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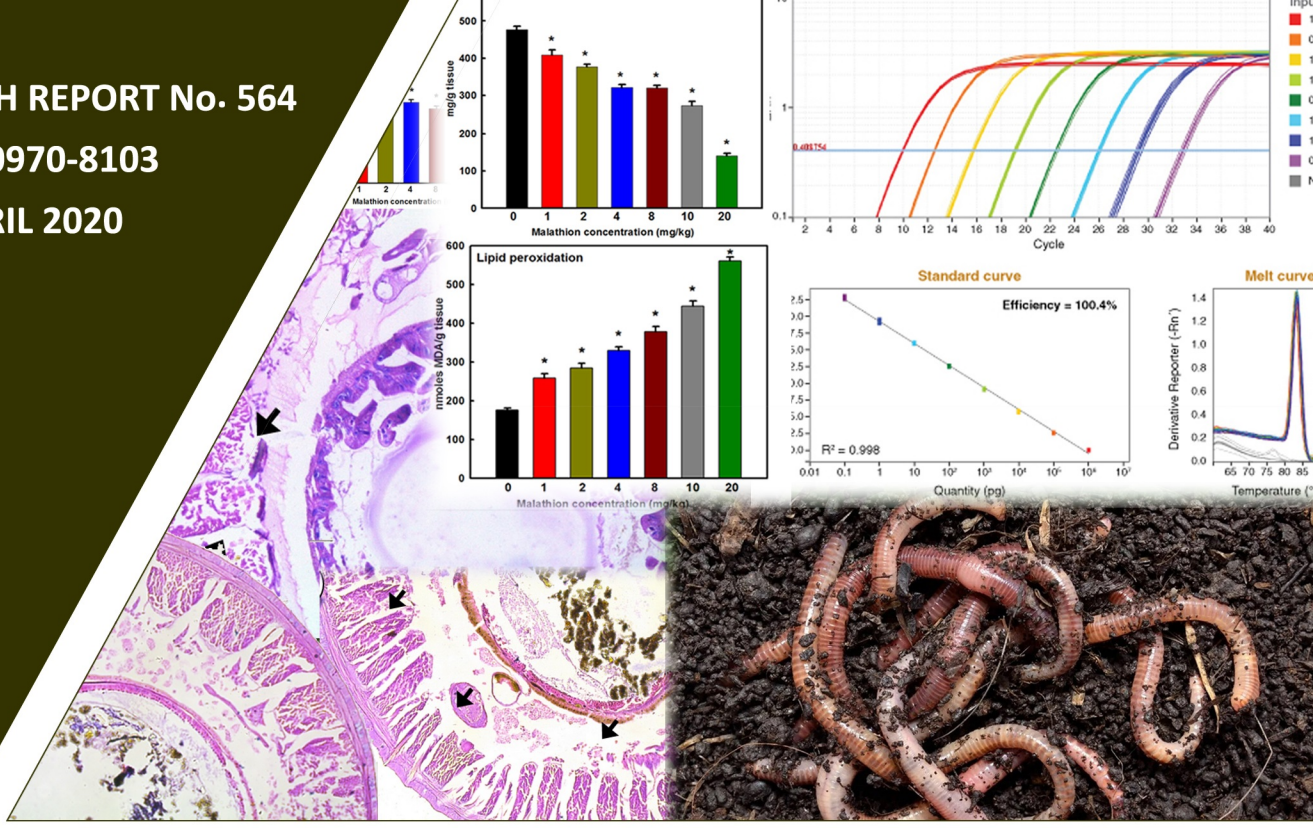

DEVELOPMENT OF BIOMARKERS AS A PREDICTIVE TOOL FOR ORGANOPHOSPHATE TOXICITY IN TERRESTRIAL ECOSYSTEM

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DEVELOPMENT OF BIOMARKERS AS A PREDICTIVE TOOL FOR ORGANOPHOSPHATE TOXICITY IN TERRESTRIAL ECOSYSTEM

FINAL REPORT KFRI RP 702/2015

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April 2020

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PROJECT DETAILS

Title of the Project	Development of biomarkers as a predictive tool for organophosphate toxicity in terrestrial ecosystem
Objectives	I. Study the biochemical toxicity of organophosphate pesticides in terrestrial invertebrates II. Analyse the response of oxidative stress parameters at gene expression level. III. Comparative analysis of acetylcholinestrase and toxicity parameters
Funding agency	Kerala State Council for Science, Technology and Environment, Government of Kerala
Investigators	Dr. R. Jayaraj Principal Investigator Senior Scientist Forest Ecology & Biodiversity Conservation Division Dr. Suma Arun Dev Co-Investigator Senior Scientist, Forest Genetics & Biotechnology Division
Project Staff	Ms. Megha. P. Nair
Duration	3 Years

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INVESTIGATORS

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ABSTRACT

The terrestrial ecosystem is the major agroecosystem in India and is predominately controlled by human activities for maximizing the food production. There are heavy inputs of chemicals including fertilizers and pesticides applied to this system for optimal agricultural production and organophospate (OP) chemicals occupies a predominant position. Many studies have clearly indicated that terrestrial organisms are severely affected by pesticide exposure. The present study tried to identify some biomarkers of organophosphate toxicity, investigating the oxidative stress pathway utilizing the earthworm *Eisenia fetida* as a model organism. Earthworms provide key soil functions that favour many positive ecosystem services and occupies 80 % of the invertebrate biomass in the terrestrial system. The present study evaluated the response of *E. fetida* against eight organophosphate pesticides; acephate, chlorpyriphos, dichlorvos, dimethoate, malathion, monocrotophos, quinalphos and triazophos. *E. fetida* showed avoidance to the pesticide contaminated soils in varying degrees. The transcript analysis of five major genes involved in the oxidative stress pathway – glutathione peroxidase (GPx), glutathione-s-transferase (GST), superoxide dismutase (SOD), catalase (CAT) and HSP-70 - showed upregulation in a dose dependant manner indicating their involvement in the OP toxicity. The evaluation of the connected biochemical events, viz, the increased lipid peroxidation, depletion of reduced glutathione and total protein confirms the involvement of oxidative stress pathway. The results are in tandem with the inhibition of classical OP biomarker acetylcholine esterase. The study identified that the evaluation of major macromolecules of oxidative stress pathway – reduced glutathione along with the alteration in enzymes GPx, GST, CAT and SOD - could function as biomarker of organaophosphate toxicity in terrestrial organisms.

