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Original Article

Assessment of Toxicity of Monocrotophos in Freshwater Bivalve, *Lamellidens marginalis*, Using Different Markers

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ABSTRACT

The present study was undertaken to evaluate the toxic effects of monocrotophos, a widely used organophosphorus pesticide, on *Lamellidens marginalis* with a wide battery of biomarkers consisting of AchE inhibition, lipid peroxidation, the levels of antioxidant enzymes, and histopathological changes. Animals were exposed to monocrotophos (52.36 mg/l) for four days. Malondialdehyde (MDA) values were measured as index of oxidation while Superoxide dismutase (SOD), Catalase (CAT), Glutathione s-Transferase (GST), and Glutathione-Reductase (GR) were measured as index of an antioxidant status. After exposure, a significant reduction of the capability to neutralize radicals was observed. Histopathological changes, such as fibrosis in gill filaments and hypertrophy in mucous cells of foot tissue, were observed after treatment.In a second series of experiment, exposed animals were thereafter transferred to clean water and kept in it up to 28 days to assess the recovery pattern. Significant recovery is observed in AchE and antioxidant enzymes. Oxidative damage observed after acute exposure indicate that mussels faced an oxidative challenge but were able to counteract, as values of anti-oxidants returned near to control values after 28 days. Altered activities in anti-oxidant enzymes due to stress recovered well after 28 days in gill and muscles as compared to foot and mantle. Overall results suggested that oxidative markers are highly sensitive and could be profitably applied to freshwater mussels for environmental quality assessment in freshwater.

Key words: Antioxidants, hypertrophy, monocrotophos, oxidative stress, recovery

INTRODUCTION

Pesticides are widely used in agriculture for pest control.[1] The pesticides that enter the aquatic system through surface run off may adversely affect the aquatic biota.[2,3] The half life of monocrotophos (MCP) in natural water (pH 7.6) at 25°C and at 35°C is 147 days and 29 days, respectively. This suggests a considerable biodegradability and hence relatively low persistence in the environment.[4] Furthermore, many authors postulate that these compounds disturb the redox processes, change the activities of anti-oxidative enzymes, and cause enhanced lipid peroxidation in many organs.[5] MCP-induced biochemical alterations are studied in *Tilapia mossambica*.[6] Hyperglycemic condition accompanied by AchE inhibition[7,8] and oxidative stress is observed in rats exposed to MCP.[9] Altered expressions of selected cytochrome P450s are observed in MCP-induced apoptosis in neuronal cells.[10]

In the context of the present study, *Lamellidens marginalis* was selected as test species as it is known to accumulate significant amount of contaminants because of its sedentary life style and long life span. Moreover, it links primary producers with higher organisms in aquatic food-chain and

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forms a part of the diet of the local population.[11] Therefore, the study aims to:

1) Investigate the effect of exposure of MCP on the tissues of *L. marginalis* and the associated histopathological changes
2) Estimate oxidative damage to the exposed tissues
3) Investigate the effect of MCP on acetylcholine esterase activity (AchE).

MATERIALS AND METHODS

Animal collection site and rearing of animals

The reservoir selected for the study is near Yedgaon dam on the river Kukadi (19°10' 59.62" N and 73°57'19.09" E). The pesticide contamination in reservoir water was assessed by Gas chromatography Mass spectrometry (GC-MS) analysis. The freshwater mussels, *L. marginalis,* were collected from reservoir (shell- length 7-9 cm), transported to laboratory, and acclimatized to laboratory condition for seven days in aged tap water. The animals were fed daily *ad libitum* with algal suspensions of spirulina[12] every day during acclimatization period. The water was renewed after every 24 hours.

Acute toxicity bioassay

The formulated pesticide toxicant (Phoskill 36%) selected for exposure was Dimethyl (E) 1-methyl-2-(methylcarbamoyl) vinyl phosphate, a polar compound whose common name is MCP.

