Genotoxic and Hematological effect of commonly used synthetic organophosphate Pesticide on non-target organism, fresh water bivalve.

Submitted by

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General information

1.	Title of the project	Genotoxic and Hematological effect of commonly used synthetic organophosphate Pesticide on non-target organism, fresh water bivalve.
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7.	Name and address of Research fellows who worked in the project (May 2013 to May 2014)	Mrs. Anju Y Mundhe G-8, Trimurti Apt, 590 Rasta peth, Near Apollo theater, Pune 411011. aymundhe@gmail.com
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CERTIFICATE

This is to certify that Dr. S. V. Pandit, Associate professor, Department of Zoology, SP Pune University, Pune, has successfully completed UGC Major Project on "Genotoxic and Hematological effect of commonly used synthetic organophosphate Pesticide on non-target organism, fresh water bivalve" during academic year 2013-2017.

This project report has not been earlier submitted to any other institute or university for the award of any degree or diploma.

Date:

Place: Pune

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INTRODUCTION

INTRODUCTION

Pesticides cause a lot of adverse effects on human health, environment, food quality and biodiversity of organisms. Pesticides and heavy metal salts are common pollutants of freshwater ecosystems that directly affect the aquatic biota. The persistence and high toxicity of insecticides make them a serious ecological concern. The increasing use of pesticides in agriculture contributes to environmental pollution. Most of the pesticides induce oxidative stress by generating reactive oxygen species (ROS) and causing imbalance of the normal antioxidant system (El-Gendy et al. 2010). Only 0.1% of pesticides reach to their specific target.

MCP is systemic OP pesticide. Systemic pesticides are enough soluble in water, which are absorbed by a plant when spread to either seeds, soil, or leaves. The chemicals then circulate through the plant's tissues to kill the insects that feed on them.

The OP chlorpyrifos (0,0-diethyl-0-3,5,6-trichloro-2-pyridyl phosphorothioate) is widely used in agriculture practices to control insect pests and reaches aquatic reservoirs through runoff (Mishra 2014). Therefore, anyone can be exposed to remains of this pesticide in agricultural products. The occurrence of chlorpyrifos (CPF) in the aquatic surroundings and its role in causing adverse effects makes it important for toxicological studies (Xing et al. 2010). The polluting substances in aquatic ecosystems affect the physiology of mollusks (Collin et al. 2010).

Rains, winds, rivers and floods may carry agrochemicals and industrial discharges to the water bodies. Contamination changes physico-chemical properties of water with toxicity. Various studies of aquatic invertebrates reveal the influence of toxic stress on different metabolic pathways (Santos and Martinez 2014, Ray et al. 2013). When the pesticides enter in the body of organism, irreversible changes may occur. Most of the agrochemicals introduced in water bodies through rain and agricultural runoff can affect many other non-target organisms. The agricultural runoff containing insecticides, even at low concentration, can kill or immobilize the animal.

The balance of catabolic and anabolic processes is homeostatically regulated in an organism. Toxicant exposure consequences make contact with the chemical and the target site in the organism and it induces a series of biochemical processes to restore the metabolic equilibrium (Aldridge 1981). If balance is not achieved, it leads to defective

biological organization at higher levels (Mazeaud et al. 1979). Metabolic pathways are interlinked systems; therefore metabolic condition of an organism can never be based on the measurement of a single enzyme.

In conclusion it is observed that a toxicant- induces interruption of the energy utilization. OPs are effective inhibitors of AchE, resulting in a number of physiological and metabolic alterations in the organism.

Protein metabolism is the central part of the organism's metabolism. Proteins are essential constituents of protoplasm and also act as growth material and building block of the body in different organisms. Decreased protein content and altered AchE activity were observed after pesticide exposure in aquatic organisms (Menendez-Helman et al. 2012).

AchE (EC 3.1.1.7) enzyme is from esterases family which degrades neurotransmitter acetylcholine in both vertebrates and invertebrates. AchE enzyme is essential for physiological activities of bivalve like filter feeding activity and opening or closing of valves. This enzyme is targeted by OP (Thompson 1999), by giving rise to functional disorders of the nervous system. The measurement of AchE activity in freshwater bivalve was used as a biomarker for environmental contamination by these compounds.

Pesticides are widely used in agriculture for pest control (Monteiro 2006). The pesticides that enter the aquatic system through surface run off which may adversely affect the aquatic biota. Furthermore, many authors postulate that these compounds disturb the redox processes, change the activities of anti-oxidative enzymes, and cause enhanced lipid peroxidation (LPO) in many organs (Lukaszewicz-Hussain 2010).

Hyperglycemic condition accompanied by AchE inhibition (Joshi et al. 2012) and oxidative stress was observed in rats exposed to MCP (Sankhwar et al. 2013). MCP-induced apoptosis in neuronal cells showed altered expressions of selected cytochrome P450s (Kashyap et al. 2011).

As a result of oxidative stress, lipid peroxides form from polyunsaturated fatty acids. The unstable lipid peroxides decomposes to form a complex series of compounds, of which the most plentiful is malondialdehyde (MDA). The level of MDA has been

widely used as an indicator of lipid peroxidation in fresh water bivalve (Irinco-Salinas et al. 2014).

Oxidative stress was recorded as biomarker when the levels of TBARS was increased in different tissues and cellular antioxidant defenses i.e. antioxidant enzyme such as SOD, CAT, GST and GR. Altered activities of those antioxidant enzymes were observed in the tissues which exposed to pesticides.

Bivalves are divided on the basis of their mobility into attached and locomotive types. Locomotive are epifauna and infauna, depending on burrowing into sediment (Gosling 2004). Foot of infaunal bivalve mostly has capabilities including locomotion and burrowing into soft substrate. Mucous secreted from foot and mantle which are supposed to functions as formation of pseudofeces, cleaning of mantle cavity and burrowing into sediments. The epithelial cells of bivalve foot, mantle and gills take appropriate actions in various environmental stimuli. Bivalves are filter feeding organisms and economically important. Bivalves are rich with protein content therefore widely used as food. Freshwater pearl culture is now emerging business in developing countries.

Biochemical and physiological reactions to pollutant in bivalve may help us to understand its health and survival (Stien et al. 1998). Oxidative stress and antioxidative enzymes are important biomarkers. Antioxidative enzyme systems including SOD, CAT, GST and GR are well-organized defensive mechanisms produced by endogenous metabolism against chemical reactive species (Srain et al. 2010).

The present study evaluates the influence of independent exposure of two pesticides on lipid peroxidation and antioxidant enzymes activities.

Distribution:

Lamellidens marginalis is found in the lower and upper Gangetic plains in India and Bangladesh, Sri Lanka, Myanmar (Ramakrishna and Dey 2007) and Terai region of Nepal.

Use of bivalve:

- It is used as food in India, Bangladesh and Nepal.
- It is also used as medicine in eastern India.
- The shells are used to extract edible lime in Terai region of Nepal.

- The shells are also used for ornament preparation.
- It is important species for freshwater pearl culture in India (Ramakrishna and Dey 2007).

Threats:

L. marginalis is assessed as least concern species by IUCN (2015), but there are no known major threats reported. It is among the most preferred food species in Nepal, India and Bangladesh. Over-harvesting, use of pesticides for fishing are some inferred threats to this species. Deforestation and sedimentation caused for clearance for agriculture and hydropower development which are the significant threats across large parts of the region. The impact of the pollution from agriculture and industrial development may not be known, on the known localities of the species.

Classification of model organism

Kingdom: Metazoa
Phylum: Mollusca
Class: Bivalvia
Sub class: Paleoheterodonta
Order: Unionoida
Super family: Unionoidea
Family: Unionidae
Subfamily: Perreysiinae
Genus: *Lamellidens*
Species: *marginalis* (Lamarck)

Higher doses of pesticide exposure caused inhibition of the filtration activity by reducing active periods and increasing the rest periods of bivalve (Koprucu 2010). OP pesticides are neurotoxic i.e. AchE inhibitors which may alter filtering, feeding and respiration activities and so the physiology of the mussels and their water-cleaning function are affected, which has an important role for maintenance of a healthy aquatic ecosystem.

No reports are available on the genotoxic effects of MCP and CPF on *L. marginalis.* The most common bivalve found in the reservoirs around Pune, which is consumed as a major food item by majority of local population.

Thus, the present study is undertaken to:

1. To determine the effect of MCP and CPF pesticides on protein content and AchE activity in gill, foot, mantle, adductor muscles and hepatopancreas (Hpt) of *L. marginalis.*
2. To investigate the oxidative potential by using TBARS activity and alterations in antioxidative enzyme activities after MCP and CPF exposure in different tissues of bivalve.
3. To investigate the genotoxic potential of pesticides, in vivo, employing the gill cells using Comet assay.
4. To explore the possible use of MN test on gill cells as a parameter for the detection of the genetic/chromosomal damage due to pesticides toxicity.

MATERIALS AND METHODS

MATERIALS AND METHODS

Bivalve collection from field:

The fresh water bivalves *L. marginalis* (7-9 cm) were collected from the Mula River (N 18° 33' and E 073° 42'). Bivalves were transported to laboratory in aerated water within 30 min.