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INTRODUCTION

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1. INTRODUCTION

1.1. Biomarkers

Biomarkers are considered as primary indicator signals to evaluate events or alterations in a biological system. It is defined as a functional measure of exposure to external stimulus that is characterized at sub-organism level of biological organizations (Adams, 2003). As per the National Research Council of US, biomarkers are indicators of events in biological systems or samples and can be a tool to clarify the relationship, if any, between exposure to a xenobiotic substance and disease (NRC, 1987). It is classified into three categories based on their relation to the exposure-disease continuum: biomarkers of exposure, effect and susceptibility (WHO, 2001). Biomarkers could be used as a sensitive early warning tool for impact assessment of various contaminants on terrestrial ecosystem. Good biomarkers are profound indices of both pollutant bioavailability and early biological responses. The success of biomarkers in evaluating ecological risk assessment depends mainly on the identification of valid biomarkers and establishing the process-level linkages between biomarkers and higher-level responses (Adams, 2003).

1.2. Soil pollution and its indicators

Soils are unconsolidated mineral or organic material on the surface of the earth that serves as a natural medium for the growth of land plants. Management of soil fertility and functioning of tropical ecosystem depends upon soil fauna; consumers and decomposers of soil ecosystem (Hendrix, 2000). A healthy soil web is necessary for sustainable use of soils. There are many organisms that make up the soil web, which includes bacteria, protozoans, nematodes, arthropods and earthworms. Soil pollution is widely increasing since the last decade due to misuse of insecticides and fertilizers in agriculture and atmospheric deposition. Hence, measurement of biomarkers in invertebrates helps us to understand the ecological impact of chemical contamination and conditions of biological restoration of damaged ecosystems (Ayesha and Irshad, 2013).

Among soil invertebrates, earthworms take a major role in maintaining the soil health. Earthworms not only consume every soil particle, they also leave behind their “castings” which is rich in organic matter and acts as a natural fertilizer (Willie and Martina, 2009). They form the greatest proportion of biomass among terrestrial invertebrates and play a vital role in structuring and increasing the nutrient content of the soil. Earthworms can bio-transform pesticides and other pollutants in the soil and clean the environment for re-development. Its body

functions as a bio-filter, they can detoxify waste water (Seema et al., 2013). Thus they can be bio-indicators of chemical contamination in the soil by providing warning signs (Yasmin and D'Souza, 2007). The use of earthworms as bio-indicators in toxicity is because they ingest large quantities of litter, manure and other organic matter in soil, and the organisms tend to indicate the presence of contaminants, if any. The behaviour and morphology of earthworms are in tune with aqueous and solid phases of soil. Several studies have reported that the skin of the earthworm is a common route of contaminant uptake and its investigation can be used in ecological risk assessment (Lord et al., 1980). The biological uptake of most organic contaminants occurs via passive diffusion across the cell membrane, the effect of these contaminants is characterized by necrosis (Campbell, 1995). Earthworms and other aquatic organisms are among the few non-target organisms that are adversely affected due to continuous exposure to chemicals released from industries and agriculture. Such exposures have decreased soil fertility and affected the richness of water (Ravi, 2011).

1.3. Soil pollution by pesticides

Pesticides are toxic substances which is non-biodegradable in the environment that persist for a very long period after its application and are also involved in certain processes like degradation, hydrolysis, photolysis and oxidation of the ecosystem (Ormad et al., 1997; Ariaz-Estevez et al., 2008). Pesticides are widely used in agriculture to protect crops from pest and other diseases. The use of these chemical agents may give quick increased yield, but long-term use may affect both biotic and abiotic factors. The industrial mining and agricultural activities lead to the release of various chemicals including pesticides and heavy metals, resulting in contamination of soil and water. In India, pesticide consumption has increased drastically from 1950s, covering 30 % of the cropped area. A large amount of the applied pesticides reaches the soil where soil building processes and nutrient cycling is accomplished, pesticides in the soil can affect the process of nutrient cycling in the ecosystem. When processes like nutrient cycling is hindered by the use of pesticides or other modes of pollution, it will lead to decline in soil fertility and productivity.

Pesticide use began in India during 1948 when DDT was used for the control of malaria and BHC. The production plant for DDT and BHC was set up in the year 1952. By the year 1958 India was producing about 5000 metric tonnes of pesticides. Currently there are about 292 registered pesticides put into use, and the production has increased to 85,000 metric tonnes. Even though the consumption pattern of pesticides in India is low i.e., 0.50 kg/ha, compared to Korea (6.60 kg/ha) and Japan (12.00 kg/ha), there is an extensive contamination

of environment and food commodities with pesticides, which is mainly due to non-judicious usage.

In India, contamination of food commodities with pesticide residues is as high as 51% and among that 20 % of the pesticide residues are above the maximum residue levels (Gupta, 2004; Agnihotri, 1999). Many health effects such as immune-suppression, hormone disruption, cancer and reproductive abnormalities are linked with prolonged and low exposure of pesticides. In worldwide consumption of pesticides, 45% is in Europe, 24% in US and 25% rest of World this amounts to almost 2 million tonnes per year (Gupta, 2004).

Among the pest control chemicals, insecticides dominate the industry with 65 per cent of consumption, followed by herbicides (16%), fungicides (15%) and others (4%). This pattern is different from global pattern where herbicides form the major share (44%), followed by fungicides (27%), insecticides (22%) and others (7%). In India, maximum use is of insecticides (61 %), then fungicides (19 %), and herbicides (17 %). Among this 54 % are used in cotton, 17 % in rice and 13 % in other fruits and vegetables. During the green revolution and post revolution periods, the consumption was at the peak (Indira Devi, 2017). In Kerala, the pesticide consumption during 1995-96 to 2007-08 was estimated to be 462.05 metric tonnes (Indira Devi, 2010). It was reported that only 1 % of the applied pesticides reaches the pest and the remaining 99 % reaches the ecosystem and affects the non-target organisms (Gavrilescu, 2005). The greater use of pesticides for high agricultural production has led to increased pollution of environmental compartments- soil, water and air. The characteristics of pesticides such as high lipophilicity, bioaccumulation, long half-life and potential of long-range transport increases the chances of contaminating air, water and soil, constantly even after many years of application.