Acute toxicity (96 hours) bioassay experiment was done by exposing ten mussels to each of the concentrations (0, 20, 40, 60, 80, 100, and 120 ppm) in quadruple to determine LC_{50}. The renewal was done after every 24 hours. Feeding was stopped during the experimental duration. LC (10, 50, and 90) 96 hours values were estimated by fitting two parameter log-logistic functions with binomial type using the DRC package,[13] in R version 3.0.0.[14] The model parameters [LC (10, 50, and 90) = median lethal concentrations] were estimated. A total of 12 animals were exposed in triplicate, to sub lethal LC_{10} (52.36 ppm) concentration of MCP along with a set of control group for 96 hours. After acute exposure, six animals were sacrificed to collect the tissues for biochemical estimations and histological studies. Remaining six animals were divided in two groups and transferred to pesticide-free water for 14 days and 28 days, respectively, to study the recovery response. The conditions during the recovery experiment were the same as those in the exposure experiment. At the end of the recovery period, tissues were isolated using the same methods as in the exposure experiment and used for further analysis. This work was designed in accordance with the guidelines of the institutional (University of Pune) norms of animal handling and care.

Estimation of protein

The protein content was measured by Lowery *et al.*[15] method.

Estimation of AchE

AchE activity was measured by Ellman *et al.*[16] method.

Estimation of thiobarbituric acid reactive substances

The TBARS were measured by Esterbauer and Cheesman[17] method to evaluate lipid peroxidation.

Estimation of SOD

SOD activity was determined by the method of Beauchamp and Fridovich.[18]

Estimation of CAT

CAT activity was measured by the method of Aebi.[19]

Estimation of GST

GST activity was measured by Habig *et al.*[20] method.

Estimation of GR

GR activity was quantified by Goldberg *et al.*[21] method.

Histopathological examination

The gill and foot tissues for histopathological analysis were fixed in Bouin's solution. 5-6 µm sections were prepared from paraffin blocks with the help of microtome. These sections were stained with Hematoxylin eosin stain, and observed under Carl Zeiss Axioscope A1 at ×10 and ×40 magnifications.

Statistical analysis

LC_{10} and LC_{50} were estimated by fitting two parameter log-logistic functions with binomial type using the DRC package,[13] in R version 3.0.0.[14] The statistical data analysis was carried out using one-way ANOVA for biochemical estimations. Data were presented as the mean ± Standard deviation (S.D.).

RESULTS

According to the results of GC-MS analysis, the pesticide concentrations in the water from the collection site were below the limit of quantification (0.01-1.01 ppb). After exposing animals to increasing concentration of MCP, LC_{50} values were calculated after 96 hour's exposure of *L. marginalis* to MCP, as shown in Table 1.

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The results of AchE activity in gill (66.15%), foot (55.38%), and muscle (42.55%) of *L. marginalis* exposed to MCP [Table 2] reveal that the AchE activity in treated animals is inhibited significantly ($P < 0.05$) when compared to control. After 28 days, significant ($P < 0.05$) recovery in AchE level was observed in gill (98.23%), foot (70.98%), and muscle (82.55%).

The levels of TBARS were estimated in the control and experimental animals. It was observed [Table 2] that lipid peroxidation was significantly increased ($P < 0.05$) in mantle (266.87%), gill (223.97%), foot (197.52%), and muscle (173.88%) in treated animals as compared to control ones. TBARS level significantly decreased ($P < 0.05$) in mantle (73.90%) after 14 days. Significant ($P < 0.05$) recovery was observed in mantle (60.15%), gill (33.95%) and muscle (36.77%) after 28 days.

After toxicant exposure, trend of CAT inhibition [Table 2] observed was mantle (88.55%) >muscle (32.11%) >gill (24.87%) with respect to control animals. After 14 days, 100% recovery in CAT activity was observed in only muscle tissue. Significant recovery in gill (92.59%) and mantle (97.48%) was observed at the end of 28 days.

The trend of increased SOD activity [Table 2] after toxicant exposure was gill (392.42%)> mantle (278.91%)> muscle (161.59%)> foot (144.11%). After recovery period of 14 days, SOD activity was reduced significantly ($P < 0.05$) in mantle (60.50%), muscle (47.83%), and gill (36.68%) except in foot (3.69%). SOD activity recovered significantly ($P < 0.05$) in muscle (81.02%)> mantle (56.02%)> gill (55.79%)> foot (37.59%) at the end of 28 days.

