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Biochemical and antioxidant alterations in fresh water bivalve, *Lamellidens marginalis* exposed to malachite green dye

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Abstract

Aim : To determine acute toxicity (96 hrs) of malachite green for *Lamellidens marginalis* and to assess its biochemical consequences and antioxidant response against control.

Methodology : Fresh water bivalve, *Lamellidens marginalis* were collected from Rajaram tank, Kolhapur and acclimatized for 7 days. To ascertain the acute effect (96 hrs) of malachite green dye, static bioassay was conducted at 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2 ppm on fresh water bivalve, *Lamellidens marginalis*. The rate of mortality was recorded and LC₀ and LC₅₀ values for 96 hrs were calculated. Estimation of biomolecules like protein, glycogen and lipid and antioxidant activity in different tissues of the organism was estimated.

Results : The LC₀ and LC₅₀ values were determined by static bioassay method. The effects of LC₀ and LC₅₀ concentrations of MG were evaluated by examining the biochemical profile and antioxidant response against control in different tissues like gill, mantle, hepatopancreas and gonad. The maximum significant decrease in glycogen (-29%) (-40.86%) and protein (-31.73%) (-43.63%) content was observed in gills at both the concentrations LC₀ and LC₅₀ respectively as compared to other tissues. There was highly significant depletion in protein content of all the tissues. Lipid content in all tissues at LC₀ concentration showed moderately significant depletion ($p < 0.01$), while at LC₅₀ concentration highly significant depletion ($p < 0.001$) was recorded. From SOD, CAT and GPx enzymes, SOD enzyme activity increased significantly ($p < 0.001$) in all tissues at LC₅₀ concentration. Gill and hepatopancreas showed significant increase in antioxidant enzymes response at both, LC₀ and LC₅₀ groups.

Interpretation : Malachite dye induces toxicity, by lowering biochemical content and inducing antioxidant enzyme activity.

Key words: Antioxidant enzymes, Hepatopancreas, *Lamellidens marginalis*, Malachite green, Static bioassay

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Introduction

Dyes are substances that impart colour to the materials. They are widely used in textile, paper, rubber, plastic, paints, drugs, pisciculture, food and cosmetics industries. Dye residues directly released into water bodies, cause severe pollution and also alter the physico-chemical parameter of water bodies, thereby affecting the aquatic biota of that system. The breakdown products of these dyes are harmful to both aquatic biota and human life (Van Der Zee *et al.*, 2001; Seesurivachan *et al.*, 2007). High concentration of textile dyes in water bodies decreases reoxygenation of water and ceases the penetration of sun light in water (Zaharia and Suteu, 2012).

Malachite green is easily soluble in water and is mostly used as food coloring agent, food additive, food disinfectant and also as dye in cotton, silk, wool, jute and leather industries. In aquaculture industries, malachite green dye is generally used as disinfectant for fungal infection since 1936 and for other ectoparasites like protozoan, trematodes and larvae of crustaceans (Sudova *et al.*, 2007). After exposure, malachite green is absorbed by aquatic animals and undergoes biotransformation into leucomalachite green. The residues of these molecules are stable both in, environment and aquatic animals (Srivastava *et al.*, 2004; Mitrowska *et al.*, 2007). Malachite green has already been reported as carcinogenic, mutagenic, chromosomal fracture, teratogenic, reduced fertility, developmental abnormality and other physiological changes in animals (Schuetze *et al.*, 2008; Pourreza *et al.*, 2010). The carcinogenic effects of malachite green have been verified in different tissues like liver, lung, kidney, ovary and thyroid of experimental animals (Culp *et al.*, 2006; Srivastava *et al.*, 2004). Due to extreme toxicity and ecotoxicity hazards associated with malachite green, restrictions have been implemented in many countries since 2000. However, it is still being used in several parts of the world due to low cost, ready availability and efficacy. Considerable amount of research is being devoted to work out the wide spectrum of biological effects it exerts on different animals and mankind.

