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Genotoxicity and oxidative stress as biomarkers in fresh water mussel, *Lamellidens marginalis* (Lam.) exposed to monocrotophos

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Monocrotophos (MCP) is an organophosphate pesticide widely used in India for controlling various pests. In this study, we evaluated the oxidative stress and genotoxic potential of MCP on the freshwater mussel *Lamellidens marginalis* (Lamarck) after 7 days exposure and repair of the damaged DNA after 4 days recovery. The bivalves were exposed to 5.25 mg/L of MCP for 7 days and then allowed to recover for 4 days in pesticide-free water. Increase in the levels of thiobarbituric acid reactive substances was recorded in the gill, muscle, foot and mantle tissues. Cellular antioxidant defences i.e. antioxidant enzyme activities like catalase, superoxide dismutase, glutathione reductase and glutathione-S-transferase were used as biomarkers of oxidative stress. Altered activities of antioxidant enzymes were observed after exposure. There was a significant recovery in the antioxidative enzymes in the tissues after the recovery period. To monitor genotoxicity of MCP, we used micronucleus and comet assay. Increase in Olive tail moment in the gill cells of exposed mussels as compared to that of control ones indicated significant DNA damage. Our findings suggest that the MCP-induced oxidative stress may be contributing partly to genotoxic damage of gill cells. Thus, these biomarkers are found to be useful in evaluating the toxicity of MCP in mussels.

Keywords: Aquatic pollution, Bivalves, DNA damage, Environmental contamination, Oxidative stress, Pesticides toxicity, Phoskill

Excessive use of pesticides in modern agriculture enters into aquatic habitats either from direct application, terrestrial runoff or wind borne drift. This, over the years, has gradually increased the level of several pesticides and their metabolites in water column and then into benthic sediments, contaminating the aquatic environment[1]. The genotoxic effects of several chemical groups of pesticides such as organochlorine, organophosphorus (OP) and pyrethroid, etc. have been shown by *in-vivo* and *in vitro* experiments[2-4]. In addition to cholinesterase inhibition, induction of oxidative stress has been reported as the main mechanism of OP pesticide's toxicity in different exposure conditions[2]. The most susceptible targets for oxidative stress are polyunsaturated fatty acids of the cell membrane, which undergo peroxidation rapidly[5]. Lipid peroxidation has been suggested as one of the molecular mechanisms involved in pesticide-induced toxicity[6]. The pro-oxidants (environmental contaminants) and antioxidant defences (enzymatic and non enzymatic) in biological systems can be used to evaluate the toxic effects in stressful environmental conditions, hence the environmental toxicology studies are primarily focused on oxidative stress[7].

It is well established that toxicants with oxidative stress potential, attack DNA result in clastogenic and molecular damages[8,9]. Besides being potent source of ROS, OP compounds also exhibit alkylating properties, known to cause DNA damage[10]. OP pesticides are reported as genotoxic in various organisms including mussels, and fishes[11,12]. Both, *in vivo* and *in vitro* studies have demonstrated monocrotophos (MCP) to be genotoxic in various animals[13], including chick[14], human lymphocytes[15], etc.; however information available about genotoxic effect of MCP on mussels like *Lamellidens marginalis* (Lamarck, 1819) (Bivalvia: Unionidae) is inadequate. In ecotoxicological studies, genotoxic biomarkers are widely considered as molecular toxic endpoints of major environmental pollutants[16]. Various genotoxic biomarkers including MN test[17,18] and comet assay[12,17] are employed in ecotoxicology for biomonitoring of aquatic ecosystems for

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pollutants. MN test was employed in the present study as it is fast, sensitive and able to detect the genomic damage. In molluscs, MN assay is mostly performed on haemocytes or gill cells. Several studies report dose-dependent increase in MN frequency in response to contaminants[19,20].

Aquatic organisms, being an important source of food for humans, can be a major health risk if exposed to environmental toxicants like genotoxic and carcinogenic substances[21,22]. Mussels are ecologically important fauna because they are used as sensitive biomarkers of aquatic pollution[23,24] on account of their wide geographical distribution, ease in collection, sessile habitat and filter-feeding mechanism which may be exposed to large amount of chemical pollutants. Further, mussels are capable of accumulating and tolerating high concentrations of organic and inorganic pollutants in their tissues[25-27].