GST activity [Table 2] showed significant ($P < 0.05$) inhibition in gill (36.58%), while significant induction was observed in foot (133.33%) and muscle (216.13%) after 96 hours of exposure. After 14 days, significant ($P < 0.05$) twofold recovery in muscle (51.87%) was observed. Significant ($P < 0.05$) recovery in GST activity was observed in gill (86.10%), muscle (54.10%), and foot (23.60%) at the end of 28 days.

After acute exposure, significant ($P < 0.05$) decrease in GR activity [Table 2] was observed in mantle (57.14%) and muscle (30.43%), while significant ($P < 0.05$) twofold increase was observed in gill. After 14 days, muscle (100%) and mantle (69.04%) recovered significantly ($P < 0.05$). Significant ($P < 0.05$) recovery was observed in gill (66.30%), muscle (108.69%), and mantle (85.71%) at the end of 28 days.

In the present investigation, as a result of acute exposure of MCP, alteration in tissue architectures was observed in gill and foot. Regular arrangement of columnar epithelial cells with uniform distribution of cilia was observed in Transverse

Table 1: Lethal concentrations of MCP for over 96 hours

LC	Estimate	Lower	Upper
10	52.36	11.13	93.59
50	75.40	43.54	107.26
90	108.57	35.45	181.69

MCP = Monocrotophos, LC = Lethal concentration

Table 2: Alterations in AchE, TBARS, CAT, SOD, GST, and GR activities in *L. marginalis* exposed to MCP (52.36 ppm) for 96 hours

Tissue exposed	Gill	Foot	Muscle	Mantle
Ache activity (moles/ml)				
Control	4.52±0.56	10.13±0.79	5.50±0.89	
Treated	1.53±0.61[a]	4.52±1.44[a]	3.16±1.64[a]	
14 D recovery	3.42±0.28[b]	4.95±0.64	3.16±0.41	
28 D recovery	4.44±0.21[c, e]	7.19±0.98	4.54±0.29	
TBARS activity (nmol/mg protein)				
Control	4.59±1.89	3.63±0.08	5.13±1.46	5.04±0.88
Treated	10.28±1.3[a]	7.17±3.14	8.92±2.51[a]	13.45±1.11[a]
14 D recovery	8.98±0.57	8.90±0.7	8.01±0.3	9.94±0.97[b]
28 D recovery	6.79±0.31[c]	9.16±0.44	5.64±0.31[c]	5.36±0.35[c, e]
CAT activity (unit/mg protein)				
Control	63±6.93	51.45±5.5	39.86±2.68	115.30±2.6
Treated	47.33±4.04[a]	96.81±15.9[a]	27.06±3.02[a]	13.2±2.3[a]
14 D recovery	30.67±8.08[b]	62.04±12.1	40.26±6.95[b]	9.55±3.5
28 D recovery	58.33±3.51[e]	53.52±7.6[c]	37.03±7.53[c]	112.39±15[c, e]
SOD activity (unit/mg protein)				
Control	1.32±0.12	6.96±0.12	4.14±0.39	1.28±0.06
Treated	5.18±0.11[a]	10.03±2.13[a]	6.69±0.52[a]	3.57±0.19[a]
14 D recovery	3.28±0.32[b]	9.66±0.25	3.49±0.22[b]	1.41±0.21[b]
28 D recovery	2.29±2.29[c, e]	6.26±0.49[c, e]	1.27±0.45[c, e]	1.57±0.17[c]
GST activity (unit/mg protein)				
Control	6.26±0.48	2.67±0.44	2.48±0.19	3.46±0.26
Treated	3.97±0.26[a]	3.56±0.37[a]	5.36±0.29[a]	21.92±1.02
14 D recovery	5.34±0.53[b]	3.53±0.24	2.58±0.08[b]	15.19±2.27
28 D recovery	5.39±0.27[c]	2.72±0.11[c, e]	2.46±0.12[c]	9.64±0.6
GR activity (unit/mg protein)				
Control	0.43±0.02	0.19±0.07	0.23±0.01	0.42±0.03
Treated	0.92±0.03[a]	0.22±0.07	0.16±0.02[a]	0.18±0.02[a]
14 D recovery	0.9±0.08	0.21±0.06	0.24±0.03[b]	0.29±0.01[b]
28 D recovery	0.61±0.03[c, e]	0.18±0.02	0.25±0.04[c]	0.36±0.02[c]