Toxicological effects of this dye has been reported in cat fish, channel cat fish, Salmon, tilapia, snake head barb and carp (Sudova *et al.*, 2007). A wide range of both organic and inorganic residues accumulate in animal tissues altering the biochemical content of tissues and inducing reactive oxygen species. This may change normal physiology of animal. Bivalves have been used as sentinel organisms in pollution monitoring programmes (Moreira and Guilhermino, 2005). Many molluscan species such as *L. marginalis*, *L. corriance*, *B. bengalasis* are used as bioindicator depending on their tolerance power (Oehlmann *et al.*, 2002; Druart *et al.*, 2011). *Lamellidens marginalis* is considered as one of the best bioindicator of pollution in aquatic environment (Kumar *et al.*, 2017). It has been reported that pollutants exhibit toxicity on molluscs at lower environmental concentrations than other invertebrates or vertebrates (Chakraborty *et al.*, 2010). In the view of the above, the aim of the present study was to assess

acute toxicity of malachite green dye and its impact on biochemical parameters and antioxidative enzymes of *L. marginalis*.

Materials and Methods

Fresh water bivalve *Lamellidens marginalis* of about 7 to 8 cm in size were collected from Rajaram tank, Kolhapur and brought to laboratory and cleaned to remove fouling algal biomass and mud. The animals were acclimatized for 7 days in glass aquaria containing dechlorinated tap water. Malachite green oxalate salt (*N,N,N',N'*-Tetramethyl-4,4'-diaminotriphenylcarbenium oxalate), CAS number – 2437-29-8 purchased from Sigma Pvt. Ltd. USA, was used for preparing stock solution. The stock solution was prepared by dissolving 10 mg powder in 10 ml distilled water and diluted up to desired concentration.

Preliminary tests were carried out to determine the LC_0 and LC_{50} values of malachite green in bivalves for 96 hrs by static bioassay method. To determine LC_0 and LC_{50} values, 10 bivalves were kept in each container containing 10 l tap water at different concentrations of malachite green (0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1 and 1.2 ppm) for 96 hrs. Water was changed every 24 hrs and the mortality of animal was noted after each change. Bivalves kept in container with tap water was referred as control. The percentage mortality with respect to concentration of malachite green was recorded (Table 1) after 96 hrs. The concentration at which 0 % mortality occurred was taken and taken as LC_0 whereas the concentration at which 50 % mortality occurred was taken as LC_{50} . After determining the LC_0 and LC_{50} values, animals of only three groups *i.e.* control, LC_0 and LC_{50} were used for further study.

After exposure period of 96 hrs bivalves from three groups, *i.e.*, control, LC_0 and LC_{50} , were sacrificed and different tissues like gill, mantle, hepatopancrease and gonad were separated to study various biochemical parameters. Glycogen was estimated by using anthron reagent (DeZwaan and Zandee, 1972), total protein content was estimated by the method of Lowry (1956) and lipid content was estimated by vanillin reagent (Barnes and Blackstock, 1973).

The activity of antioxidant enzymes like SOD, CAT and Gpx were measured spectrophotometrically after 96 hr exposure to malachite green dye. SOD activity was measured by the method of Beauchamp and Fridovic (1971) with slight modification. Catalase activity was estimated by the method of Luck (1974) where as GPx activity was measured following the method of Beers and Sizor (1952), respectively

For acute toxicity tests, the mortality data was statistically analyzed by Probit Analysis (Finney *et al.*, 1971). The data on biochemical profile and antioxidant response was analyzed for statistical significance between control and experimental bivalve groups by Student's *t*-test. Significant differences were established at *p* values of 0.05, 0.01 and 0.001.

Results and Discussion

The relation between the concentration of malachite green and mortality rate of fresh water bivalve *Lamellidens marginalis* is shown in Table. 1. During experimental period, bivalves showed no mortality at 0.3 ppm concentration. The observed LC_{50} value was 0.9 ppm and the calculated LC_{50} value was 0.88 ppm. The regression equation established was $Y = 7.016x + 5.365$. Presence of malachite green and its metabolized and transformable form, i.e., leucomalachite green accumulate in tissues of aquatic animals (Zlatka Bajcl *et al.*, 2007; Hidayah *et al.*, 2013; Srivastava *et al.*, 2015). Cooking fish at high temperature, the leucomalachite form is stable. This leucomalachite form persist in tissues for several days, which affects the metabolism of that animal. Some workers have reported its LC_{50} values for fishes (Clarke and Anliker, 1980, Srivastava *et al.*, 2004). Different LC_{50} values for different fishes were found for same dye. Srivastava *et al.*, 2007; Sudova *et al.*, 2007 determined the LC_{50} value for fishes like Silver barb (0.2mg l⁻¹), Snake head fish (0.17mg l⁻¹), Channel catfish (0.975 mg l⁻¹), Rainbow trout (1.4 mg l⁻¹), Carp (0.19 mg l⁻¹), Atlantic salmon (1.0 mg l⁻¹), Nile Tilapia (0.45 mg l⁻¹). It has been observed that some fishes are sensitive towards the toxicity caused by textile dye while some are less sensitive to same dye.