Lamellidens marginalis, the most common bivalve found in the reservoirs around Pune, is consumed as a major food item by majority of local population. The present study was undertaken to: (i) investigate the genotoxic potential of MCP in gill cells using Comet assay;(ii) oxidative potential of MCP in gill, foot, mantle and muscles of *Lamellidens marginalis*; and (iii) explore the possible use of MN test on gill cells as a parameter for detection of genetic/chromosomal damage.

Materials and Methods

Phoskill 36% [Dimethyl (E) 1-methyl-2-(methylcarbamoyl) vinyl phosphate] was procured from Sudarshan chemicals, Pune for experimental purpose.

Lamellidens marginalis (length: 7-9 cm, weight: 22-28 g**,** bisexual) were collected from the Mula River (N 18° 33' and E 073° 42') transported to the laboratory in aerated water within 30 min. Water from collection site was checked for pesticide residue contamination with the help of GC-MS. Water quality parameters such as pH, temperature, chlorophyll content, DO, BOD, COD and nitrate were determined following the standard procedure[28].

On the day of collection, 10 specimens were used to determine Condition Index (CI) to assess their physiological status[29]. Bivalves were dissected and whole soft tissues and shells were kept in oven at 60°C and then weighed after 96 h. The ratio of dry flesh weight to dry shell weight (FW/SW × 100) was used to determine CI for each individual. In the laboratory, prior to the experiment, 36 bivalves were maintained in dechlorinated tap water (1.5 L per individual) for 15 days for acclimatization. During this period bivalves were fed ad libitum with spirulina powder[30]. Animal experiments were performed after approval of Institutional Animal Ethics Committee (IAEC) with the ethical standards provided by CPCSEA.

Eighteen bivalves (6 specimens in 9L of water per aquarium) were exposed to 5.25 ppm of ($1/10^{th}$ of LC_{50}, determined from earlier studies[31]) formulated MCP for seven days. The specimens were not fed during the experimental period[4]. After 7 days of exposure, half of the (n=9) specimens were transferred to toxicant free water and maintained for 4 days for assessment of recovery. Control specimens (n=18) were maintained in MCP free water in two parallel sets.

At the end of MCP exposure period, 9 specimens were dissected separately to collect the gill, foot, mantle and muscle tissues. The tissues were then homogenized separately over ice in respective phosphate buffer. Homogenates were centrifuged at 4°C, 8000 rpm for 20 min. Supernatants were separated and stored at −80°C. Biochemical estimations were carried out using standard methods within 7 days. After 4 days of recovery period remaining 9 specimens were dissected separately and same procedure was carried out for tissue preparation in all the four groups.

The protein content of the samples was measured by the method of Lowry *et al.*[32]. The thiobarbituric acid reactive substances (TBARS) assay was used to evaluate the peroxidation of lipids[33]. Super oxide dismutase (SOD) activity was assessed by the method of Beauchamp[34]. Catalase (CAT) activity was determined by the method of Aebi[35]. Glutathione S-transferase (GST) activity was measured by Habig *et al.*[36] method. Glutathione reductase (GR) activity was quantified by Goldberg *et al.*[37] method.

For MN test, single cell suspension of gill tissue was used for preparation of smears (PBS pH 7). Smears were prepared on glass slides and dried in the dark for 24 h. Slides were stained using May- Grunwald Giemsa staining technique[38]. Slides were observed at 1000X magnification using Carl Zeiss Axiovision microscope. Normal and micro nucleated intact gill cells were scored. Micronuclei were identified according to criteria followed by Klobucar *et al.*[39]

For standardization of the comet assay protocol, fresh single cell suspension of gill was treated with

H_2O_2 (1, 10, 25 and 50 mM) in PBS for 5 min. The control cells were incubated in PBS without H_2O_2. Three replicates per condition were performed[40].

