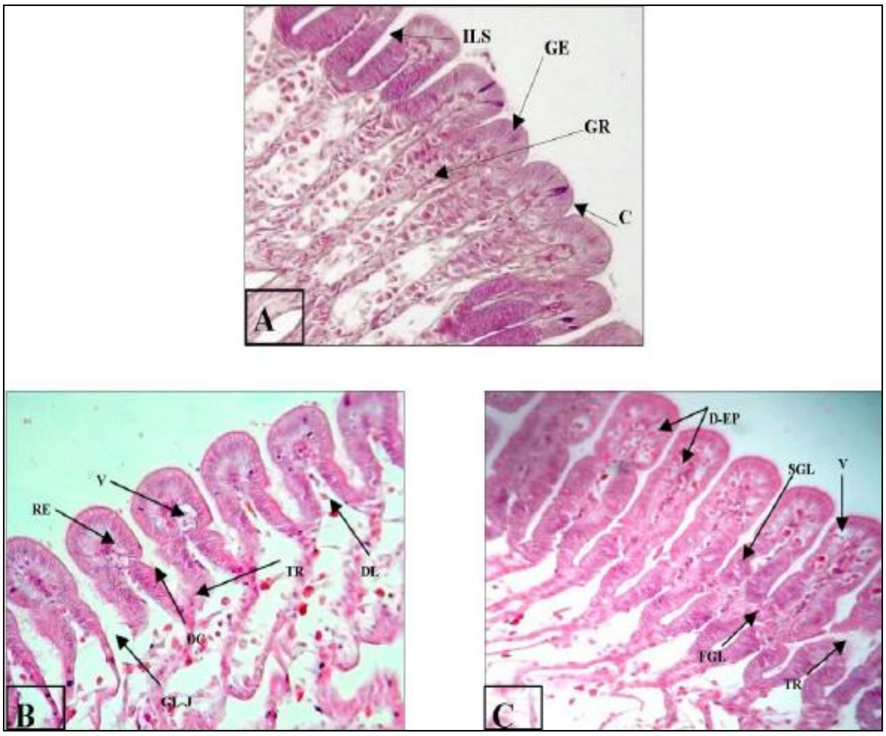


Plate 1

Fig A: Section passing through gill of fresh water bivalve *Lamellidens marginalis* from control group (400X).
Fig B: Effect of 0.3 ppm (LC0) of Malachite green on gill of fresh water bivalve *Lamellidens marginalis* after 96 hours exposure (400X). Fig C: Effect of 0.9 ppm (LC50) of Malachite green on gill of fresh water bivalve *Lamellidens marginalis* after 96 hours exposure (400X)
In Figures GE – Gill Epithelium, GR – Gill Rod, C – Cilia, ILS – Interlamellar Space, V – Vaccuolation, RE – Ruptured Epithelia, DC - Damaged Cilia, TR- Tissue Rupture, GL-J -- Junction of adjacent Gill Lamellae, D-EP - Damaged Epithelium, SGL- Swollen Gill Lamellae, FGL- Fused Gill Lamellae, DL=Dilate sinus.