Glycogen serves as an energy storage compound in animals, and also plays an important role in energy metabolism. In the present study, is a significant decrease in glycogen content was found in all tissues exposed to malachite green (Fig. 2). The gill showed highly significant depletion of glycogen in animals exposed to LC_0 (-29%) and LC_{50} (-40.86%) concentration as compared to control. The mantle and hepatopancrease showed moderately significant decrease of glycogen at both LC_0 (-7%); (-6.87%) and LC_{50} (-17.23%); (-21.5%) concentrations with respect to control workers reported that, the alteration in glycogen content was due to toxicological stress when exposed to toxicants. Decrease in glycogen content in all tissues may be due to the utilization of carbohydrates for energy production and induced hypoxia by

toxicants (Somaiha *et al.*, 2016). In the present study, gills showed maximum decrease in glycogen content as compared to other tissues, which may be due to direct exposure of toxicant to the gills.

Protein plays a major role in cell metabolism because of the proteinaceous nature of all enzymes which mediates various metabolic pathways. The protein content in different tissues of fresh water bivalve, *Lamellidens marginalis* of control group was in the order : gonads > hepatopancrease > mantle > gills. In present finding, all four tissues showed significant depletion in protein content at both, LC_0 and LC_{50} concentrations of malachite green after 96 hrs. The maximum decrease was observed in gills at both LC_0 and LC_{50} concentrations (-43.13% and -31.73%). Similar trend was observed in mantle (-26.08%) and hepatopancrease (-26.1%) at LC_{50} concentration. In gonads, depletion in protein content was less at both LC_0 (-18%) and LC_{50} (-25.54%) concentrations. Depletion in protein content suggests an increased proteolysis and possible utilization of degraded products for metabolic reaction (Khare *et al.*, 2002; Rao *et al.*, 2000). The decrease in protein content may be due to increased catabolism and decreased anabolism of proteins or inhibition of protein synthesis (Muley *et al.*, 2007).

Malachite green induced toxicity decreased in lipid content in different tissues of *L. marginalis*. The total lipid content in control group was in the following order : gonads> hepatopancrease>mantle> gills. Comparatively a high significant ($p < 0.001$) decrease in lipid content was observed in all tissues studied at 0.9 ppm (LC_{50}) concentration, while moderately significant decrease in lipid content was noticed at 0.3 ppm (LC_0) concentration in all tissues. The maximum decrease in lipid content was observed in gills (-35.10%) at LC_{50} . Alterations in lipid content in all target organs are due to utilization of lipid component at cellular level, further increased lipolysis or mitochondrial structural damage impair the function of citric acid and fatty acid mechanism (Dhanpackium *et al.*, 2000). In the present study, at LC_0 concentration the decrease in lipid content was moderately significant, while at LC_{50} concentration the

Table 1 : Acute toxicity of malachite green dye in fresh water bivalve *L. marginalis*

Concentration of malachite green (mg l ⁻¹)	Log of concentration	No. of bivalves exposed	No. of bivalves died at 96 hrs	Probit kill	Percent kill (%)
0.3	-0.5228	10	00	00	00
0.4	-0.7184	10	01	3.72	10
0.5	-0.3010	10	02	4.15	20
0.6	-0.2218	10	03	4.48	30
0.7	-0.1549	10	03	4.48	30
0.8	-0.0969	10	04	4.75	40
0.9	-0.0457	10	05	5.00	50
1.0	00	10	05	5.00	50
1.1	0.04139	10	06	5.25	60
1.2	0.07918	10	06	5.25	60

Fig. 1 : Glycogen content in fresh water bivalve *Lamellidens marginalis* exposed to LC₀ and LC₅₀ concentrations of malachite green.