The alkaline comet assay was performed as described by Singh *et al.*[41] with minor modifications. Microscopic slides were pre-coated with 1% NMP agarose on pre-cleaned and methanol treated dry slides. Then 30 µL of gill cell suspension was gently mixed with 70 µL of 0.1% LMP agarose and covered with a coverslip and kept for 5 min at 4ºC and then slides were processed. The slides were examined with a fluorescence microscope (Carl Zeiss Axiovision, 400X, excitation filter 510-560 nm, barrier filter 590 nm). Fifty nuclei were analyzed per slide. All experiments were carried out in triplicates.

Statistical analysis

The statistical data analysis was carried out using one-way ANOVA; Tukey pair wise-multiple comparison test was used for biochemical estimations and micronuclei assay. Data was presented as the mean ± Standard Deviation (SD). Microscopic images of comets were scored using Comet IV Computer software. This data was presented as the mean ± Standard Error (SE).

Results

According to the results of GC-MS analysis, the concentrations of pesticides in reservoir water from collection site were below the limit of quantification (0.01-1.01 ppb). The water quality parameters of collection site were analyzed for pH, temperature, chlorophyll content, DO, BOD, COD and nitrate (Table 1). Bivalves that were collected from site showed CI ranging from 7.2 to 12.4. Evidently, physiological condition of the mussels was healthy at the time of collection.

Seven days of MCP exposure significantly (P <0.05) induced lipid peroxidation (Table 2) in gill (1197) > foot (1042) > mantle (867) > muscle (830%), as compared to control. While after four days of recovery period, foot (74), muscle (68.1), mantle (67.9) and gill (54%) recovered significantly (P <0.05).

Significant (P <0.05) elevation of SOD activity after MCP exposure (Table 2) was observed in gill (1982%) followed by foot (1169%) and mantle (741%) but four days recovery period was found to be sufficient for mantle (86) > foot (80) >gill (74%) to recover significantly (P <0.05). Gill showed maximum elevation and comparatively lower recovery as compared to other tissues.

CAT activity (Table 2) increased significantly (P <0.05) in muscle (977), gill (646), foot (282) and mantle (206%) tissues after MCP exposure. The trend of significant (P <0.05) recovery observed after four days of recovery period was as follows: muscle (36%) > gill (31%), >mantle (29%) >foot (13%). It is observed that the muscle tissue which showed

Table 1—Physicochemical parameters of water at collection site, Mula River (N 18° 33' and E 073° 42'), Pune, Maharashtra, India.

pH	Temperature	Chlorophyll content µg L^{-1}	DO mg L^{-1}	BOD mg L^{-1}	COD mg L^{-1}	Nitrate mg L^{-1}
9.29	30°C	0.94	7	122.7	39.5	8.0

Table 2—Changes in the TBARS, CAT, SOD, GST and GR activity in *L. marginalis* when exposed to MCP (5.25 mg/lit).

Tissue exposed		Gill	Foot	Muscle	Mantle
TBARS activity (thiobarbituric	Control	3.37±0.12	3.68±0.21	3.13±0.30	6.52±0.25
acid reactive substances)	7 DE	40.37±8.32[a]	38.36±7.32[a]	26±2.92[a]	27.15±6.28[a]
(nmol/mg protein)	4 DR	18.40±2.4[b]	9.89±2.84[b]	8.16±2.9[b]	8.73±1.95[b]
CAT (catalase) activity	Control	12.28±5.27	16.65±4.7	3.43±0.46	17.57±1.48
(unit/mg protein)	7 DE	79.36±5.16[a]	47.09±1.6[a]	33.50±4.62[a]	36.13±7.62[a]
	4 DR	54.97±2.32[b]	41.15±3.2	21.32±6.03[b]	25.49±4.71[b]
SOD (Super oxide dismutase)	Control	2.57±0.69	2.04±0.18	1.02±0.02	3.62±0.75
activity (unit/mg protein)	7 DE	50.94±2.03[a]	23.86±10.96[a]	0.61±0.24	26.84±4.64[a]
	4 DR	13.44±0.62[b]	4.66±1.69[b]	4.14±0.90	3.85±0.19[b]
GST (Glutathione S-transferase)	Control	2.33±0.14	4.14±0.69	1.98±0.14	3.65±1.55
activity (unit/mg protein)	7 DE	14.36±3.86[a]	11.64±3.17[a]	8.73±2.38[a]	8.90±1.04[a]
	4 DR	9.39±4.66	9.14±3.19	6.41±1.01	6.27±1.93[b]
GR (Glutathione reductase)	Control	0.14±0.06	0.18±0.06	0.19±0.13	0.38±0.02
activity (unit/mg protein)	7 DE	0.24±0.01[a]	0.15±0.001	0.12±0.01[a]	0.12±0.01[a]
	4 DR	0.20±0.01	0.16±0.001[b]	0.13±0.01	0.14±0.01