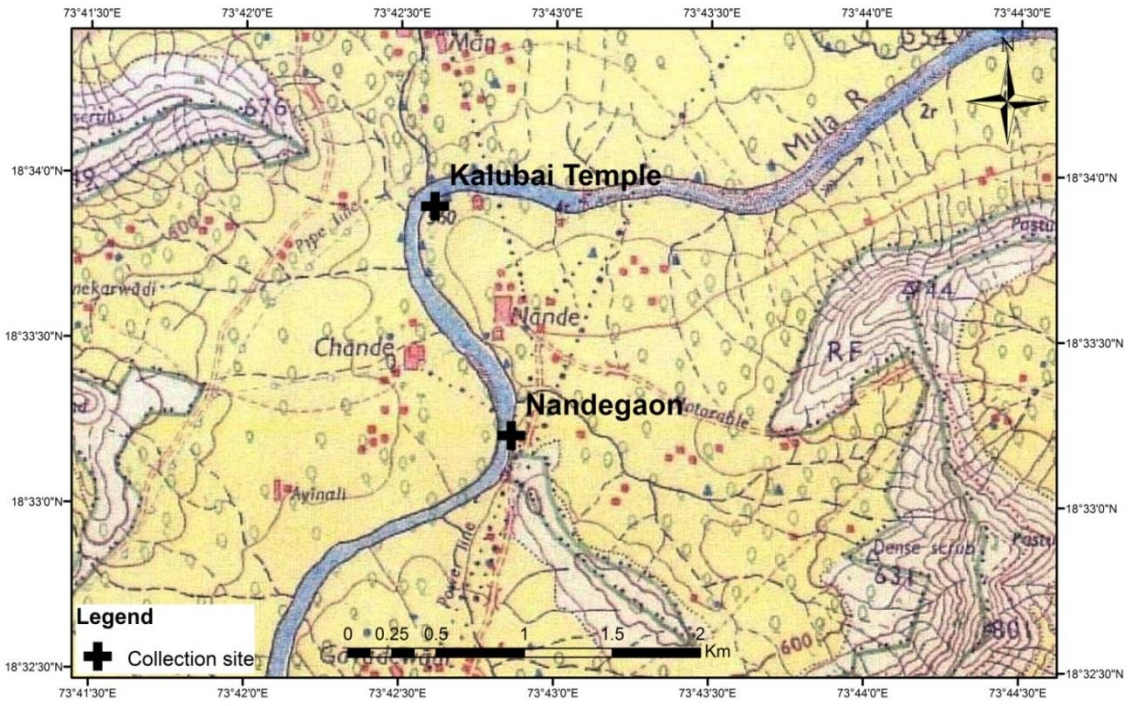


Figure 1: Map showing bivalve collection sites (plus) (Source: Survey of India)

Water parameter analysis:

The water from the collection site was checked to ascertain any traces of pesticide contamination with the help of GC-MS analysis. Physical parameters like pH, electrical conductivity, temperature, salinity and total dissolve solid were checked on site using EU-tech pocket tester. Other parameters like total nitrogen, nitrate, biological oxygen demand, dissolved oxygen, total phosphorus and chlorophyll content were checked to know the quality of water.

Sr. No.	Analysis	Method/instrument
1	Chlorophyll	Acetone extraction spectrophotometric
2	T. Phosphorus	Digestion and ascorbic acid spectrophotometer
3	T. K. Nitrogen	EDTA-Titrimetery
4	Total hardness	EDTA-Titrimetery
5	Dissolved Oxygen	Winklers method
6	COD	Open reflux
7	BOD	Digestion and ascorbic acid spectrophotometer
8	Nitrate	UV visible spectrophotometer

Table 1: Analytical methods used for physico-chemical parameters

Condition Index (CI):

On the day of collection, ten individuals used for the determination of Condition Index (CI) which was useful to check the physiological status of the bivalve (Filgueira et al. 2013). Bivalves were dissected and the whole soft tissues and the shells of bivalves were kept in oven at 60 ºC and then weighed after 96 h. The ratio of the weight of dry flesh and the weight of dry shell (FW/SW X 100) was used to determine CI for each individual.

	Condition Index (CI) (FW/SW X 100)		
Sr no.	24/09/2013	03/12/2013	07/05/2014
1	11.15	13.78	10.09
2	7.52	13.78	14.13
3	7.23	13.13	8.35
4	9.29	9.79	19.17
5	12.42	13.13	13.41
Mean	9.52	12.72	13.03

Table 2: Condition index

Acclimatization of bivalves to laboratory conditions:

Bivalves were acclimatized to laboratory conditions for seven days in dechlorinated stored water. The bivalves were fed every day, according to the method of Amanullah (2010). The water was renewed after every 24 hours.

Toxicants:

The pesticide toxicants selected for exposure were-

1. **Common name:** Monocrotophos (Phoskill 36%)

   Chemical name: Dimethyl (E) 1-methyl-2- (methylcarbamoyl) vinyl phosphate,

   Mechanism of action: MCP is systemic and contact OP pesticide is used to control common mites, ticks and spiders.

   Target enzyme: AchE enzyme

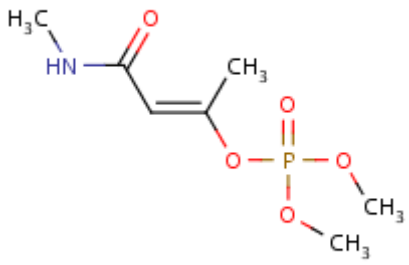


Figure 2: Chemical structure of MCP

2. **Common name:** Chlorpyrifos

   Chemical name: 0, 0-diethyl-0-3, 5, 6-trichloro-2-pyridyl phosphorothioate

   Mechanism of action: CPF is non systemic pesticide. It is effective by direct contact, ingestion and inhalation. It is used to control termites, mosquitoes and round worm.

   Target enzyme: AchE enzyme

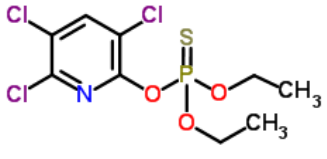


Figure 3: Chemical structure of CPF

Working solution preparation:

1. Formulated MCP i.e. Phoskill is water soluble.
2. Technical grade CPF was procured from AIMCO Pesticide Limited.The stock solution was prepared by mixing the required quantity CPF in acetone (carrier solvent).Test solution is obtained from stock solution as per the desired concentration.

Experimental design:

MCP

Fresh water bivalve *L. marginalis* were exposed to 5.25mg/lit concentration for seven days followed by recovery for four days. The control group was kept without addition of pesticide for 7 days.

The conditions during the recovery experiment were the same as those of in the exposure experiment. At the end of the recovery period, tissues were isolated by using the same method as in the exposure experiment and which were used for further analysis.

CPF

Fresh water bivalve *L. marginalis* were exposed to 0.4 mg/lit environmental relevant concentration of CPF for 7 days, 14 days (T1 and T2) and four days for recovery (R1 and R2) following each exposure group.

Animals were divided into 5 groups, with 6 animals in each group

Group I: Bivalves were maintained as control group without addition of pesticide.

Group II (T1): Bivalves were exposed to CPF (0.4 mg/lit) up to 7 days.

Group III (R1): Bivalves were exposed to CPF (0.4 mg/lit) up to 7 days and then they were transferred to water without toxicant, up to 4 days for the assessment recovery.

Group IV (T2): Bivalves were exposed to CPF (0.04 mg/lit) up to 7 days.

Group V (R2): Bivalves were exposed to CPF (0.04 mg/lit) up to 7 days and then they were transferred to water without toxicant, up to 4 days for the assessment recovery.

Biochemical estimations

1. **Protein estimation:**

The protein content of the samples was measured by the method of Lowery et al. (1951).

Principle:

The -CO-NH (peptide bonds) in polypeptide chain reacts with copper sulphate in an alkaline medium to give a blue colored complex.

Method:

At the end of respective exposure period, shells were cleaned with distilled water and bivalves were sacrificed to collect gill, foot, mantle, adductor muscle and Hpt tissues. The tissues were homogenized in phosphate buffer over ice, and then centrifuged for 20 minute at 8000 rpm at 4ºC. TCA (20%) was added to the supernatants and kept at 4°C for 30 minutes. After centrifugation of mixture at 8000 rpm for 20 minutes at 4°C, supernatant was discarded. Pellet was washed with acetone, and then centrifuged and supernatant was discarded. Pellet was dissolved in 1 ml of 0.1 N NaOH, and then 5 ml freshly prepared alkaline copper sulphate reagent was added and kept for 10 minutes for incubation. Foline reagent (0.5ml) was added to the reaction mixture and absorbance recorded at 660nm.

2. **Acetylcholinesterase (AchE) activity :**

AchE activity was measured by Ellman et al. (1961) method.

Principle:

Acetylthiocholine iodide (158.5 nmol) was used as substrate. The AchE in the sample catalyzes the DTNB [5, 5′ Dithiobis (2 nitrobenzoic acid)] and forming color product 5 thio 2 nitrobenzoate having maximum absorbance at 412 nm. The activity of the enzyme could be measured by following the increase in absorbance for 5 minutes.

Method:

At the end of respective exposure period, shells were cleaned with distilled water and bivalves were sacrificed to collect gill, foot, mantle, adductor muscle and Hpt tissues. The tissues were homogenized in phosphate buffer over ice, and then centrifuged

for 20 minute at 8000 rpm at 4ºC. Supernatants were used to measure AchE activity. Supernatant (50µl) was added to the sodium phosphate buffer (3 ml) and incubated for 5 minutes at room temperature. DTNB (10 µl) and Acetylthiocholine iodide (20 µl) was added to the reaction mixture. Then increasing absorbance was recorded at 412nm for 5 minutes.

3. **Lipid peroxidation (LPO):**

The thiobarbituric acid reactive substances (TBARS) assay was used to evaluate the peroxidation of lipids (Esterbauer and Cheesman 1990).

Principle:

MDA reacts with TBA and produces fluorescent product which can be measured at 532nm.

Method:

At the end of respective exposure period, shells were cleaned with distilled water and bivalves were sacrificed to collect gill, foot, mantle, adductor muscle and Hpt tissues. The tissues were homogenized in phosphate buffer over ice, and then centrifuged for 20 minute at 8000 rpm at 4ºC. Supernatants were used to measure TBARS activity. The supernatant (500 µl) and TBA reagent (500 µl) were added. That mixture was boiled in water bath for 10 minutes. Then absorbance of mixture was checked at 532nm.

4. **Super oxide dismutase (SOD) activity:**

SOD activity was determined by the method of Beauchamp and Fridovich (1971).

Principle:

The super oxide radicals reduced nitroblue tertrazolium to blue colored formazon which can be measured at 560nm.

Method:

At the end of respective exposure period, shells were cleaned with distilled water and bivalves were sacrificed to collect gill, foot, mantle, adductor muscle and Hpt tissues. The tissues were homogenized in phosphate buffer over ice, and then centrifuged for 20 minute at 8000 rpm at 4ºC. Supernatants were used to measure SOD activity.

Sr. No.	Components	Blank	Sample	Final conc
1.	Phosaphate buffer pH7.8 (0.1 M)	1.5 ml	1.5 ml	50mM
2.	Methionine (65mM)	0.6ml	0.6ml	13mM
3.	NBT (750µM)	0.3ml	0.3ml	75 µM
4.	Riboflavin (2mM)	0.3ml	0.3ml	20 µM
5.	EDTA (0.001M)	1µl	1µl	2µM
6.	Homogenate	--	50 µl	--
7.	Distilled water	0.56 ml	0.51ml	--
	Total	3.261ml	3.261ml	--

Table 3: SOD enzyme assay additions

All components were added to blank and sample, except that riboflavin was added in the last. The mixture was mixed properly. One set of mixture was illuminated under the light source for 30 minutes at a distance of 20 cm. While another set of mixture was kept in the dark for 20 minutes. Then absorbance of above mixtures was checked at 560nm.