Among different pesticides in use, organophosphates (OP) are considered to be one of the most successful chemical agents. Organophosphates contain carbon which is derived from acid containing phosphorus. The lethality of OPs arises due to their neurotoxicity, by the way of inhibiting enzyme cholinesterase in the nervous system. The neurotransmission is stimulated when acetylcholine is broken down by cholinesterase. During OP pesticide exposure, these OPs bind with cholinesterase and inhibit the enzyme from degrading acetylcholine. This results in accumulation of acetylcholine in synapse and causes a state of over stimulation, which is followed by paralysis of synaptic transmission (Grob & Harvey, 1953; Nambat et al., 1971). The phosphoryl moiety of OPs reacts with serine hydroxyl group of cholinesterases (ChEs) to form a stable complex. At this stage, the phosphorylated enzymes can undergo three main pathways: spontaneous reactivation, oxime-induced reactivation or ageing. Spontaneous

reactivation occurs in presence of water, but it is a slow process, the enzyme does not recover its full normal activity (Thompson & Richardson, 2004). OPs can enter the body through any route: orally, via inhalation or by absorption through skin. Lowered levels of plasma cholinesterase are considered to be the most sensitive indicator of OP exposure.

1.4. Earthworms as soil pollution indicators

Earthworm avoidance test was one of the methods developed for finding out the favourable environment of survival of terrestrial organisms. The acute earthworm avoidance test was first developed in 1996 (Yeardley et al., 1996). International Standards Organization (ISO) had further established it as a test for soil functions and developed as a method for rapid screening and evaluation of soil function and influence of contaminants and chemicals on earthworm behaviour (ISO, 2008). These tests were accepted across the globe for screening of contaminated soil and soil functions (Environment Canada, 2004; Schaefer, 2004; Yeardley et al., 1996). Earthworms were opted as test-organisms in these tests because they are common in a wide range of soils, representing 60-80 % of the total soil animal/invertebrate biomass. In the present study, the experiment was conducted with soils from the grids identified with presence of pesticides and earthworms have shown considerable avoidance to these soils compared to control soils.

1.4.1. Pollution induced metabolic changes in earthworms

Earthworms when exposed to xenobiotics (pesticides, other pollutants) cause oxidative stress by the production of Reactive Oxygen Species (ROS) i.e. free radicals including superoxide radicals (O_2*-), hydrogen peroxide (H_2O_2), single molecular oxygen [O^-] that are able to cause oxidative damage to lipids, proteins, nucleic acids and disturb normal function of cells. At stress conditions the levels of ROS get increased. This stress causes changes in structure and function of important cellular components like DNA and protein due to enhanced lipid peroxidation in mitochondria, which further leads to the production of cytochrome C, which in turn causes apoptosis, or DNA damage leading to cell death (Gonzalez et al., 2005).

1.4.2. Stress induced enzyme changes as biomarkers

To overcome oxidative stress, cells possess certain antioxidant defences and ROS enzymes such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH). GSH plays a vital role in the maintenance of intercellular redox and antioxidant enzyme functions; it acts as reducing agent and plays a vital role in detoxification. Depletion of GSH may reduce ability of cells to reduce

free radicals and ROS; therefore, its depletion is considered as an important biomarker of oxidative stress (Almar et al., 1998; Pena-Llopsis et al., 2001). SOD is an enzyme which acts against oxidative stress by catalysing the dismutation of superoxide anion radicals (O^{2*-}) to hydrogen peroxide (H_2O_2) and oxygen. CAT also acts by degrading H_2O_2 to water and oxygen, thereby protecting the organism from harmful effects of ROS (Scandalios, 2005). These antioxidant enzymes have been considered as good biomarkers of pesticide mediated oxidative stress to reflect the magnitude responses in various populations exposed to xenobiotics. Gene expression analysis could be helpful in protein level detection as the mRNA levels depict a snapshot of cell activity. However, there is a dearth of information of OP pesticide response on antioxidant enzymes and transcriptional regulation of antioxidant and stress responsive genes with respect to earthworms. One of the characteristic features of OPs is its inhibition to the enzyme acetylcholinesterase (AChE). The inhibition of this enzyme affects neurotransmission and is reported to cause muscular discoordination (Gupta and Sundararaman, 1991). AChE may serve as a valuable tool to detect OP exposure and in investigation of relationship between OP and oximes. This enzyme could be a valuable biomarker to assess environmental contamination by such pesticides.

1.5. Aim of the present study

The present study aims to understand various toxicological mechanisms such as oxidative stress, pesticide induced stress and resistance in terrestrial invertebrates specifically on earthworms to delineate early biomarkers of OP toxicity.

The objectives of the study are

I. Study the biochemical toxicity of organophosphate pesticides in terrestrial invertebrates
II. Analyse the response of oxidative stress parameters at gene expression level.
III. Comparative analysis of acetylcholinestrase and toxicity parameters

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REVIEW OF LITERATURE

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2. REVIEW OF LITERATURE

2.1. Relevance of biomarkers

The presence of a chemical in the environment is always a risk for living organisms. However, to establish its adverse effects, there is a need to detect toxicity to the living organisms and prove intoxication with clinical manifestations. The relationship between toxic levels within the organism and toxic response depends on several factors, namely toxicokinetic and genetic factors. One of the methods to quantify the exposure to xenobiotics and its potential impact on living organisms, including human being, is monitoring by the use of biomarkers.

The National Academy of Sciences, USA defined biomarker or biological marker as a xenobiotically induced alteration in cellular or biochemical components or processes, structures, or functions that is measurable in a biological system or sample (ENTOX/TIWET, 1996). Biomarkers/ biological markers can also be defined as physiological signals that reflect exposure, early cellular response or inherent or acquired susceptibilities, which provide a new strategy for resolving some toxicological problems (Silbergeld and Davis, 1994).

The advantages of biological monitoring over environmental monitoring are; measurement of internal dose of a compound, individual differences in absorption, bioavailability, excretion, molecular changes, etc. Additionally, individual changes, as consequence of certain pathophysiological variations happening in a definite period of time, should also be considered. The organism functions as an integrator of exposure and several physiological factors, which modulate the uptake and metabolism of xenobiotic. Thus, we may state that a collective cannot be assimilated as a homogeneous group of individuals exposed to a xenobiotic of physicochemical properties under reproducible and standard conditions.

A balanced biological monitoring is only possible when adequate toxicological information has been assembled on the mechanism of action and on the toxicokinetic of xenobiotics including absorption, distribution, metabolism and excretion (Lauwerys, 1991). An ideal biomarker is expected to have following characteristics; a) sample collection and analysis are simple and reliable; b) the biomarker is specific for a particular type of exposure; c) the biomarker only reflects a subclinical and reversible change; d) relevant intervention or other preventive effort can be considered; and e) use of the biomarker is regarded as ethically acceptable (Grandjean et al., 1994).