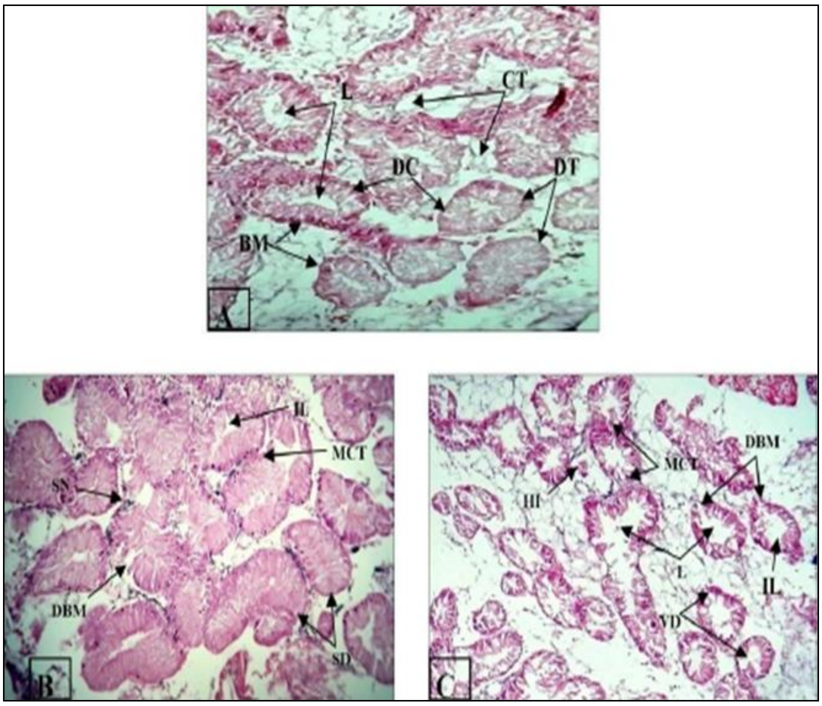


Plate II

Fig A: Section passing through hepatopancreas of fresh water bivalve *Lamellidens marginalis* from control group(400X). Fig B: Effect of 0.3 ppm (LC0) of Malachite green on hepatopancreas of fresh water bivalve *Lamellidens marginalis* after 96 hours exposure (400X). Fig 3 C: Effect of 0.9 ppm (LC50) of Malachite green on hepatopancreas of fresh water bivalve *Lamellidens marginalis* after 96 hours exposure (400X).
In Figures 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 Tubules, HI – Hemocyte Infilteration, DBM - Disintegration of Basement Membrane, SN - formation of Syncytium layer of Nuclei, MCT - Mixing of Cellular contents of different Tubules Membrane

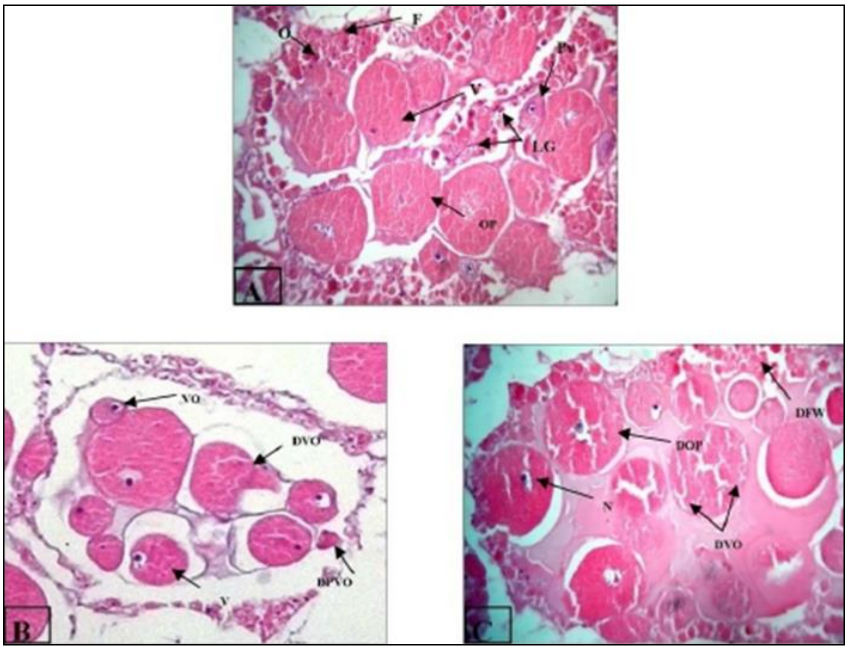


Plate III

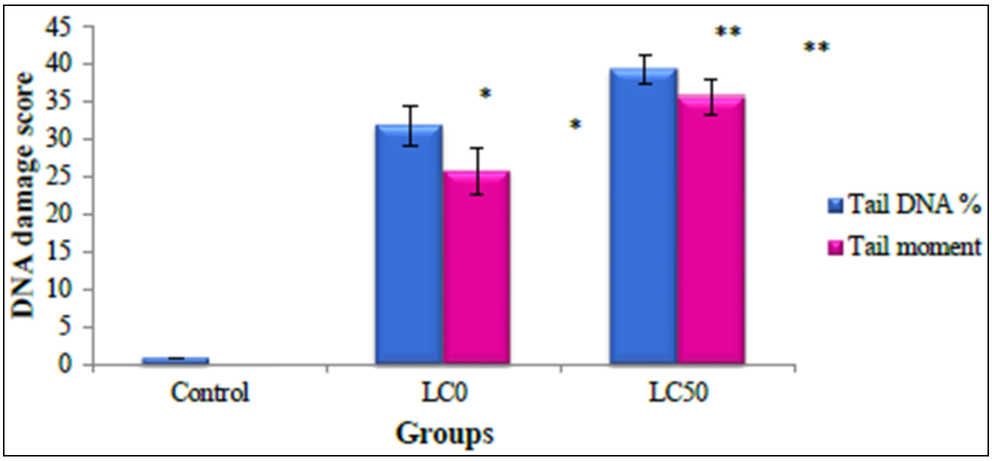
Fig A: Section passing through gonad of fresh water bivalve *Lamellidens marginalis* from control group(400X). Fig B: Effect of 0.3 ppm (LC0) of Malachite green on gonad of fresh water bivalve *Lamellidens marginalis* after 96 hours exposure (400X). Fig C: Effect of 0.9 ppm (LC50) of Malachite green on gonad of fresh water bivalve *Lamellidens marginalis* after 96 hours exposure (400X).
In Figures, 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, DPVO - Degeneration of previtellogenic Oocyte.