[a]There are significant differences ($P<0.05$) between the control and treated groups, [b]There are significant differences ($P<0.05$) between the treated and 14 day recovery, [c]There are significant differences ($P<0.05$) between the treated and 28 day recovery, [e]There are significant differences ($P<0.05$) between the14 and 28 day recovery. D: day, TBARS = Thiobarbituric acid reactive substances, CAT = Catalase, SOD = Superoxide dismutase, GST = Glutathione s-Transferase, GR = Glutathione-Reductase, MCP = Monocrotophos

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Section (T. S.) of foot of control animal [Figure 1a] while fragmented columnar cells with empty areas or vacuoles, disruption of normal arrangement of cilia and hypertrophy in mucous cells were observed in T.S. of foot of a treated animal [Figure 1b].



Figure 1a: Regular arrangement of columnar epithelial cells in control. cec: columnar epithelial cells



Figure 2a: Control. sl: secondary lamellae, t: tip

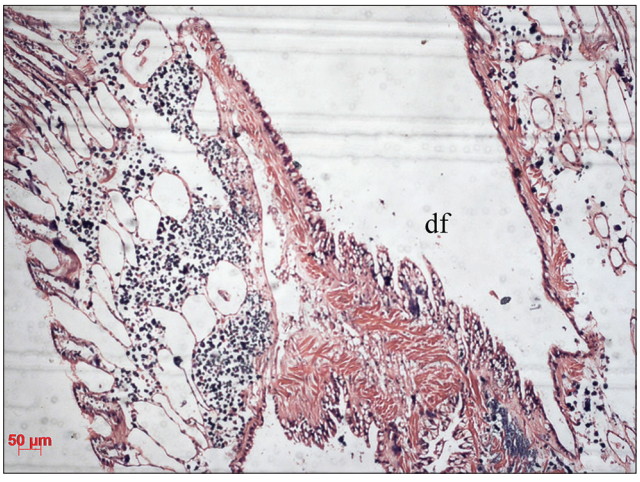


Figure 2c: Dense fragmentation in treated animal. df: dense fibrosis, fsl: Fusion of secondary lamellae

T. S. of gill [Figure 2a] in control group showed regular arrangement of lamellae. T. S. of gills of treated animal [Figure 2b-d] exhibited dense fibrosis within the

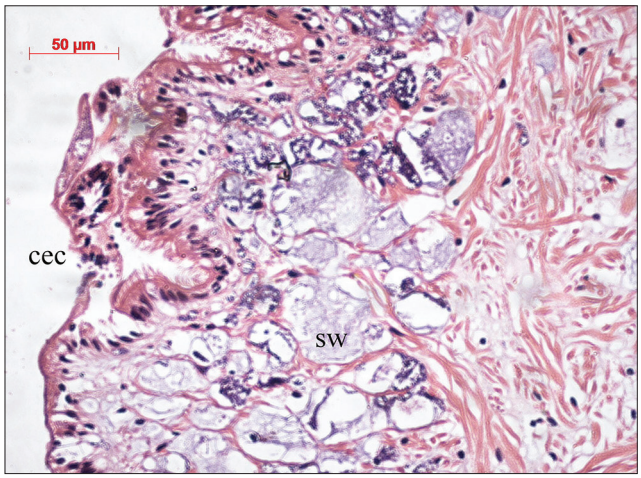


Figure 1b: Swelling due to hypertrophy of mucous cells in treated animals. cec: columnar epithelial cells, sw: swelling