[7DE, 7 days exposure; 4DR, 4 days recovery. Values are mean ± SD from 9 bivalves in each group. P values (P <0.05). a, between control and exposed groups; and b, between exposed and 4 day recovery]

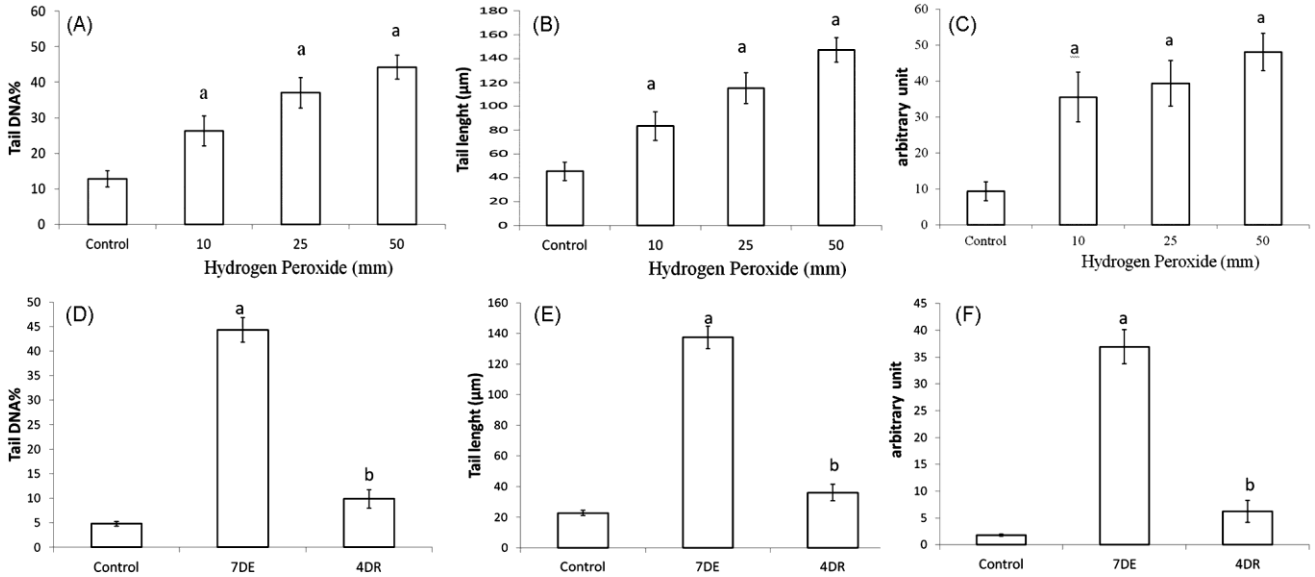

Fig.1— DNA strand breaks in gill cells of *L. marginalis* exposed to H_2O_2 (A-C); and MCP (5.25 mg/L) (D-F) for 7 day exposed (7DE) and 4 days recovery (4DR). [*Comet parameters were reported as mean ± standard division. a: significant differences (P <0.05) between the control and exposed groups, b: significant differences (P <0.05) between the exposed and 4 day recovery]