Table 1: Comet assay, tail DNA % and tail moment after acute exposure to Malachite green in *L. marginalis*

Group	Tail DNA %	Tail moment
Control	0.905 ± 0.0034	0.0272 ± 0.0138
LC0	31.8473±2.54*	25.9232±3.11*
LC50	39.3254±1.98**	35.7861±2.32**

Values are mean ± S.D., *, ** indicates significance level P < 0.05, P < 0.01 (n = 70)



Graph 1: DNA strand breaks in gill cells of *Lamellidens marginalis* after acute exposure to Malachite greenin *L. marginalis*.

Plate IV A: Photomicrogragh of gill cell of *Lamellidens marginalis* for 96 hours exposure; degree of damage class 0
B: Photomicrogragh of gill cell of *Lamellidens marginalis* for 96 hours exposure to 0.3 ppm concentration of Malachite green; degree of damage class I
C: Photomicrogragh of gill cell of *Lamellidens marginalis* for 96 hours exposure to 0.9 ppm concentration of Malachite green; degree of damage class I

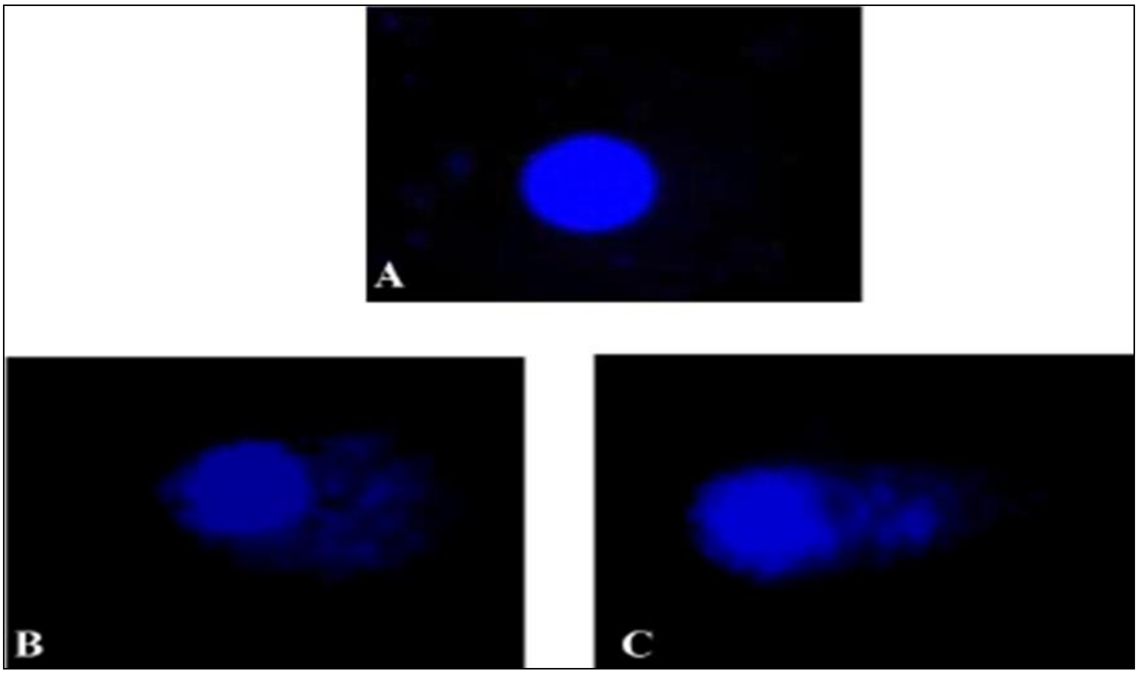


Plate IV

Discussion

Though Malachite green was banned in several countries for treatment for aquatic animals; due to readily availible and low cost MG is being used. MG and its reduced form Leucomalachite green both are toxic to aquatic organisms. The MG which dissolved in water can be easily absorbed and converted into leucomalachite green which can higher persistent than MG (Bauer *et al*., 1988). The toxicity of MG was reported in many fish species. This dye was reported as mutagenic, carcinogenic, teratogenic and chromosomal fracture while in some fish species it was act as respiratory enzyme poison which damage the cells who lose the ability to produce metabolic processes (Srivastava *et al*.,2004; Mitrowska *et al*., 2005; Stammati *et al*., 2005) [15, 29, 30].

Gill, Hepatopancrease and gonad are the important vital organs of bivalves involved in respiration, digestion and reproduction. Our study revealed that there were histopathological changes in selected organs after exposure to 0.3ppm and 0.9 ppm concentration of Malachite green. In bivalves, the gill is the first organ which contactswith any toxicant after exposure. The toxicant has potential to cause maximum damage to the organ that contact first (Timbrell *et al*., 1991) [32]. The ciliary loss was observed in treated group which can adversely affect the growth of bivalve (Qasim *et al*., 1977: Sivalingam, 1977) [2]. The gill of *Heteropneustesfossilis*showed necrosis, karyolysis, desquamation of epithelial lining cells and vacuolisation after exposure to MG (Srivastava *et al*., 1998b). 