Biomarkers are generally classified into three groups: exposure, effects, and susceptibility (Silbergeld and Davis, 1994; Repetto, 1997). Response or effect of biomarkers is indicative of biochemical changes within an organism as a result of xenobiotic exposure. The ideal biomarker should be early detected and be able to show adverse effects before they are irreversible. Those are the most studied biomarkers and they include modifications in some parameters of blood composition, alterations of specific enzyme activities, DNA-adducts appearance, localised mRNA and protein increases, and appearance of specific antibodies (auto antiboides) against a xenobiotic or a particular cellular fraction (Repetto, 1997)

The major factors in delineating biomarkers are; (a) It is necessary to investigate the toxicodynamics (i.e., mechanism of toxicity at the target site) of chemicals to develop new, sensitive, and reliable biomarkers; (b) Biomarkers should be examined under field conditions to validate them as early warning indicators of negative ecological consequences; (c) Biomarker responses must be linked to adverse effects on life cycle traits (cocoon production rate or changes in body weight) under laboratory bioassays: and (d) It is necessary to assess the impact of environmental factors (e.g., temperature, pH, osmotic stress, organic matter content, or photoperiod) and biological variables (e.g., reproductive cycle, nutritional status) on the biomarkers (van Gestel and Weeks, 2004). Biomarkers have also been applied in standardized toxicity tests using earthworms. Most of these studies have tried to link biomarker responses to adverse effects on life cycle traits. Ideally, the biomarker should show a concentration - dependent response to pollutants, particularly under stable experimental conditions.

2.2. Earthworms as bioindicators

Earthworms are important members in the agro-ecosystem because of their beneficial contribution to soil structure and function. Despite this, laboratory and field studies involving biomarkers for assessing pesticide impact on earthworms are still scarce in comparison to other organisms (Scott-Fordsm and Weeks, 2000). Organophosphorus (OP) and carbamates (CB), commonly named anticholinesterase (anti-ChE) pesticides, are important groups of agrochemicals widely used in modern agriculture.

Pesticides are toxic compounds used by humans that have effect on soil and aquatic systems. Pesticides have been used since centuries to control pests. When these pesticides enter the soil and water ecosystems it affects the non-target organisms. Several studies indicate the biochemical assays of acetyl cholinestrase and butyl cholinestrase as biomarkers of organophosphate exposure. Pesticide

exposure can produce an increased amount of reactive oxygen species in organisms. Biochemical evaluation of superoxide dismutase, catalase, glutathione-S-transferase, glutathione peroxidase and lipid peroxidation can serve as effective biomarkers for contaminants like pesticides and heavy metals.
Earlier studies showed that a considerable number of cholinesterase inhibiting pesticides have been assayed in earthworms but that the potential use of ChEs as biomarkers of pesticide exposure has not been sufficiently explored (Scott-Fordsmand and Weeks, 2000). Correlating molecular biomarkers to behavioural changes, with direct ecological implications, could be one of the impending challenges in earthworm ecotoxicology. For example, the relationship between brain AChE inhibition by organophosphate insecticides and behavioural disturbances in vertebrates were well established (Peakall, 1992; Sanchez-Hernandez 2001; Hill, 2003; Bain et al., 2004). However, no such studies on disturbance of earthworm behaviour by pesticides exist and cannot thus be drawn any conclusions about this well-established relationship.

2.3. Organophosphorus compounds and their mechanism of action

Organophosphorus compounds (OPs) are a class of insecticides, derived from phosphoric and thiophosphoric acids. These classes of compounds consist of organophosphates and also organophosphorodithiolates, organophosphoro-thiolates and organophosphorothionates which contain sulphur as well as phosphorus. On the basis of structural characteristics, they are divided into at least 13 types, including phosphates, phosphonates, phosphinates, phosphorothioates (S=), phosphonothioates (S=), phosphorothioates (S substituted), phosphonothioates (S substituted), phosphorodithioates, phosphorotrithioates and phosphoramidothioates (Gupta, 2006). OPs are the most widely used pesticides worldwide and their metabolites are widespread across different populations (Aprea et al., 2000; Barr et al., 2004; Curl et al., 2003).
OP compounds owe their toxic effect to the inhibition of cholinesterase enzyme activity in the nervous tissue. There are different types of cholinesterases in the human body, which differ in their location in tissues, substrate affinity and physiological function. According to the current status, organophosphorus pesticides are used in the control of weed and pests due to their less persistence. The first organophosphorus insecticide named tetraethyl pyrophosphate was developed and used in 1937, during that time to other OPs named Tabun and Sarin, commonly used as nerve agents were produced and used. Pesticides even at very low concentration are found to affect the basal metabolism. OP poisoning has reported to cause about 2,00,000 deaths annually. These compounds cause

severe nervous and muscular disorders in humans. Agrochemicals play a vital role in decline of population of amphibians and pollinators. Prolonged use of same pesticide without any crop or pesticide rotation for several years leads to failure in control of target organisms. OPs are widely used in agriculture and domestic purpose to control pests. Organophosphorus insecticides have replaced organochlorides due to their rapid breakdown in water and low persistence in the environment. Reports show that OPs interact with various membrane events including neurotransmission, plasma membrane and organelle enzyme activities (Binder et al., 1976: Rosenstock et al., 1990; Schneider, 1975).