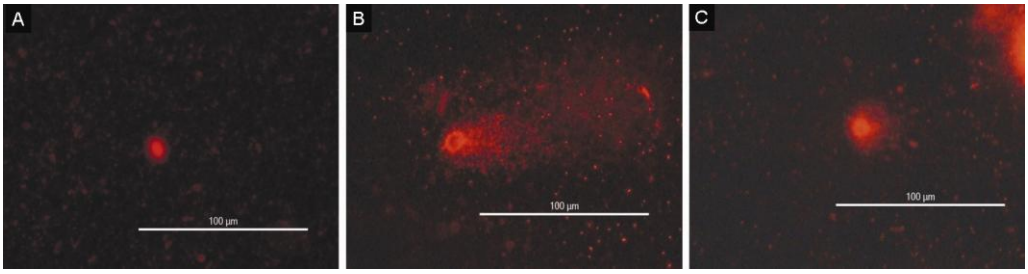

Fig. 2—DNA damage in (A) control; (B) exposed; and (C) recovered gill cells [400X], after MCP exposure (5.25 mg/L).

maximum elevation of CAT activity also showed maximum recovery.

Significant (P <0.05) induction of GST activity (Table 2) was observed in gill (616%), muscle (441%), foot (281%) and mantle (244%) tissues after MCP exposure. However, after recovery period, maximum recovery was observed in gill (35%) followed by mantle (30), muscle (27) and foot (21%). Though maximum elevation of GST was experienced by gill, it could recover better than other tissues.

Induction of GR activity (Table 2) was observed only in gill (171%) while trend of inhibition at the end of exposure period observed was as follows mantle (68%) > muscle (37%) > foot (17%). GR activity changed significantly (P <0.05) in MCP exposed bivalves. Significant (P <0.05) recovery was observed in foot tissue. It is observed that the foot tissue experienced minimum stress and showed significant recovery.

H_2O_2 (5, 10, 25, 50 mM) treated gill cells of *L. marginalis* showed a significant (P <0.05) dose-dependent increase in the percentage tail DNA (Fig. 1A), tail length (TL) (Fig. 1B) and olive tail moment (OTM) (Fig. 1C).

After 7 days of MCP exposure, tail DNA percentage (Fig. 1D), TL (Fig. 1E) and OTM (Fig. 1F) increased significantly (P <0.05) in gill cells, as compared to control. After four days of recovery, significant (P <0.05) repair was observed in TL, tail DNA percentage and OTM in gill cells of *Lamellidens. marginalis* (Fig. 2). MCP induced significant (P <0.05) MN formation in exposed bivalves, in comparison with control ones (Fig. 3).

Discussion

After 7 days of exposure to MCP, significant (P <0.05) increase in lipid peroxidation (LPO) showed oxidative stress. Enhancement of LPO in all the tissues of mussel suggested the participation of free radical-induced oxidative cell injury triggered due to the toxicity of MCP. The most affected tissue

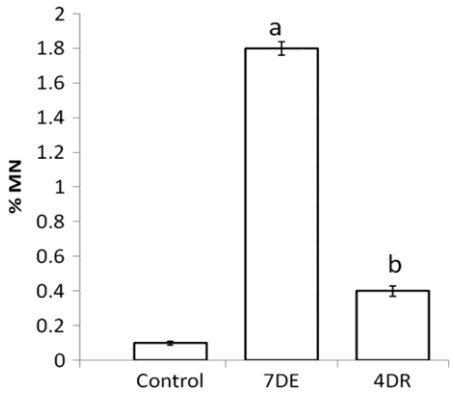

Fig. 3—Frequency of micronuclei in control, 7 day exposed (7DE) and 4 day recovery (4DR) in gill cells, after MCP exposure (5.25 mg/L). [*a indicates significant differences (P <0.05) between the control and exposed groups; and b indicates significant differences (P <0.05) between the exposed and 4 day recovery]

was gill and the least affected were mantle and muscle. Increased LPO content in gills and digestive glands of the freshwater mussels exposed to the pesticide was reported by Kopriicu *et al.*[42] Results suggested that as compared to the other tissues, gill was more susceptible to oxidative stress, owing to its filter feeding mechanism[43]. Although foot was the second highest sensitive tissue in case of lipid peroxidation, it showed maximum significant (P <0.05) recovery followed by muscle, mantle and gill.