5. Catalase (CAT) activity:

CAT activity was measured by the method of Aebi (1984).

Principle:

CAT converts the hydrogen peroxide into water and oxygen. The disappearance of hydrogen peroxide was measured spectrophotometrically at 240nm.

Method:

At the end of respective exposure period, shells were cleaned with distilled water and bivalves were sacrificed to collect gill, foot, mantle, adductor muscle and Hpt tissues. The tissues were homogenized in phosphate buffer over ice, and then centrifuged for 20 minute at 8000 rpm at 4ºC. The supernatants were used to measure CAT activity. Decreasing absorbance was monitored for three minutes at 240nm.

6. Glutathione S-transferase (GST) activity:

GST activity was measured according to Habig et al. (1974) method.

Principle:

GST catalyzes the conjugation of reduced glutathione and 1-chloro-2, 4-dinitrobenzene (CDNB) produce dinitrophenyl thioether which can be detected.

Method:

At the end of respective exposure period, shells were cleaned with distilled water and bivalves were sacrificed to collect gill, foot, mantle, adductor muscle and Hpt tissues. The tissues were homogenized in phosphate buffer over ice, and then centrifuged for 20 minute at 8000 rpm at 4ºC. Supernatants were used to measure GST activity. Increasing absorbance was measured at 340nm.

7. Comet assay:

For standardization of the comet assay protocol, gill's fresh single cell suspension was treated with H_2O_2 (1, 10, 25 and 50 mM) in PBS for 5 minutes. The controlled, cells were incubated in PBS without H_2O_2. Three replicates were performed per condition (Sarkar et al. 2013).

The alkaline comet assay was performed as described by Singh et al. (1988) 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 minutes at 4ºC, then coverslip was removed and the slides were transferred to freshly made pre-chilled lysis buffer (2.5M NaCl, 0.1 M di-sodium EDTA, 0.01M Tris Buffer, 0.2 M NaOH) at 4ºC for one hour. Slides were placed in an electrophoresis chamber containing alkaline electrophoresis buffer (300mM NaOH, 1mM EDTA, pH 13) for 20 minutes to unwind DNA. Electrophoresis was carried out at 0.8V/cm and 300mA for 30 minutes at 4ºC. After electrophoresis, the slides were placed into cold (4ºC) neutralizing buffer (0.4M Tris, pH 7.5) which was freshly made, for 15 minutes. Staining was performed with 20 µL of ethidium bromide (20mg/ml) per slide. The slides were examined with a fluorescence microscope (Carl Zeiss Axiovision, 400X, excitation filter 510-560nm, barrier filter 590nm). Microscopic images of comets were scored using Comet IV Computer software. Fifty nuclei were analyzed per slide. All experiments were carried out in triplicate.

8. Micronuclei (MN) assay:

For MN test, single cell suspension of gill tissue was used for preparation of smears (PBS pH 7). Smear was prepared on a glass slide and dried in the dark for 24 hrs. Slides were stained with May-Grunwald Giemsa staining technique as follows: Initially stained for 3 minutes in May- Grunwald (0.25% in methanol), followed by diluted May Grunwald solution (1:1:: May Grunwald: distilled water) for 5minutes, rinsed in distilled water thrice (5 min each) and stained with diluted Giemsa (1:6 of the Giemsa stock: distilled water) for 10 minutes, rinsed in distilled water thoroughly. The slides were dried, cleared for 5 minutes in xylene and mounted in DPX (Malladi et al. 2007). 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. (2003).

Statistical analysis:

The statistical data analysis was carried out using one-way ANOVA; Tukey pair wise-multiple comparison test was used for biochemical estimations. Data were presented as the mean ± Standard deviation (S.D.).

RESULTS

RESULTS

1. Protein content:

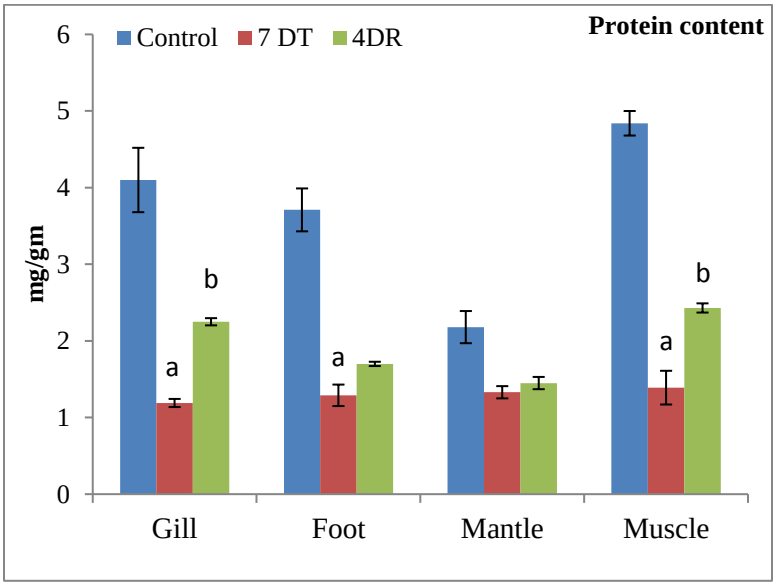


Figure 4: Changes in the Protein content in different tissues of *L. marginalis* when exposed to MCP (5.25mg/lit) for 7 days followed by 4 days recovery. 7dt: 7 days treated, 4dr: 4 days recovery (mean ± s.d.) a there are significant differences (p< 0.05) between the control and treated groups, b there are significant differences (p< 0.05) between the treated and 4 days recovery

Decreased protein content (Figure 4) was observed after 7 days of MCP exposure in gill (71%), foot (65%), mantle (39%) and adductor muscle (71%) tissues. Except mantle, all other tissues showed significantly ($P< 0.05$) decreased protein content. After 4 days of recovery gill (55%), foot (43%), mantle (67%) and adductor muscle (50%) tissues showed somewhat recovery. Gill and adductor muscle tissues showed significantly ($P< 0.05$) decreased protein content followed by significant ($P< 0.05$) recovery.

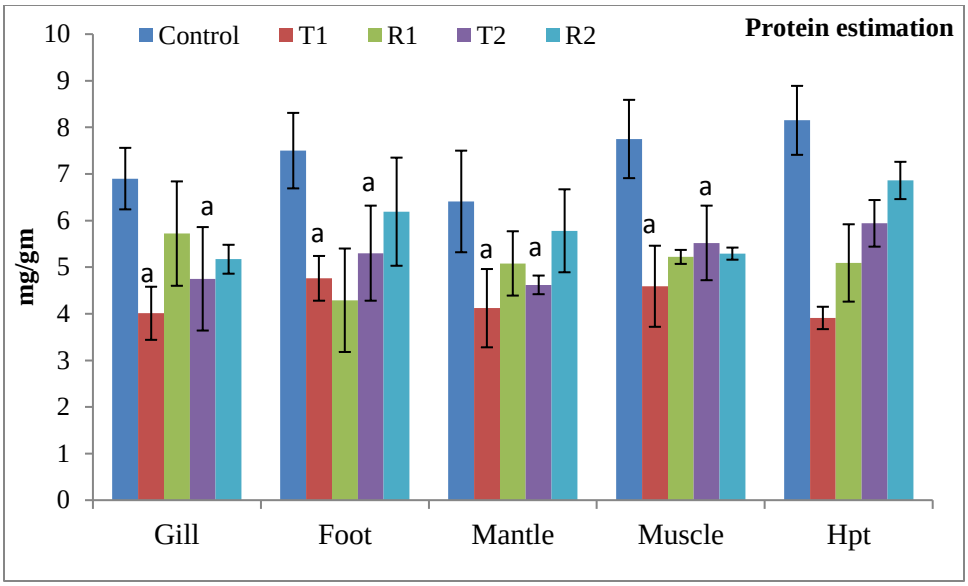


Figure 5: Changes in the protein content in different tissues of *L. marginalis* when exposed to CPF for 7 days in T1 (0.4mg/lit) and T2 (0.04mg/lit) groups followed by 4 days recovery in R1 and R2 groups. 7dt: 7 days treated, 4dr: 4 days recovery (mean ± s.d.) a there are significant differences (p< 0.05) between the control and treated groups, b there are significant differences (p< 0.05) between the treated and 4 days recovery

CPF caused significant ($P < 0.05$) reduction of protein content (Figure 5) in T1 (0.4mg/lit) group. The trend of reduction was Hpt (51.98%) > gill (41.83%) > adductor muscle (40.77%) > foot (36.46%) > mantle (34.13%). Significant recovery was not observed after 4 days in any of the observed tissue.

In the T2 (0.04mg/lit) group, significant ($P < 0.05$) depletion of protein was observed in the gill (31.15%), foot (29.30%), adductor muscle (28.77%) and Hpt (27.15%) followed by a non-significant recovery R2 ($P < 0.05$) after 4 days.