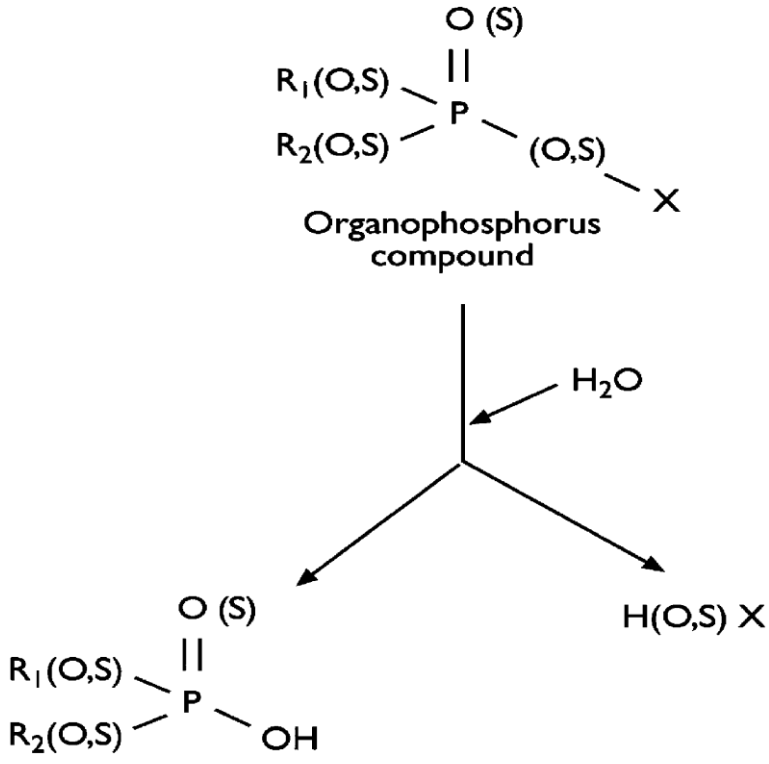
Acetyl cholinesterase (AChE) is required for impulse transmission from the nerve fibers to smooth, skeletal muscle cells, ganglia and central nervous system (CNS). The symptoms of OP poisoning occur immediately after a major portion of tissue enzyme is inactivated by phosphorylation. The enzyme inhibition causes accumulation of acetyl choline at cholinergic neuro-effector junctions (muscarinic effects), skeletal nerve-muscle junctions and autominic ganglia (nicotinic effects). High concentration of acetyl choline at cholinergic nerve junction with smooth muscle and secretory cells causes muscle contraction and secretion. High levels of acetyl choline may be excitatory, causing muscle twitching or depolarising leading to paralysis of the cell. Excess acetyl choline causes sensory and behavioural disturbances, incoordination and depressed motor function in the CNS. The extreme conditions of OP poisoning may lead to pulmonary secretion coupled with respiratory failure. The most common symptoms of OP toxicity include headache, vomiting, nausea, tachycardia, muscle twitching, seizures and depressed RBC acetylcholinesterase or butrylcholinesterase levels. The most common treatment undertaken is the administration of atropine sulphate or glycopyrolate. Several investigations have provided evidence that OP insecticides through lipid interaction interfere with allosteric behaviour of enzymes (Domenech et al., 1977). Acute exposure to OP causes irreversible inhibition of AChE leading to muscarinic and nicotinic effects.

Most OPs are ester or thiol derivatives of phosphoric, phosphonic or phosphoradamic acid. The general formula is represented in Figure - 2.1. R1 and R2 shown in the figure represent the aryl or alkyl group which could be easily attached to phosphorus atom (phosphinates) or by oxygen (phosphates) or by sulphur atom (phosphothioates). At times R1 is directly bounded with phosphorus and R2 with oxygen or sulphur atom. One of these groups is attached with un-, mono- or di-saturated amino groups in phosphormidates. The X-group could be aromatic, alicyclic or heterocyclic group. The X group is called leaving group because on hydrolysis of ester bond, it is released from phosphorus. OPs inhibit the activity of AChE by covalent bonding and thereby

modifying the structure and function. They bind to serine 203 active site of AChE. The leaving group binds to positive hydrogen group of His-447 and breaks off the phosphate, leaving the enzyme phosphorylated. After the organophosphate pesticide gets exposed at the junction, the enzyme-phosphoyrl bond is strengthened by loss of one alkyl group from the phosphoryl adduct. This process is called as ageing. The bond formed is permanent. The time of ageing varies from minutes to days. Depending upon the time some phosphorylated acetyl cholinesterase can be re-phosphorylated or reactivated by a compound oxime. Pralidoxime is the only FDA approved oxime in USA. HI-6 and obidoxime are some of the oximes used in Europe and Asia (de Kort et al., 1988).

Some OPs act by causing damage to the afferent fibers of the peripheral and central nerves along with the inhibition of the enzyme neuropathy target esterase (NTE). Certain OPs being stored in fat tissues cause organophosphate-induced delayed neuropathy (OPIDN). A condition of intermediate poisoning is also being reported along with OPIDN and acute poisoning. It occurs after 24 – 96 hr after exposure. It is characterised by respiratory paralysis, weakness in facial, neck and proximal limb muscles. The pesticides dimethoate, methyl-parathion and fenthion act by causing intermediate syndrome (Bleecker et al., 1993).

Figure-2.1. General formula and pathway of degradation of OP compounds



OPs like phophomidon, dichlrovos is reported to cause oxidative stress *in vitro* and *in vivo* in hepatocytes by malaondialdehyde (MDA) production (Yamano and Mortia, 1992). The most widely used pesticide monocrotophos is found to generate oxidative stress and gene toxicity in rat tissues (Santosh et al., 2010). Dimethoate exhibited enhanced lipid peroxidation levels in brain of rats as it contains large amount of polyunsaturated fatty acids and consumes 20% of body's oxygen (Travacio et al., 2000). Reports shows that dicrotophos are mutagenic to the bacterium *S. typhii* and increased the sister chromatid exchange in chicken hamster ovarian (CHO) cells, thereby causing cytotoxicity and genotoxicity.

During chronic or sub chronic exposure, OP also induces toxicity by oxidative stress. The ROS is produced as a metabolism of cytochrome P450. ROS generation is also caused by increased high energy consumption coupled with inhibition of oxidative phosphorylation and glycogenolysis in the liver and subsequent release of ATP to meet the energy requirement (Ranjbar et al., 2005). Acute organophosphate poisoning leads to three type of paralysis in humans (I) cholinergic crisis in 24h (II) intermediate syndrome and respiratory muscle weakness and (III) delayed neuropathy. Intermediate syndrome may be due to reactive oxygen species that leads to muscle damage (Dandapani et al., 2003).

Carboxylases are the other class of enzymes inhibited by OP's during xenobiotic exposure. It acts by the sulphur atom that rises from desulphuration in phase I of the metabolism and inhibits cytochrome P40 enzymes. Organophosphates also inhibit lipases which play an important role in cell-signalling. Chlorpyriphos inhibit DAG-lipase and indirectly activates ERK kinases that regulate cell proliferation and differentiation (Bomser et al., 2002). OP mediated ROS formation and oxidative stress is also reported to be associated with apoptosis. Malathion inhibits lysyl oxidase in *Xenopus* embryos which alters post translational modification of collagen (Snawder and Chambers, 1993).