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 (Neweshy *et al*., 2011). The cytoplasm was also disintegrated because of swelling of epithelium which resulted into reduced interlamellar space (Sonwane *et al*., 2015). Jegede (2016) [12] reported that similar damages were observed in Tilapia after exposure to MG.The hepatopancreas in mollusc is the largest digestive gland formed by vast number of tubules, with different size and shape. After treatment with malachite green for 0.3ppm 0.9ppm concentration the degeneration of basement membrane, swelling and degeneration of digestive tubules were observed. The heavy metal, copper exposure caused cellular degeneration, vacuolization and rupturing of cells in digestive gland and foot of *Halotisrufescens* (Vaint *et al*., 2002). The acute exposure of copper and zinc showed cellular histopathological alteration like, rupturing of hepatopancreatic luminal cells and gland cells of the snail *Turritella sp.* (Heng *et al*., 2004). The digestive gland of bivalve *Crenomytilusgrayanus* showed vacuolization of digestive cells, lipofuschin, necrosis and lysis of cells in connective tissue due to polluted water (Usheva *et al*., 2006). Yasmeen (2012) observed the histopathological damages like distortion of tubules, karyolytic and necrotic condition of hepatopancreas after acute exposure to cadmium in *Lamellidens marginalis*. To ecotoxicology, the gill cells are used as a cellular model which is constantly exposed to dissolved pollutant and able to metabolize mutagens and carcinogens into active products (Mitchelmore *et al*., 1998; Wilson *et al*., 1998) [14] Genotoxicity is one of the important tool in testing chemical toxicity and risk assessment (Hayashi *et al*., 2005). Rank (2005) [22] found the significant corelation between the DNA tail moment and chromium concentraion in haemocytes of *Mytilus edulis*. Grazeffe (2008) classified the 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 (Sarkar *et al*., 2013).

All the histological changes indicated that exposure to Malachite green to acute concentration caused degenerative effects such as damaged and fused epithelial cells, ruptured tissue, swollen gill lamellae in gill tissue while the hepatopancrease showed swollen and damaged hepatic tubules, vacuolization. In gonadal tissue degeneration of oocytes were observed. These changes in normal structure of the tissue lead to loss of normal physiology of animals. While tail DNA % and DNA damage were significantly noticed in both 0.3 ppm and 0.9ppm concentration. The genotoxic effect of malachite green dye was first recorded in fresh water bivalve.

Conclusion

With accordance to the results of present study, it can be concluded that Malachite green dye having potential to accumulate in the targeted tissues of *Lamellidens marginalis*. Malachite green forms granuloma in the cells. After observing the histology, it can be said that the tissues gill, hepatopancrease and gonad showed drastical structural changes. At both the concentration the DNA damage was significantly observed in bivalve. After exposure to the small concentration of Malachite green dye for short period is cytotoxic, damage to internal physiology of bivalves. Thus Malachite green is highly toxic to small concentration to fresh water bivalves.

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