2. AchE activity:

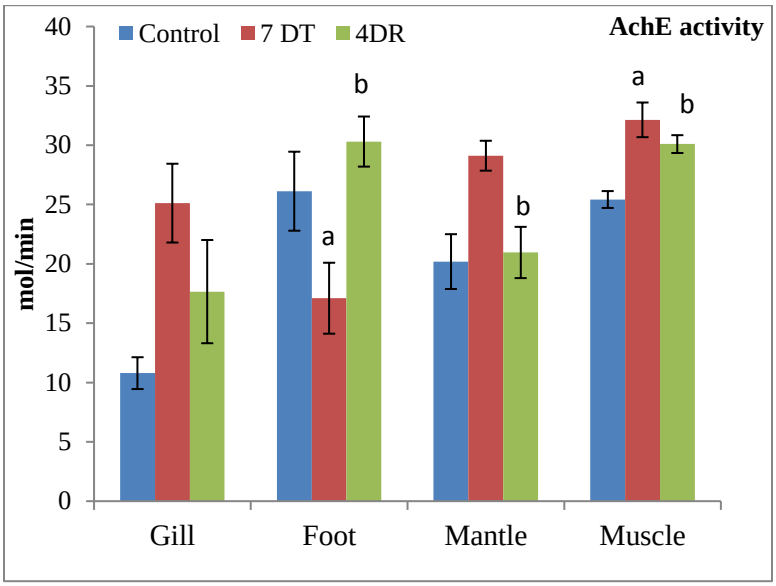


Figure 6: Alterations of AchE activity in different tissues of *L. marginalis* when exposed to MCP (5.25mg/lit) for 7 days followed by 4 days recovery. 7dt: 7 days treated, 4dr: 4 days recovery (mean ± s.d.) a there are significant differences (p< 0.05) between the control and treated groups, b there are significant differences (p< 0.05) between the treated and 4 days recovery

Foot (35%) tissue showed significantly (*P*< 0.05) inhibited AchE activity (Figure 6) after 7 days of exposure followed by significant (*P*< 0.05) recovery. In gill (233%), mantle (144%) and adductor muscle (126%) tissues AchE activity increased after toxicant exposure. Though gill (164%) recovered to some extent, mantle (104%) and adductor muscle (118%) showed significant recovery.

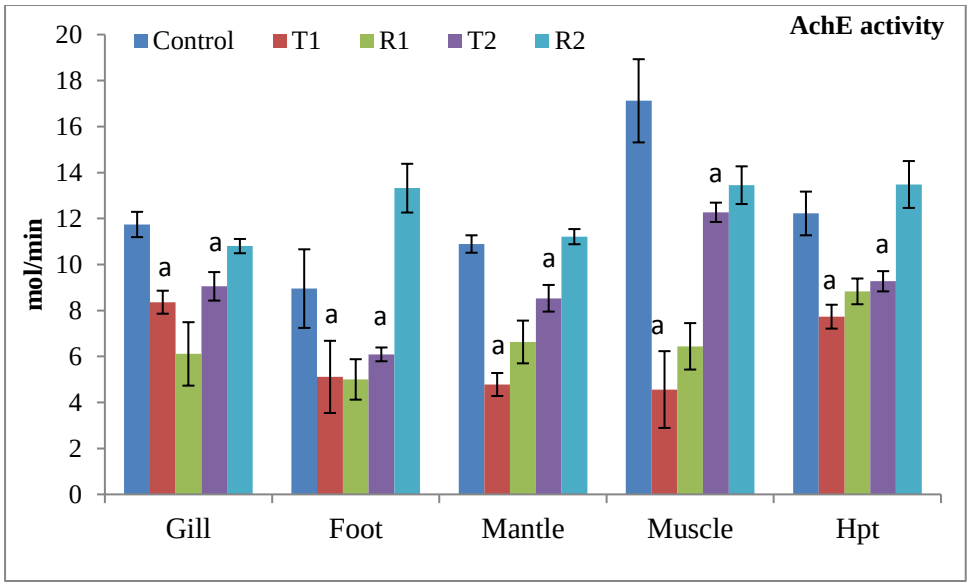


Figure 7: Changes in the AchE activity in different tissues of *L. marginalis* when exposed to CPF for 7 days in T1 (0.4mg/lit) and T2 (0.04mg/lit) groups followed by 4 days recovery in R1 and R2 groups. 7dt: 7 days treated, 4dr: 4 days recovery (mean ± s.d.) a there are significant differences (p< 0.05) between the control and treated groups, b there are significant differences (p< 0.05) between the treated and 4 days recovery

L. marginalis exposed to CPF T1 (0.4mg/lit) revealed that the AchE activity (Figure 7) in treated animals was inhibited significantly after 7 days of treatment, ($P < 0.05$) as compared to respective control. The trend of inhibition of AchE activity was like adductor muscle (73.63%), mantle (56.13%), Hpt(36.76%), foot (35.73%), gill (28.83%) followed by significant ($P < 0.05$) recovery only in mantle (38.72%), whereas Hpt, gill, foot and adductor muscle were not recovered significantly ($P < 0.05$).

In the T2 group (0.04 mg/lit), CPF revealed the significant ($P < 0.05$) depletion of AchE in adductor muscle (28.99%), Hpt (24.16%), foot (23.42%), gill (22.93%) and mantle (21.64%). The significant recovery ($P < 0.05$) was observed in descending order in foot (69.45%), Hpt (45.37%), mantle (31.32%), gill (19.88%) and adductor muscle (9.62%).

3. TBARS activity:

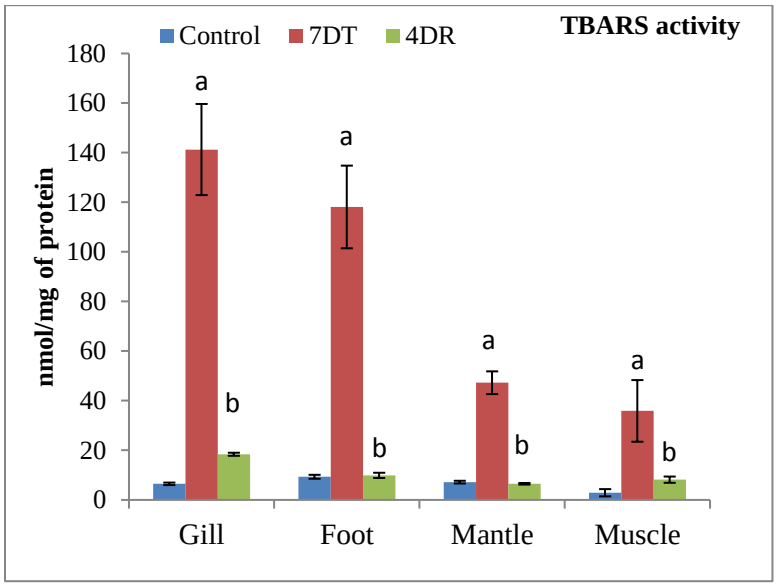


Figure 8: Changes in the TBARS activity in different tissues of *L. marginalis* when exposed to MCP (5.25mg/lit) for 7 days followed by 4 days of recovery. 7dt: 7 days treated, 4dr: 4 days recovery (mean ± s.d.) a there are significant differences (p< 0.05) between the control and treated groups, b there are significant differences (p< 0.05) between the treated and 4 days recovery

In the exposure period of seven days of MCP, lipid peroxidation (Figure 8) was significantly (*P*< 0.05) induced in gill (1197%)> foot (1042%)>mantle (867%)>adductor muscle (830%), as compared to controlled group. But in the recovery period of four days, lipid peroxidation was recovered significantly (*P*< 0.05) in foot (74%), adductor muscle (68%), mantle (68%) and gill (54%).

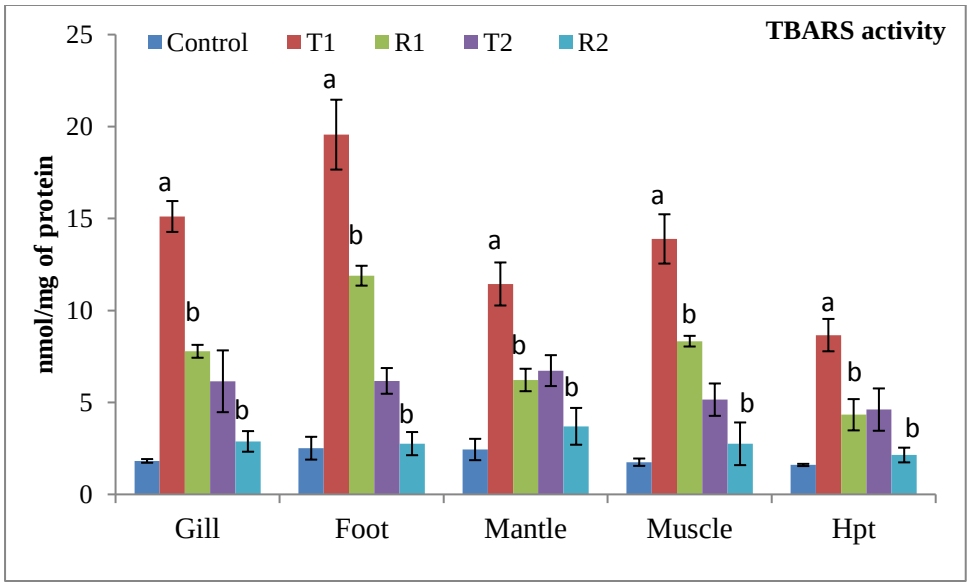


Figure 9: Changes in the TBARS activity in different tissues of *L. marginalis* when exposed to CPF for 7 days in T1 (0.4mg/lit) and T2 (0.04mg/lit) groups followed by 4 days recovery in R1 and R2 groups. 7dt: 7 days treated, 4dr: 4 days recovery (mean ± s.d.) a there are significant differences (p< 0.05) between the control and treated groups, b there are significant differences (p< 0.05) between the treated and 4 days recovery

The levels of TBARS (Figure 9) were estimated in the control and experimental animals. It was observed that TBARS level was significantly increased ($P < 0.05$) in T1 group (0.4mg/lit) after 7 days of exposure in gill (828.70%), foot (779.15%), adductor muscle (792.20%), Hpt (540.33%), and mantle (468.98%). TBARS level decreased after 4 days of recovery R1 in all the tissues as follows: Hpt (50.05%), gill (48.53%), mantle (45.64%), adductor muscle (40%) and foot (39.20%).

In T2 group, 0.04mg/lit concentration of CPF caused significant ($P < 0.05$) increment of TBARS in gill (337.47%),foot (245.81%), adductor muscle (293.53%), Hpt (287.73%) and mantle (275.81%) followed by the significant ($P < 0.05$) recovery of TBARS in gill (53.19%), foot (55.26%), mantle (45.07%), adductor muscle (46.56%) and Hpt (53.61%) after 4 days of recovery period.