Exposure to OPs has been linked to non-Hodgkin lymphoma and various types of leukemia. OPs induced genotoxicity in different ways, including DNA damage in peripheral lymphocytes *in vitro* and *in vivo* in occupational workers (Garaj-Vrhovac and Zeljezic, 2000); chromosomal and sister chromatid exchange (Galloway et al., 1987); induce micronuclei formation in bone marrow (Mathew et al., 1990) and responsible for sperm abnormalities in mice (Mathew et al., 1992). Genotoxicity study of parathion, paraoxon and dimefox in *in vitro* experimental model with HepG2 cells demonstrated OPs induced changes in phosphorylation of kinases, low concentration of paraoxon and parathion are genotoxic, dimefox is a mitogen (Hreljac et al., 2008).

2.4. Earthworms for environmental monitoring

The model species' in soil ecotoxicology are usually chosen from species that are bred in laboratory conditions, easy to maintain and for which molecular tools are available. They need not essentially occur naturally on polluted soils. Considering soil ecotoxicology in oligochaete annelids, model species' are mostly from the genus *Eisenia* mainly *E. fetida* and *E. andrei*, are used in toxicological studies (Sanchez-Hernandez, 2006). Also species from the *Lumbricus* genus are increasingly employed (Morgan et al., 2007). Mainly, *E. fetida* is considered as reference earthworm in international toxicity tests (Nahmani et al., 2007a, b, OECD, 1984). Off late, ecotoxicological investigations have advanced significantly by the development of molecular biology techniques, leading to a better understanding of the mechanisms of contaminant action at molecular level (Brulle et al., 2010).

In addition to the harmful effects caused to humans, the pesticides are harmful to other organisms also. It badly affects the environment leading to loss in biodiversity. Soil biota is one of the majorly affected classes of organisms. Among soil organisms, earthworms occupy the major part as it occupies 70 % of soil biomass. Earthworms participate in number of activities including, soil aggregation, water infiltration and also function as a primary member of food web. Assessment of earthworm's behavioural and biochemical status could be used to evaluate the soil and environmental quality.

A larger proportion (above 80 %) of biomass of terrestrial invertebrates is represented by earthworms which play vital roles in drainage, structuring, aeration and increasing the nutrient content of the soil. Through feeding, burrowing and faecal decomposition, earthworms directly and indirectly effect the microbial communities of the soil (Ali et al., 2006). Since earthworms are in complete contact with the substrate in which they live and consume large volumes of the substrate, these organisms are exposed to chemicals present in the soil environment which affects their growth, fecundity, behaviour and also leads to death.

Because of their favourable effects on soil structure and function, earthworms are considered to be an important component of the soil system (Paoletti, 1999; Jongmans et al., 2003). Their activities contribute to augment water infiltration, soil aeration, and stabilization of soil aggregates. Earthworms also help to increase soil fertility by formation of an organic matter layer in topsoil. These organisms consume huge amounts of soil, or organic matter fractions of soil, hence getting continuously exposed to chemicals or other contaminants through their digestive system (Morgan et al., 2004). In addition, earthworm skin also

plays significant role in contaminant uptake (Saxe et al., 2001; Jager et al., 2003; Vijver et al., 2005).

There are many toxicity testing systems developed based on several earthworm species (e.g., *Eisenia fetida* and *E. andrei*) (OECD, 1984). The key goals of these tests are (i) the assessment of potential toxicity of new chemicals to be introduced into the environment, and (ii) the risk assessment for toxic effects of previously contaminated soils. Alteration in biomass, abundance or species richness of natural populations were considered to be common ecological endpoints to identify the causes of contamination or pollution (Dunger and Voigtländer, 2005; Vandecasteele et al., 2004). The earthworms may accumulate chemical agents which are present in the soil environment (Hillel, 1971; Ali et al., 2002). The tolerance ability of earthworms towards highly metal-contaminated soils and capability to accumulate higher concentrations of heavy metals in their tissues have led to the use of earthworms as sentinel species (Lukkari et al., 2004; Carpené et al., 2006). Since the earthworms play crucial role in microbial decomposition process, they can be bio-indicators of chemical contamination of the soil in terrestrial ecosystems and can function as an early warning system for deterioration in soil quality (Sorour and Larink, 2001; Bustos-Obreg and Goicochea, 2002; Ali et al., 2009).

This feature makes earthworms attractive for monitoring the impact of contaminants in soils. Being a part of food web, earthworms play crucial roles in transferring these contaminants to many invertebrates and thus transmitted to higher tropic levels (Ali et al., 2007). In ecotoxicology of earthworms, a range of biomarkers are existing for toxic compounds, including biomarkers from the molecular to the organism level like metallothioneins, stress proteins, cholinesterases, detoxification enzymes, parameters of oxidative stress and others (Novais et al., 2011).

2.5. Effect of environmental contamination on earthworms

There are many reports on the toxicity effect of environmental contaminants on earthworms. Earthworms in general are highly resistant to many chemical contaminants including heavy metals and organic pollutants in soil and have been reported to bio-accumulate them in their tissues. After the Seveso chemical plant explosion in 1976 in Italy, when vast inhabited area was contaminated with certain chemicals including the extremely toxic TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), several faunas perished but for the earthworms that were alone able to survive. Earthworms which ingested TCDD contaminated soils were shown to bioaccumulate dioxin in their tissues and concentrate it on average 14.5-fold (Satchell, 1983).

A number of biomarkers have been developed in earthworms for effective monitoring of environmental contamination including pesticide and heavy metal contaminations. There are many biomarkers identified for heavy metal toxicity. Studies report ethoxyresorufin-O-deethylase (EROD) and CYP3A4 as biomarkers of heavy metal (Cd, Cu, Pb and Zn) contamination in soil. There was a dose-dependent variation in the activity of these enzymes over heavy metal exposure. Among the four heavy metals cadmium was most potent in inducing the activities of EROD and CYP3A4. The report suggests EROD as a biomarker for heavy metal pollution (Cao et al., 2010). Exposure to heavy metals (copper, zinc, cadmium, lead and nickel) to *E. fetida* affected the growth and development of the organism. Insecticides like polyvalent, polyfor (fungicide) and capsicol was found to be moderately toxic to *E. fetida* and microorganisms (Toualy et al., 2017). Reports suggest that cadmium due to its high bioavailability decreases the soil pH and bio accumulates in earthworms which in turn affects the food web (Ma, 1982). The combined toxicity of lead and cadmium in *E. fetida* increases cellulase activity and DNA damage. The combined toxicological effects may be due to adsorption of both metals in the soil and bioavailability (Wu et al., 2012).