Fig. 2 : Protein content in fresh water bivalve *Lamellidens marginalis* exposed to LC₀ and LC₅₀ concentrations of malachite green.

depletion was highly significant in all tissues, i.e., the concentration of malachite green was directly proportional to depletion in lipid content. In response to catalyzing ROS, three antioxidant enzymes, (SOD, CAT, GPx) were positively correlated with each other (Manduzio *et al.*, 2005). There was highly significant increase in the activities of SOD, CAT and GPx in *L. marginalis* when exposed to LC₀ and LC₅₀ concentration of malachite green dye for 96 hrs. The superoxide dismutase activity increased significantly in tissues at 0.05 and 0.001 level of

significance (Fig 4). The increase in enzyme activity was in the following order : gills > hepatopancrease > gonads > mantle at both (LC₀ and LC₅₀) concentrations as compared to control group. Gonad and mantle showed less significant increase at $p < 0.05$ in LC₀ group, while gills and hepatopancrease showed more significant increase at $p < 0.001$ in LC₀ group against the control. Pollutants or their metabolites may exert harmful effects by catalyzing ROS production. If the balance between pro-oxidants and antioxidants is disrupted, it may result in oxidative stress and

Fig. 3 : Lipid content in fresh water bivalve *Lamellidens marginalis* exposed to LC₀ and LC₅₀ concentrations of malachite green.

Fig. 4 : SOD activity in fresh water bivalve *Lamellidens marginalis* exposed to LC₀ and LC₅₀ concentrations of malachite green.

induce further damage tissues due to ROS and impair cellular functions, alter the physico-chemical properties of cell membranes and finally disrupt vital functions of the body (Manduzio *et al.*, 2005). Dyuthi (2010) have observed increased concentration of antioxidant enzymes like SOD, CAT and GPx in *Oreochromis massambicus* when exposed to malachite green. SOD acts as a primary defence against oxygen toxicity by catalysing the conversion of superoxide anion to oxygen and hydrogen peroxide (Alscher *et al.*, 2002; Manduzio *et al.*, 2005; Miller *et al.*, 2008).

Catalase activity changed at both the concentrations after 96 hrs exposure. At LC₀ concentration, the gonads and mantle

showed non significant increase in CAT activity, while gill (12.55%) and hepatopancrease (14.51%) showed significant increase at $p < 0.05$ as compared to control. At LC₅₀ concentration, there was highly significant increase ($p < 0.001$) in catalase activity in gills (29.14%). This was due to stimulation of antioxidant defence mechanism in gills which may be due to direct contact with toxicant (Monteiro *et al.*, 2006). SOD catalyses dismutation of superoxide anion radicals ($O_2^{\cdot-}$) to H_2O_2 , which is reduced to water and molecular oxygen by CAT or by GSH-dependant pathway through GPx. Increased activity of SOD generated during oxidative stress inhibits CAT activity (Kono *et al.*, 1982). The

Fig. 5 : CAT activity in fresh water bivalve *Lamellidens marginalis* exposed to LC₀ and LC₅₀ concentrations of malachite green.

Fig. 6 : GPx activity in fresh water bivalve *Lamellidens marginalis* exposed to LC₀ and LC₅₀ concentrations of malachite green.

activities of antioxidant enzymes can vary, depending on the concentration and duration of stress applied to the organism and on susceptibility of exposed species (Ballesteros *et al.*, 2009). Comparing the activity of antioxidant enzymes, SOD activity was more significant than CAT and GPx. GPx activity was more in exposed bivalves compared to that of control. Gonads and mantle showed non significant increase at both, LC₀ and LC₅₀ concentration. Gills and hepatopancrease showed significant increase at both LC₀ (24.35% and 22.83%) and LC₅₀ (28.48% and

29.46%) concentration at $p < 0.05$ and $p < 0.01$. The increased activity of GPx in gills and hepatopancrease is the most sensitive biomarker as a function of xenobiotic concentration (Moreira *et al.*, 2005). GPx removes hydrogen peroxide and lipid hydroperoxides in mammals, while its role has been recently investigated in different aquatic animals (Sole *et al.*, 1995). The role of GPx is probably to maintain normal cell functions whereas SOD and CAT acquires importance in response to oxidative stress (Vidal-Linan., 2010). The increased activity of SOD and CAT was

observed in *Perna perna* exposed to different metals (Almeida *et al.*, 2004). Vlahogianni (2007) reported increased activity of antioxidant enzymes exposed to metals in bivalve *Mytilus galloprovincialis*. The effect of textile dyes with alterations in its biochemical content and antioxidant response in fresh water bivalve has not been reported so far. Malachite green affects the biochemical content and antioxidant enzyme activity of bivalves which was not studied yet on any experimental animal. Our investigation suggests that, like metals, pesticides and textile dyes are also harmful to aquatic biota.

A small concentration of malachite green affect the biochemical content of bivalve and it also increases the activity of antioxidant enzymes within period of 96 hrs of exposure. From the above results, it can be concluded that malachite green alters the metabolism of bivalve.

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