4. SOD activity:

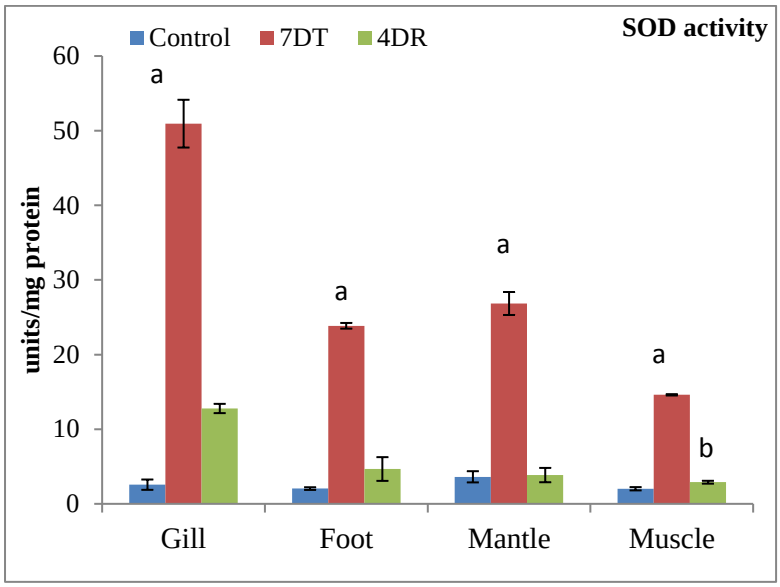


Figure 10: Changes in the SOD activity in different tissues of *L. marginalis* when exposed to MCP (5.25mg/lit) for 7 days followed by 4 days of recovery. 7dt: 7 days treated, 4dr: 4 days recovery (mean ± s.d.) a there are significant differences (p< 0.05) between the control and treated groups, b there are significant differences (p< 0.05) between the treated and 4 days recovery

Significant (*P*< 0.05) elevation of SOD activity after MCP exposure (Figure 10) was observed in gill (1982%) followed by foot (1169%), mantle (741%) and adductor muscle (1819%) tissues but four days recovery period was found to be sufficient to recover significantly (*P*< 0.05) for adductor muscle (90%)> mantle (86%)> foot (80%)>gill (74%). Gill showed maximum elevation of SOD activity and comparatively slower recovery as compared to other tissues.

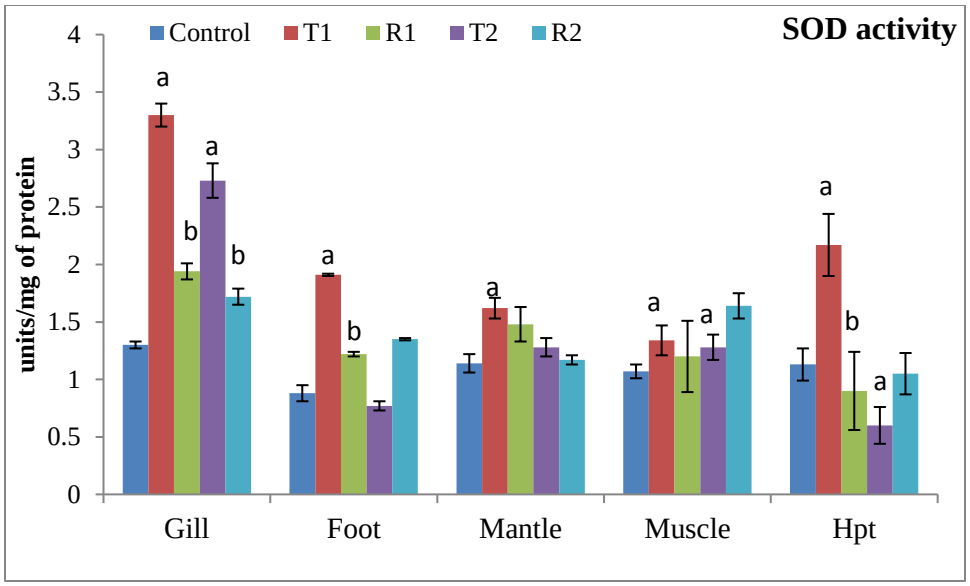


Figure 11: Changes in the SOD activity in different tissues of *L. marginalis* when exposed to CPF for 7 days in T1 (0.4mg/lit) and T2 (0.04mg/lit) groups followed by 4 days recovery in R1 and R2 groups. 7dt: 7 days treated, 4dr: 4 days recovery (mean ± s.d.) a there are significant differences ($p< 0.05$) between the control and treated groups, b there are significant differences ($p< 0.05$) between the treated and 4 days recovery

The T1 group displayed significant ($P < 0.05$) increase of SOD (Figure 11) in gill (331.02%), foot (285.22%), Hpt (217.04%), mantle (124.53%) and foot (51.26%). After 4 days of recovery period, gill (54.91%) and Hpt (58.74%) recovered better than adductor muscle and mantle.

Significant elevation of SOD activity was observed in T2 (0.04mg/lit) group after exposure for 7 days, in adductor muscle (119.25%), gill (87.87%), and Hpt (52.95%), however significant recovery ($P < 0.05$) was found only in gill (36.79%).

5. CAT activity:

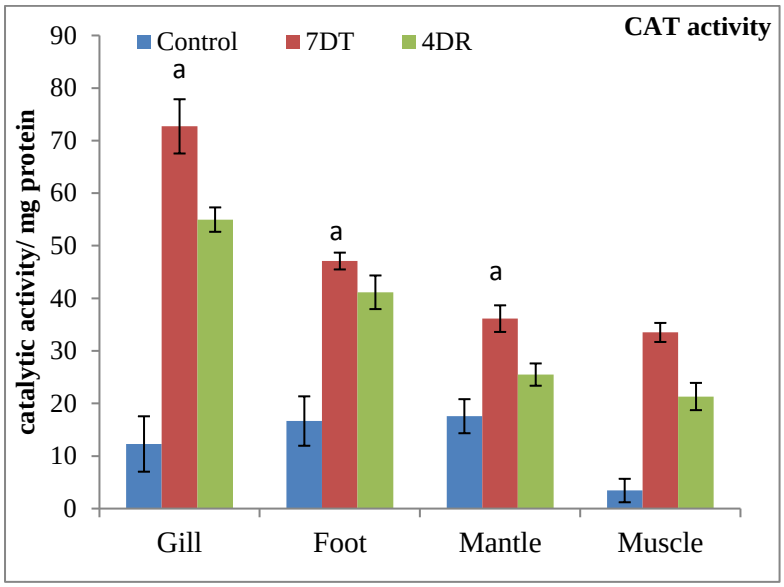


Figure 12: Changes in the CAT activity in different tissues of *L. marginalis* when exposed to MCP (5.25mg/lit) for 7 days followed by 4 days of recovery. 7dt: 7 days treated, 4dr: 4 days recovery, (mean ± s.d.) a there are significant differences (p< 0.05) between the control and treated groups, b there are significant differences (p< 0.05) between the treated and 4 days recovery

After MCP exposure, CAT activity (Figure 12) was increased significantly (*P*< 0.05) in adductor muscle (977%), gill (646%), foot (282%) and mantle (206%) tissues. The trend of significant (*P*< 0.05) recovery was observed after four days of recovery period which was as follows; adductor muscle (36%)> gill (31%), >mantle (29%)>foot (13%).

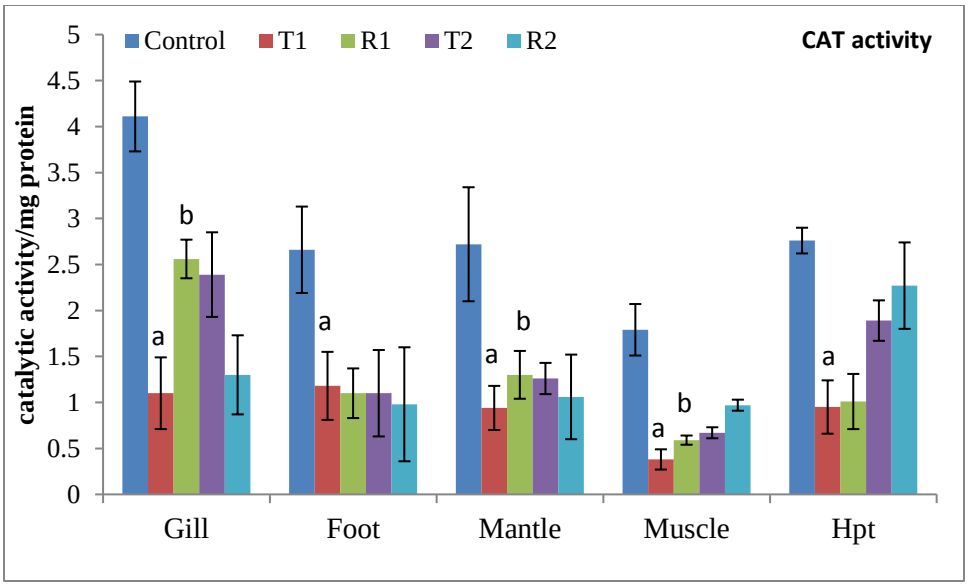


Figure 13: Changes in the CAT activity in different tissues of *L. marginalis* when exposed to CPF for 7 days in T1 (0.4mg/lit) and T2 (0.04mg/lit) groups followed by 4 days recovery in R1 and R2 groups. 7dt: 7 days treated, 4dr: 4 days recovery (mean ± s.d.) a there are significant differences ($p< 0.05$) between the control and treated groups, b there are significant differences ($p< 0.05$) between the treated and 4 days recovery

Reduction of CAT activity (Figure 13) was observed in T1 (0.4mg/lit) treated group in adductor muscle (78.54%), gill (73.21%), Hpt (65.65%), mantle (65.27%) and foot (55.70%). The recovery was observed in the adductor muscle (54.78%), mantle (37.80%) and in gill (7.12%).

T2 (0.04mg/lit) group showed significant ($P < 0.05$) decrease of CAT activity in Hpt (31.43%), gill (41.88%), mantle (53.61%), adductor muscle (62.68%) and foot (58.72%) however the significant recovery was not observed in any of the observed tissue.