Titanium oxide (TiO_2) by-products caused toxicity in *E. fetida* by increasing the expression of metallothionein and SOD and induction of apoptotic activity. TiO_2 caused decrease in phagocytosis at a concentration of 0.1 mg/l (Bigorgne et al., 2011; Caceres et al., 2011) reported the degradation of fenamiphos in alkaline soils leading to decrease the biomass in *E. fetida*.

It is also reported that *E. fetida* helps in reduction of toxic compounds from the tannery effluent and improves the nutrient availability through vermicomposting (Selladurai et al., 2010). In addition, *E. fetida* and *E. eugeniae* are reported to recycle the industrial wastes - fly ash and vinasse - and found that equal ratio (1:1) of fly ash and vinasse is an optimum concentration to obtain good quality vermicompost (Pramanik and Chung, 2011). The phytotoxicity of olive mill waste waters was reduced by application of *E. fetida* in the presence of oat seedlings. Earthworms decreased the total organic carbon, total nitrogen, dehydrogenase activity and increased humic substances and available nitrogen. The bioaccumulation studies reported that fluroranthene concentration and bioconcentration factor in *E. fetida* intestine was higher than in the epidermis, suggesting earthworm accumulates fluroranthene by ingesting it with soil particles. The activities of SOD, MDA decreased but CAT activity decreased initially and later increased indicating mild oxidative stress (Ma et al., 2013).

Studies in *E. fetida* and *L. terrestris* has shown that cadmium (100mg/kg) caused significant reduction in membrane fragility in *E. fetida.* The metalothionin (Mt) expression was up regulated in both the species of earthworms (Asensio et al.,

2007). The dermal exposure to heavy metals affected the immunocompetent cells in *E.fetida* along with alterations in Ca/K balance. The heavy metals also induced up regulation of stress proteins (HSP 70, HSP 72). There was an up regulation of caspase 3 leading to apoptosis. The uptake of metals by ceolomytes was accompanied by Ca/P and S/P ratios. Ca/P ratio increased within 2h of copper or cadmium exposure but the levels decreased after 3day exposure of lead (Homa et al., 2007). Perchlorate, a chemical used as explosive and as an oxidiser in rocket propellant, persists in soil and water several decades and found to affect the survival, reproduction success in *E. Fetida* (Landrum et al., 2006).

2.6. Effect of chemical agents on the biochemical mechanisms in earthworms

In a recent report, toxicity of 24 pesticides of six chemical categories on *E. fetida* was studied. It was reported that neonicotinoids were extremely toxic to *E. fetida*, pyrethroids were very toxic and insect growth regulators were moderately toxic to *E. fetida.* Antibiotics, carbamates and organophosphate pesticides showed varying degrees of toxicity and were very to extremely toxic (48h LC_{50} values ranged from 3.64 to 75.75µg/cm^{-2}) in *E. fetida* (Wang et al., 2012). It was also reported that, *in vitro* use of chlorpyrifos affects the antioxidant enzymes and lipid peroxidation by the generation of free radicals (Glutekin et al., 2000). The study showed malondialdehyde (MDA) levels increased with increased dose of chlorpyriphos correspondingly super oxide dismutase (SOD) and catalase (CAT) decreased in the erythrocytes.

Changes in the chemical composition of soil affect non-target organisms, particularly earthworms. Exposure to pesticides has shown to affect the growth of earthworms. Earthworms exposed to chlorpyiphos spiked samples showed high degree of conduritol B epioxide (CbE) than Cholinesterase (ChE) irrespective of the tissue used for measuring esterase (Collange et al., 2010; González Vejares et al., 2010). Studies with *L. terrestris* showed the occurrence of multiple CbE isoenzymes with slight variation in substrate specificity in gastrointestinal, reproductive and muscle tissues. Uranium exposed soils causes DNA damage in earthworms after 24h, 7days and 56 days of exposure, which may be due to production and accumulation of free radicals. The fungicides folpet, myclobutanil, metalaxyl and fosetyl-Al and the pesticides chlorpyriphos-ethyl and α-cyhalothrin caused an increase in GST and CAT due to metabolisation of these pesticides causing production of ROS. The cholinesterase activity also inhibited after few days of exposure indicating neurotoxicity in earthworm *A. caliginosa*, addition to bioaccumulation in earthworm tissue even at higher concentration (Schreck et al., 2007). Endosulphan exposed *E. fetida* has shown decrease in weight in a dose dependent manner. In addition to affecting growth, endosulphan was found to affect cocoon production and hatching

successes in *E. fetida* (Sameena and Ayesha, 2011). Studies suggested that caudal regeneration capacity of earthworms was decreased by endosulphan (Kulkarni and Wakale, 2012). Similarly, studies on *C. elegans* have showed cypermethrin induced oxidative stress along with decreased life span and brood size by alternating the permeability of nerve membranes and disturbing the phospholipid orientation. Cleavage of cypermethrin also releases cynohydrins, which are unstable under physiological stress and cause oxidative stress (Shivaiah and Rajini, 2011). GST induced detoxification system has been reported in *Pheretima posthuma* exposed to organochlorine pesticides (Hans et al., 1993). Activities of the antioxidant enzymes SOD, CAT, POD (guaiacol peroxidase), ROS and MDA levels was increased in *E. fetida* due to low doses of the herbicide formasen. The adverse effect of formasen gradually disappeared and restoration of antioxidant enzymes occurred as the exposure time was prolonged (Zhang et al., 2013).

A retardant used in television industry - decabromodiphenyl ether (BDE- 209) is reported to affect soil organisms. Antioxidant responses after 7 days of exposure was studied in BDE-209 exposed earthworm *E. fetida*. The GSH/GSSH ratio was decreased; SOD and CAT activities were increased at 0.1-1 and 5mg/kg of exposure. The levels of MDA and protein carbonyl (PCO) were significantly increased. The study indicates very low levels of BDE induced severe oxidative stress in *E. fetida*. Similarly BDE-209 did not affect the growth, survival and reproduction in earthworms at a concentration of 0.1-1000 mg/kg. However, there was a decrease in number of juveniles hatched per cocoon in artificial soils. The results indicate the tolerance of *E. fetida* towards BDE-209 (Chuan et al., 2013). Adult *E. fetida* when exposed to sub lethal concentration of 1,2,4 trichlorobenzene effect of antioxidant enzyme SOD was stimulated and the activity of AChE was significantly inhibited (Wu et al., 2012). A neonicotinoid insecticide guadipyr is found to cause oxidative stress, inhibition of AChE activity and DNA damage in *E. fetida.* (Wang et al., 2015). The pesticides dimethoate, carbendazim and glyphosate were found to have adverse effects on *E. fetida.* The weight of the earthworms was reduced when exposed to these pesticides in a dose dependent manner. The reproductive output was significantly decreased during pesticide exposure. The findings suggest that carbendazim and dimethoate was more harmful to *E. fetida* when compared to glyphosate (Yasmin and D'Souza, 2007). Exposure to imidacloprid inhibits worm growth, reproduction and increases mortality in *E. fetida* (Wang et al., 2016). Imidacloprid also affected the microorganisms in soil by reducing the dehydrogenase activity.