The observed trend of increased SOD activity in the exposed bivalves was (Gill>Foot>Mantle). The trend of significant recovery observed was in reverse manner (Mantle>Foot>Gill). It was observed that tissues which were under lower stress recovered better and mantle recovered the most[44]. CAT activity was significantly (P <0.05) increased in all the tissues after seven days of exposure. Muscle showed the highest CAT activity, but also showed the most efficient recovery. Increased SOD and CAT activities indicated generation of superoxide radical and hydrogen peroxide during MCP exposure in *L. marginalis*. SOD catalyzed the dismutation reaction of the superoxide anion radical-O_2^- to form the less-reactive molecular oxygen and then CAT converted H_2O_2 to H_2O and O_2 in order to prevent oxidative damage and maintain cell homeostasis[45]. Overall, the percentage of increase in SOD activity was higher as compared to that of the CAT activity in MCP exposed bivalves. The positive relationship between SOD and CAT activities was observed in the exposed and recovery groups.

Significant (P <0.05) increase in activity of GST was observed in all the tissues of exposed bivalves. Significant (P <0.05) recovery was observed only in the mantle tissue while other tissues recovered to a certain extent. The marked increase in GST activity suggested active involvement of this enzyme in the detoxification of MCP as a part of the phase II biotransformation[46]. Increased GST activity which served as a detoxification enzyme[47] might be considered as an indicator of chemical stress[48]. Our results were in confirmation with Kaaya *et al.*[49]

In the present study GR activity was tissue specific**,** the induction of GR activity in gill and its inhibition in other tissues suggested an organ specific response of mussel to MCP. The induction might be due to the increased production of oxidized glutathione (GSSG) as suggested by Zhang *et al.*[50] Inhibition of GR activity reflected its utilization in reduction of the effect of free radicals generated by TRZ. Similar trend of inhibition of antioxidant enzymes was observed in different tissues of marine bivalve upon exposure to chlorpyrifos[51].

It was observed by Banu *et al.*[13] that Phosphorus group of MCP acted as a good substrate for nucleophilic attack, it might cause phosphorylation of DNA, which is an evidence of DNA damage. MCP might have a potential for methylation of DNA which could cause mutation. Both acute and chronic exposure of MCP could induce DNA damage in rats. The results of present study showed significantly (P <0.05) increased levels of percentage of tail DNA, OTM and TL in exposed animals, which demonstrated the genotoxicity of MCP in *L. marginalis*. Our results are in accordance with Sarkar *et al.*[40]. Significant recovery of comet parameters revealed the great capacity of bivalve to repair DNA damage within 4 days. Our results are in accordance with observations of Fedato *et al.*[18]

MN and abnormal nuclei formation was observed in gill cells after 7 days of exposure. MCP induced significant (P <0.05) MN formation in exposed bivalves, in comparison with control ones. Results from comet assay supported the MN assay observations in exposed gill cells indicating high intensity of DNA damage. These biomarkers have opened a broad perspective in aquatic toxicology. It was established that gill is the most sensitive tissue[52] because of its constant exposure to environmental

pollutants due to its filter feeding mechanism. The results of this study might help in protecting the environment through judicious and careful use of MCP in agriculture.

Conclusion

Administration of MCP promoted induction of lipid peroxidation causing fluctuations in the anti-oxidative systems in various tissues and induction of micronuclei as well as increase in comet parameters (percentage of tail DNA, tail length and olive tail moment) in gill cells. Oxidative and DNA damage were experienced by mussels; however anti-oxidant enzymes recovered well, the levels of micronuclei decreased (P <0.05) and also repair in terms of comet parameters was observed after four days. We suggest that DNA strand breakage and micronucleus formation in mussel gill cells can potentially be used as convenient biomarkers of exposure to genotoxicants in the aquatic environment. Thus, fresh water mussels can be considered as suitable bio-indicators for assessment of quality of freshwater environment by using appropriate markers.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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