6. GST activity:

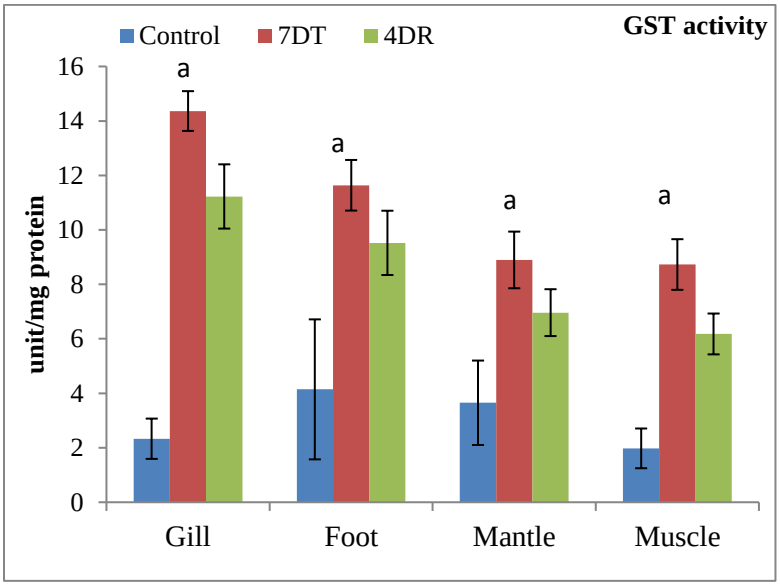


Figure 14: Changes in the GST activity in different tissues of *L. marginalis* when exposed to MCP (5.25 mg/lit) for 7 days followed by 4 days of recovery. 7dt: 7 days treated, 4dr: 4 days recovery, (mean ± s.d.) a there are significant differences ($p< 0.05$) between the control and treated groups, b there are significant differences ($p< 0.05$) between the treated and 4 days recovery

After MCP exposure, significant ($P< 0.05$) induction of GST activity (Figure 14) was observed in gill (616%), adductor muscle (441%), foot (281%) and mantle (244%) tissues. After the recovery period, maximum recovery was observed in gill (35%) followed by mantle (30%), adductor muscle (27%) and foot (21%) tissues.

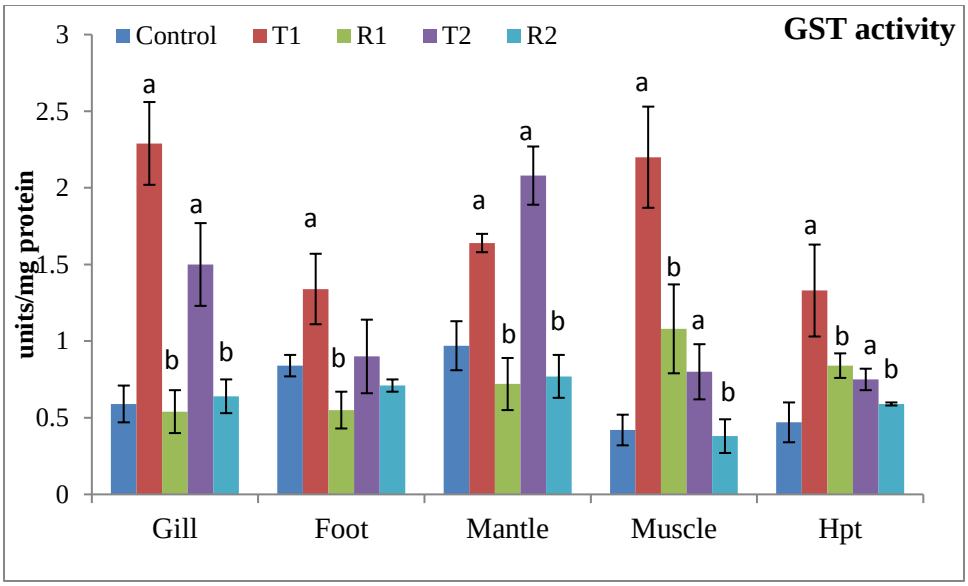


Figure 15: Changes in the GST activity in different tissues of *L. marginalis* when exposed to CPF for 7 days in T1 (0.4mg/lit) and T2 (0.04mg/lit) groups followed by 4 days recovery in R1 and R2 groups. 7dt: 7 days treated, 4dr: 4 days recovery (mean ± s.d.) a there are significant differences (p< 0.05) between the control and treated groups, b there are significant differences (p< 0.05) between the treated and 4 days recovery

Seven days exposure of 0.4mg/lit (T1) of CPF revealed significant ($P < 0.05$) elevation of GST (Figure 15) in adductor muscle (528%), gill (389.77%), Hpt (280.98%), mantle (168.15%), foot (159.52%) followed by significant ($P < 0.05$) recovery in a manner like- gill (76.53%), foot (58.95%), mantle (56%), adductor muscle (51.06%) and Hpt (37.09%).

Significantly increased activity of GST enzyme was observed in 0.04 mg/lit treated group (T2) after 7 days of exposure. Highest elevation was marked in gill (255.68%) followed by mantle (214.04%), adductor muscle (191.2%) and Hpt (158.45). Significant ($P < 0.05$) recovery in gill (57.11%), mantle (63.2%), adductor muscle (51.88%) and Hpt (12.88%) was seen after the recovery period.

Comet assay

Standardization of the comet assay:

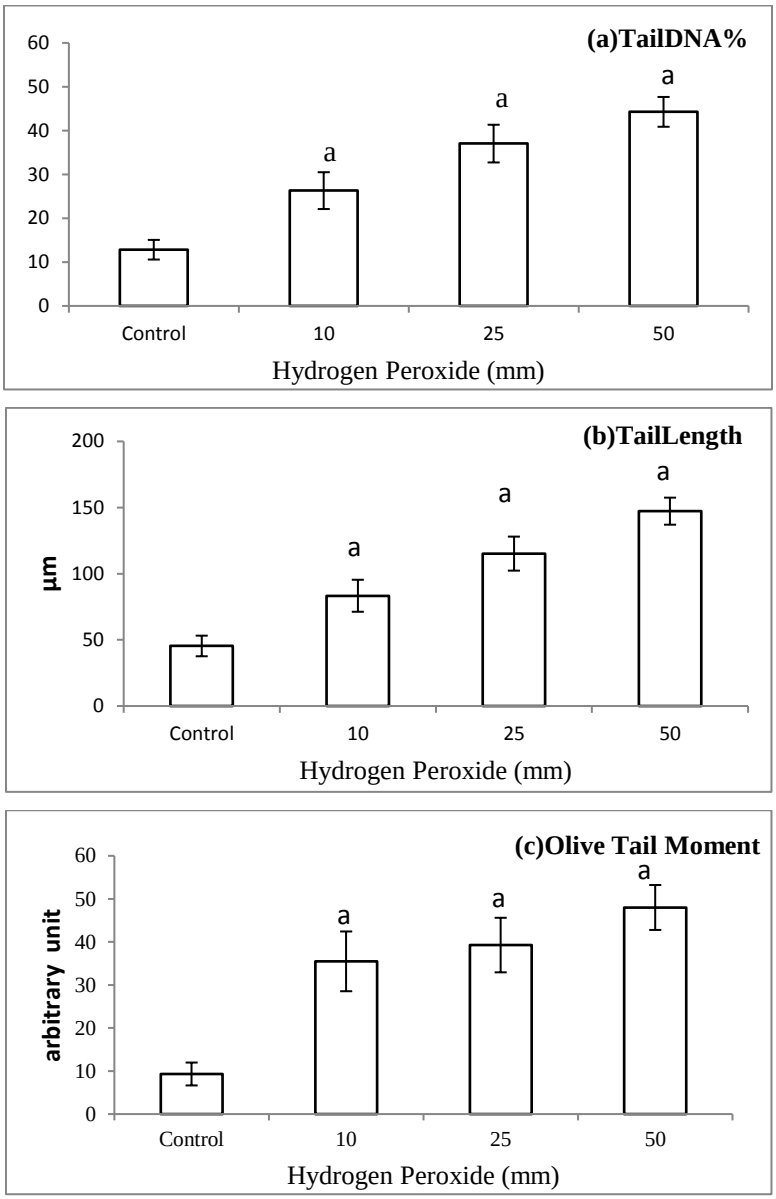


Figure 16: DNA strand breaks in gill cells of *L. marginalis*, exposed to H_2O_2 (a, b & c)

Comet parameters were reported as mean ± standard error. a: significant differences (p<0.05) between the control and treated groups, b: significant differences (p<0.05) between the treated and 4 days recovery

Gill cells of *L. marginalis* treated with different concentrations of H_2O_2 (5, 10, 25 and 50 mM) that showed a significant (P<0.05) dose-dependent increase in the

percentage tail DNA (Figure 16a), tail length (TL) (Figure 16b) and olive tail moment (OTM) (Figure 16c).

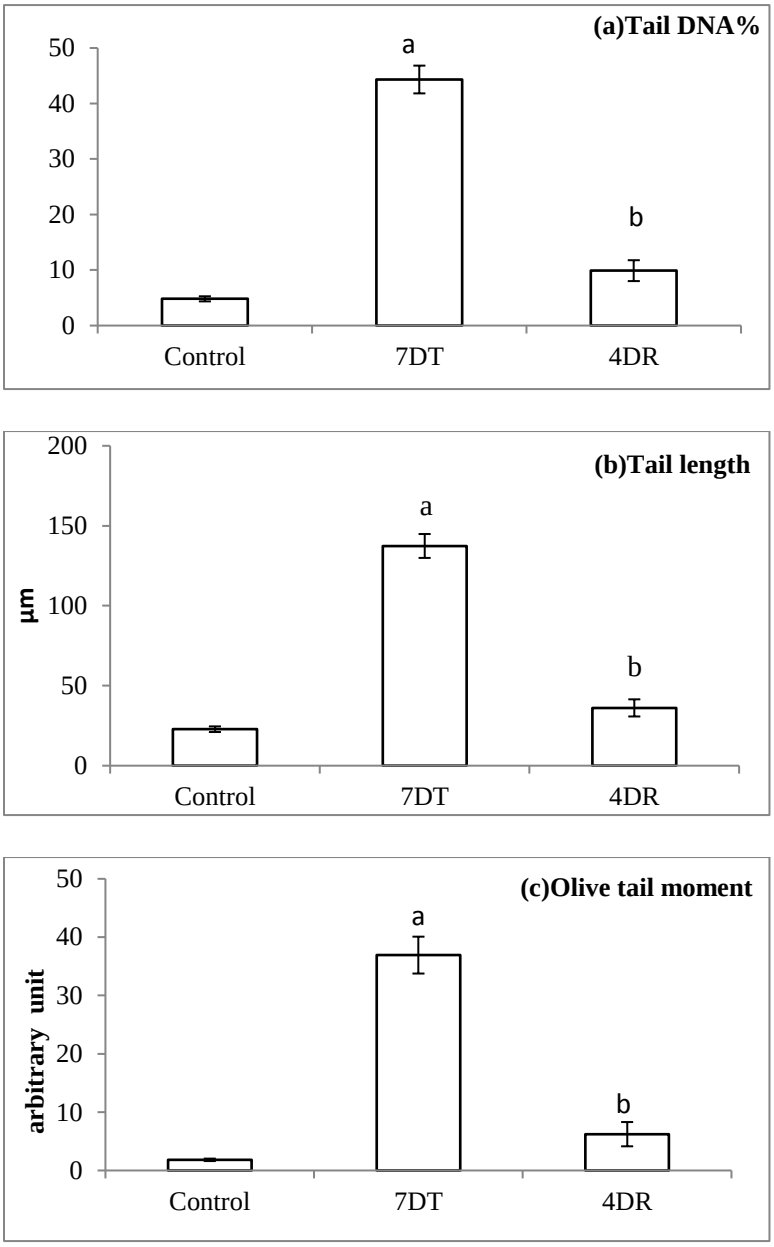


Figure 17: DNA strand breaks in gill cells of *L. marginalis* exposed to MCP (5.25mg/lit) (a, b & c) for 7 days treated (7DT) and 4 days recovery (4DR). Comet parameters were reported as mean ± standard division. a: significant differences (p<0.05) between the control and treated groups, b: significant differences (p<0.05) between the treated and 4 days recovery