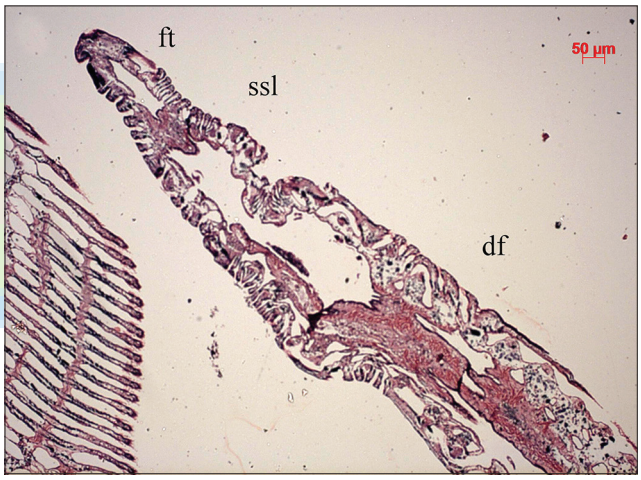


Figure 2b: Treated. df: dense fibrosis, ssl: shortening of secondary lamellae, ft: fused tip

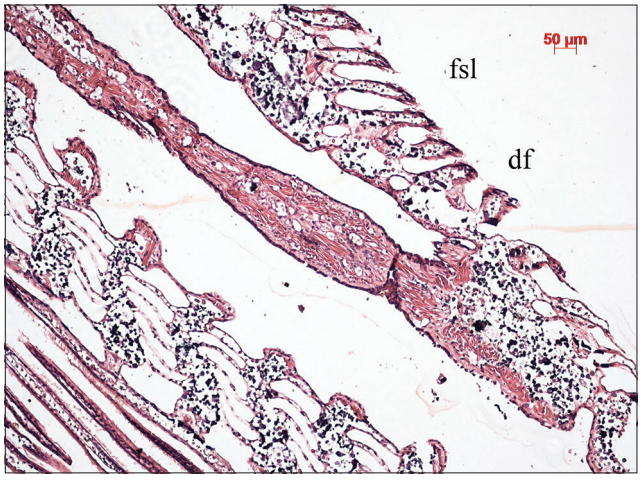


Figure 2d: Dense fragmentation in treated animal. df: dense fibrosis

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matrix of gill filaments and secondary lamellar fusion. The epithelial linings at the tip of gill filaments were disintegrated.

DISCUSSION

Lethargy was observed in animals with increasing concentrations of MCP during 96 hour's exposure LC_{50} experiment.[22,23] 76% recovery in AchE activity was observed in gill tissue after 14 days, and 98% recovery after 28 days, which indicate that the gill has maximum ability to overcome the stress of toxicant.[3,12] Elevated levels of lipid peroxidation observed in the present study are in accordance with previous studies.[1,5,24] Significantly increased CAT activities in gill could be due to stimulation of antioxidant defense mechanism in gill which may be due to its direct contact with toxicant.[25] Decreased CAT activities in mantle, foot, and muscle could be due to superoxide radicals generated during oxidative stress, which have been reported to inhibit CAT activity.[26] The trend of CAT and SOD activities are in accordance with the previous studies.[1,27] The trend of SOD strengthens the results of CAT and TBARS of the present study. Increase in SOD activity can be explained by the stimulation of antioxidant defense system in all the tissues studied. Antioxidant enzymes such as GR and GST are activated to counteract the negative effect of the ROS.[28,29] The exposed bivalves exhibited significant induction in GST activity, may be to reduce the pesticide-induced stress.[30]

The present study suggests that a period of 96 hours of exposure to MCP (52.36 ppm) in *L. marginalis* was enough to generate reactive oxygen species (ROS), which alters antioxidant enzyme activities such as SOD, CAT, GST, and GR as a first line of defense against oxygen radicals. The altered levels of antioxidant enzymes probably demonstrate a pollutant-induced toxic response in molluscs.[11]

All the histological observations indicated that exposure to sublethal concentration of MCP caused degenerative effects such as dense fibrosis of gill filaments,[11] fusion and shortening of secondary lamellae,[31,32] fragmented columnar cells, and hypertrophy in mucous cells of foot tissue. These changes in normal tissue architecture lead to loss of normal physiological functions of animal.

In conclusion, acute exposure to MCP caused oxidative stress in mussels. However, mussels were able to recover, as displayed by antioxidant enzyme activities that recovered well after 28 days. In addition, gill which is in direct contact with these pollutants appeared to be the most sensitive tissue. Thus, evaluation of these biomarkers seemed to aid for the estimation of the effects of agricultural pollution on freshwater invertebrates.

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