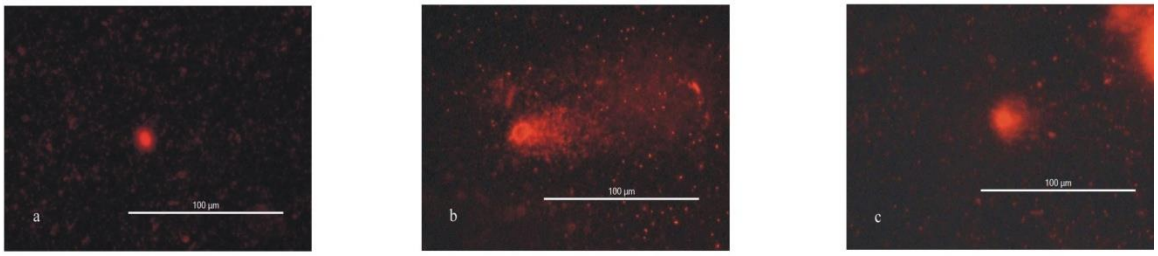


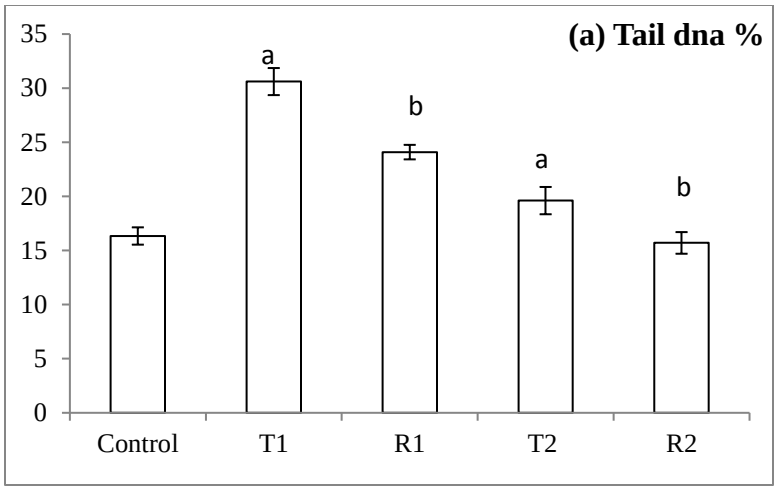
Figure 18: DNA strand breaks in gill cells of *L. marginalis* exposed to MCP (5.25mg/lit), stained with ethidium bromide (a) control, (b) treated and (c) recovery.

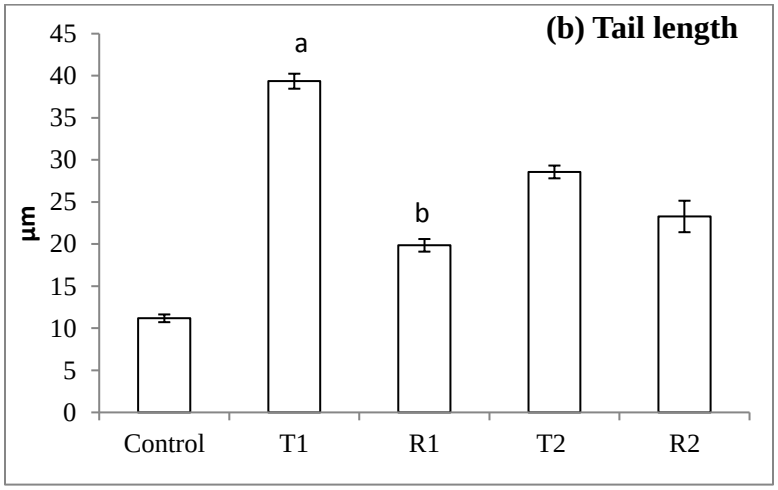
After 7 days of MCP exposure, tail DNA percentage (920%) (Figure 17a), tail length (603%) (Figure 17b), and OTM (2028%) (Figure 17c) were significantly (P <0.05) increased in gill cells, as compared to control. After four days of recovery period, significant (P<0.05) recovery was observed in tail length (74%), tail DNA percentage (78%) and OTM (83%) in gill cells of *L. marginalis*.

Figure 18 represents observation of comet assay in gill cells *L. marginalis*. Gill cells from control (Figure 18 a) showed round shape of stained DNA without tail. Comet shape of nuclear DNA with head and long tail was observed in treated (Figure 18 b) gill cells. Comparatively small tail was observed in gill cells (Figure 18 c) after recovery period.

As compared with the control samples, significant increase in tail DNA % (P < 0.05) were observed due to CPF exposure in T1 (187%). Tail DNA % was found to have increased by almost double fold in treated gill cells. In R1 group (147%), after the 4 days of recovery period, significant reduction (P < 0.05) was observed in tail DNA %. In T2 (0.04 mg/lit) (120%) group the same trend of significant increase (P < 0.05) in tail DNA % was observed as compared to control. Significant decrease was obtained in R2 (96%) recovery group when compared to (T2) treated group.

An increase in TL was observed in T1 (351%) treated gill cells in comparison with control. Significant increase in TL was observed in exposed cells as compared to control followed by significant decrease in R1 (178%) recovered gill cells. The increased TL was observed in T2 (256%) group in comparison with control followed by recovery in R2 (208%) group.





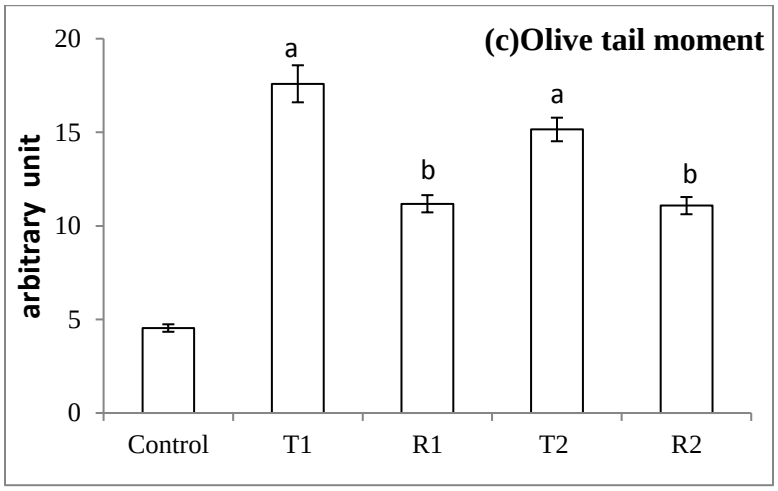


Figure 19: DNA damage in gill cells of control, treated T1 (0.4mg/lit) & T2 (0.04mg/lit) and recovery group (R1 & R2), in *L. marginalis* after CPF exposure. Comet parameters were

reported as mean ± standard error. a: significant differences (p<0.05) between the control and treated groups, b: significant differences (p<0.05) between the treated and 4 day recovery

As far as OTM (Figure 19c) is concerned, there was significant elevation ($p < 0.05$) in T1 (387%) treated gill cells as compared to control ones followed by significant recovery R1 (246%) after 4 days as compared to T1group. In 0.04mg/lit CPF T2 group same pattern was observed, significant increase in OTM in T2 (334%) as compared to control, whereas significant decline in OTM was reported from R2 (244%) recovery group as compared to treated (T2).

Micronuclei assay

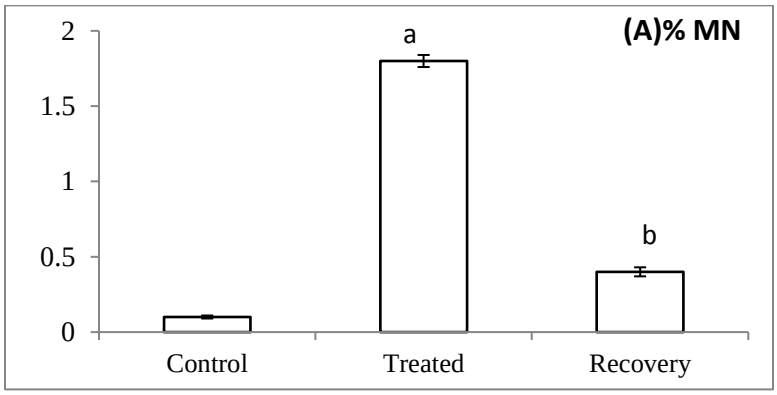


Figure 20: Micronuclei formation in gill tissue of *L. marginalis* when exposed to MCP (5.25mg/lit), for 7 (T1) days followed by 4 days of recovery (R1). 7dt: 7 days treated, 4dr: 4 days recovery (mean ± s.d.) a there are significant differences ($p< 0.05$) between the control and treated groups, b there are significant differences ($p< 0.05$) between the treated and 4 days recovery

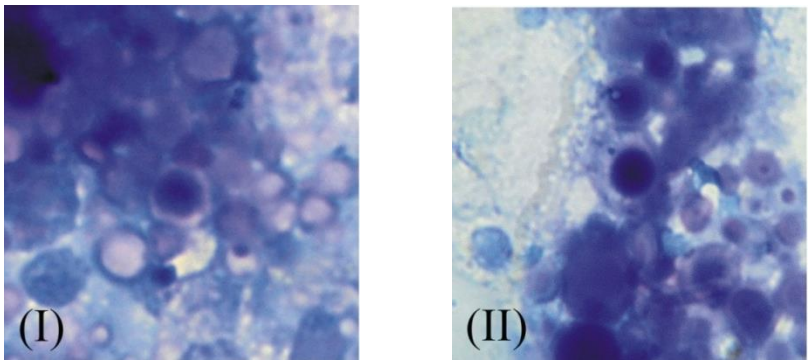


Figure 21: Morphological abnormalities in *L. marginalis* gill cells nuclei after MCP & CHL exposure (I) Normal cell, (II)cell with micronuclei (1000X).

MCP (Figure 20 A) induced significant ($P<0.05$) MN formation in treated bivalves, in comparison with control ones. Also four days recovery duration was found to be sufficient to recover significantly ($P<0.05$). Figure 21 (I & II) represents observations of MN. Figure 21 (I) showed control gill cell and (II) gill cell with micronuclei.

DISCUSSION

DISCUSSION:

From the above experiment of bivalves with their tissues- gill, foot, mantle, muscle and Hpt were taken for two periods i.e. exposure and recovery, treated in MCP and CPF which are OP pesticides used in agriculture field. The exposure period was 7 days while recovery period was 4 days.

Our tested concentration of MCP (5.25 mg/L) is higher than the environmental relevant concentration. If there is repeated use of pesticide, then its concentration in the aquatic ecosystem may exceed thus suggesting the importance of test concentration.

MCP exposure showed decreased protein in all the tissues after 7 days. Four days of recovery period was found to be sufficient for tissues recovery. Gill and adductor muscle tissues were sensitive to MCP exposure as well as recovered efficiently. Gill tissue always bathing in the water so it was found to be susceptible to toxicant exposure because of filter feeding purpose. Adductor muscle tissue has a number of synaptic junctions than gill and mantle tissues; therefore it may be targeted by OP toxicant easily.

Reduced protein content may also be related with enhancing proteolytic activity and may be consumed its products for metabolic function. The decrease in protein level after exposure may suggest its increase catabolism and decrease anabolism.

The results showed that the 0.4mg/lit caused more depletion in protein as compared to 0.04 mg/lit of CPF. A visible reduction in the protein levels in the tissues after treatment of CPF suggests either depletion in rate of protein synthesis or increased proteolytic activity. Decrease in protein in pollutant treated animal might be due to increased proteolytic activity. Hpt was found to be the most affected tissue which might be due to its high metabolic potency as compared to other tissues (Tambe 2014).

AchE is responsible for hydrolysis of Neurotransmitter acetylcholine into choline and acetic acid. The AchE plays crucial role in nerve conduction processes at the neuromuscular junction. The inhibition of this enzyme is directly connected to the toxicity of OP. AchE activity is widely used as biomarker of neurotoxicity in invertebrates (Rakhi et al. 2014).

AchE enzyme has many essential functions like cilliary activity of gill epithelium, cilliary activity for transport of suspended particles, valve opening and closing etc. (Corsi et al 2007).

Altered AchE activity in gill, foot, mantle and adductor muscle was observed after MCP exposure. Foot tissue showed inhibition while other tissues showed enhanced AchE activity. Foot and adductor muscle tissues showed significant altered activity upon exposure followed by significant recovery. Adductor muscle tissue and foot tissue's muscular part contained more synaptic junctions therefore they were susceptible to OP exposure.

The adductor muscle showed the most reduction in AchE activity after CPF exposure, followed by least recovery. This may be due to the presence of neuromuscular synapses in adductor muscles**.** AchE activity was found to be inhibited in the anterior adductor muscle of *Amblema plicata* upon exposure to various doses of OP pesticides (Doran et al. 2001; Fernandez-Vega2002). Result indicated that the mantle had more ability to overcome the stress of CPF in T1 group.

When bivalve exposed to pesticides, different toxic effects like altered protein content and enzyme activity were observed in different tissues. To protect themselves against the toxic effect, bivalves were activating all possible protective mechanisms therefore there may be a change in metabolic process. Animals which go through the toxic stress show a change in biochemical and physiological mechanisms.

In brief, after 7 days of exposure of MCP, bivalve experienced oxidative stress due to significant ($P< 0.05$) increased LPO. The enhancement of LPO in all the tissues of bivalves suggested that the participation of free radical-induced oxidative cell injury. In which the most affected tissue was gill and the least affected tissues were mantle and adductor muscle.

Increased LPO content in gills and digestive glands of freshwater mussel exposed to pesticide was reported by Koprucu et al. (2010). It is suggested that, gill was more susceptible to oxidative stress, as compared to other tissues due to its filter feeding mechanism (Borkovic-Mitic et al. 2011). The foot was the second highest sensitive tissue in case of lipid peroxidation, which showed significant ($P<0.05$) recovery compared to adductor muscle, mantle and gill tissues.

Pesticides may stimulate oxidative stress and lead to lipid peroxidation. According to Giordano et al. (2007), OPs may promote higher reactive oxygen species (ROS) levels and higher lipid peroxidation**.**

Elevation of lipid peroxidation after pesticide exposure in present study suggests participation of free-radical induced oxidative cell injury indicating the toxicity of pesticides.

Superoxide dismutase (SOD; EC 1.15.1.1) is an important enzyme for detoxification of reactive oxygen species in all the organisms. SOD plays an important role in protecting cells against the oxidative damage of free radicals by catalyzing the conversion of superoxide anion to oxygen (O_2) and hydrogen peroxide (H_2O_2), which is then catalyzed either by CAT or GPX enzyme.

The observed trend of increased SOD activity in treated bivalves was Gill>Foot>Mantle. The trend of recovery observed was in reverse manner (Mantle>Foot>Gill). It was observed that tissues which were under lower stress recovered better and the mantle tissue recovered the most. During MCP exposure in *L. marginalis*, SOD and CAT activities were increased which indicated generation of superoxide radical and hydrogen peroxide. Overall, the exposure of MCP in bivalves; the percentage of SOD activity was higher than the CAT activity. The positive relationship between SOD and CAT activities was observed in the treated and recovery groups.

Elevation of SOD in the present study might have occurred as a response to CPF induced superoxide anion production.

Mundhe and Pandit (2014) found inhibition of CAT activity in *L. marginalis* after treatment of MCP. Our results were in confirmation with Kaaya et al. (1999) in *Perna perna* and *Mytilus galloprovincialis*.

Oreochromis mossambicus exposed to thiol analogue of MCP (0.017mg/lit) which showed induction in GST activity followed by significant recovery in liver tissue (Rao 2006).

The main aim of estimating Glutathione S-Transferase (GST- EC. 2.5.1.18) enzyme activity in *L. marginalis* upon exposure to CPF was to evaluate the use of this enzyme as biomarker of CPF toxicity. According to Foumier et al. (1992), GST plays an important role in detoxification and protection of cells from oxidative stress. GST is involved in the detoxification of organophosphorus compounds and is crucial for insecticide resistance (Clark 1990).

The increase in GST activity suggests the involvement of this enzyme in the detoxification of CPF, hence used as indicator of stress caused by the chemical. GST activity maintains high protection against lipid peroxidation and there is an increasing correlation between susceptibility to toxic substances and GST activity.

The gills cells were chosen for comet assay because gill cells are suitable target for mutagen exposure. Comet assay can be used as early biological marker for determination of genotoxic pollutants in aquatic environments (Kushwaha et al. 2012). Gills are more prone to experience oxidative stress than other tissues because they are entry sites for toxicants, so they should have more efficient antioxidant mechanisms than other tissues.

Comet assay was standardized with hydrogen peroxide treatment in experimental bivalve that showed increase in tail DNA %, OTM and TL in dose dependant treatment.

It was observed by Banu et al. (2001) that phosphorus group of MCP acted as a good substrate for nucleophilic attack; it might cause phosphorylation of DNA, which is an instance of DNA damage. MCP might have a potential of 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 treated bivalves, which demonstrated the genotoxicity of MCP in *L. marginalis*. Our results are in accordance with Sarkar et al. (2013) in gill cells of *Nerita chamaeleon*.

Upon exposure to 0.4 mg/lit of CPF for 7 days (T1), we found significant elevation in comet parameters, such as Tail length, Tail DNA%, Olive tail moment (OTM) in treated gill cells as compared to control followed by significant recovery.

The increased lipid peroxidation leads to generation of reactive oxygen species also contribute to formation of DNA strand breaks (Banu et al. 2001).The observed DNA damage in this study may be due to reactive oxygen species formed during metabolism of the pesticide (Zeid 2014).

Conclusions

The outcome of this study proposed that MCP and CPF could induce oxidative stress and lead to impairment in acetylcholinesterase activity as well as genotoxicity in the freshwater bivalve *L. marginalis*. Oxidative and DNA damage were experienced by MCP treated mussels; however anti- oxidant enzymes recovered well, the levels of micronuclei decreased and also repair in terms of comet parameters was observed after four days.

Both the concentrations of CPF could enhance lipid peroxidation in tissues of *L. marginalis*, followed by remarkable recovery in gill and Hpt after withdrawal of stress. Induction of SOD and GST activities was observed with increase in concentration of CPF. Therefore, they can be used as valuable biomarkers in assessment of toxicity. Concentration dependent elevation of comet parameters was observed after CPF induced stress which proves genotoxic potential of CPF. However, significant recovery in comet parameters was observed after removal of stress.

Publications

1. Anju Y. Mundhe and **S. V. Pandit** (2014) Assessment of toxicity of monocrotophos in freshwater bivalve, *Lamellidens marginalis* using different markers. Toxicology international, Vol-21 (1): 51-56.
2. Mrs. Anju Yogesh Mundhe**,** Dr. Hari Bhilwade, **Dr. Sangeeta V. Pandit** (2016) Genotoxicity and oxidative stress as Biomarkers in fresh water mussels, *Lamellidens marginalis*, exposed to Monocrotophos. Indian Journal of Experimental Biology Vol 54: 822-828.

Outcome of the Project:

Mrs. Anju Yogesh Mundhe has worked as JRF for this project for one year and part of her PhD work is based on this research project work.

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