All these studies clearly indicate that the biochemical mechanisms in vertebrates as well as invertebrates are severely affected by the pesticide exposures. However, based on the parameters evaluated it was noted that there is a huge variation in the extent of response. The present study explored this gap and tried to bring out an early biomarker, investigating the oxidative stress pathway involved in organophosphate toxicity utilizing *E. fetida* as a model organism.

MATERIALS AND METHODS

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3. MATERIALS AND METHODS

3.1. Test organisms

Eisenia fetida was selected as the test species as recommended in standard guidelines (OECD, 1984). The earthworms were collected from a culture at Department of Soil Science, Kerala Agricultural University (Thrissur, Kerala, India) which were maintained at KFRI Campus at an average 22 - 24 ^{O}C in dark containers with layers of dried plants and cow-dung manure. Compost, garden soil and waste from the campus were used as food source. For experimental purposes, earthworms were brought to the Biochemistry laboratory of Division of Forest Ecology and Biodiversity Conservation, KFRI as and when required.

3.2. Chemicals

Protein estimation was carried out using Bio-Rad DC Protein assay kit (Bio-Rad, Hercules, California, USA). Thiobarbituric acid was obtained from SRL Chemicals, Mumbai. Commercially used formulations of eight pesticides viz. chlorpyriphos, quinalphos, dimethoate, triazophos, malathion, acephate, monocrotophos and dichlorovos were obtained locally. All other chemicals were purchased from M/s. Merck India Ltd, until otherwise mentioned.

3.3. Pesticide exposure

Different doses of pesticides i.e., 0, 10, 20, 40, 80 and 100 mg per kilogram of soil were taken for toxicity studies and four replicates for each dose was made. After 24 h, ten earthworms each per replica were placed in control and pesticide applied soils. The exposure time of the experiment was fixed as 24 h. Weight loss and mortality were regularly observed. At the end of time point, specimen samples were processed for biochemical assays. All the experiments and exposures were carried out at least three times.

3.4. Avoidance behaviour assay

The avoidance behaviour test can be applied as a rapid screening test to determine the bioavailability of chemical substances or of contaminants in the soil. The principle of this test is the exposure of the earthworms at the same time to the samples of non-contaminated (control) and contaminated soil. Both samples were placed in the same chamber, in different sections, separated by a divider, forming two compartments. After organizing the samples on each side, the divider was removed, forming a line where sufficient number of adult organisms (with developed clitellum and wet mass between 300 mg and 600 mg) were placed on the surface. After the observation time, the number of organisms in each section of the chamber was verified. The sample is considered to be toxic

(with the habitat function/favourable environment is limited), if more than 80 % of the total of exposed organisms was found in the control soil sample.

3.5. Biochemical assays

3.5.1. Acetyl Cholinesterase assay

Cholinesterase hydrolyzes acetylthiocholine iodide into thiocholine and acetate. Thiocholine reacts with dithiodinitrobenzoic acid (DTNB) to form thionitrobenzoic acid that can be measured spectrophotometrically at 406 nm. Reagents required are ; Phosphate Buffer: 0.05 M pH- 7.9 made by adding Sodium phosphate (dibasic), Potassium phosphate (monobasic); 5-5′ dithiobis-(2 nitrobenzoic acid) (DTNB): 2.5 $x10^4$ M in phosphate buffer p^H 7.9; Acetylthiocholine iodide : 45 mg in 5 ml distilled water (freshly prepared); Triton-X-100: 1%v/v. Assay was carried out with 200 mg of tissue. Sample is weighed and homogenized in ice cold condition using 800 µl of Triton-Buffer solution. Samples were centrifuged at 15,500 rpm for 45 minutes. Supernatant was collected and used for the assay. Supernatant was kept in ice cold condition until use. Assay was carried out with 3 ml of DTNB/buffer solution dispensed into a cuvette and 20 µl of tissue homogenate was added. The spectrophotometer was set to auto zero at 406 nm. 100 µl of the substrate was added and mixed well. The change in absorbance was recorded for one minute after the addition of substrate.

$$\text{Cholinesterase activity} = \frac{\Delta A/\text{min} \times 3.12\ \text{mL} \times 1000}{\frac{13.33\text{cm}}{\mu\text{l}} \times 1\ \text{cm} \times .02\ \text{mL} \times 200\ \text{mg} \times 0.8\text{ml}} = \mu\text{mol/min/g}$$

3.5.2. Estimation of reduced glutathione (GSH)

The reaction of GSH with Ellman's reagent (5, 5'-dithiobis-2-nitrobenzoic acid [DTNB]) gives rise to a product that can be quantified spectrophotometrically at 412 nm. This reaction is used to measure the reduction of GSSG to GSH. Reagents used were; Phosphate Buffer: 0.2M p^H 8: Made by adding Sodium phosphate (dibasic), Sodium phosphate (monobasic); 5-5′ dithio bis- (2 nitrobenzoic acid) (DTNB): 0.6 mM in phosphate buffer pH- 8; Standard GSH: 10 mg in 10 ml phosphate buffer (freshly prepared) and TCA: 5 %. Sample was prepared with 50 mg of tissue sample and homogenized in ice cold using 1 ml of phosphate buffer. The protein was precipitated by adding 250 µl TCA to 500 µl homogenized tissue sample to precipitate the protein. The rest of the sample is stored for protein estimation. The homogenized sample is centrifuged at 10,000 rpm for 10 minutes at 4° C. Supernatant was separated and 0.1 ml was used for the assay. The sample was added with 2 ml of DTNB solution and incubated for 10 min. The colour developed was measured at 412 nm. Standard GSH

concentration ranging between 10-100 µg/µl was prepared and standard graph was created. The results were expressed as mg of GSH/g protein.

3.5.3. Estimation of lipid peroxidation

Lipid peroxidation was carried out in earthworm tissue samples. Malondialdehyde (MDA) occurs in lipid peroxidation and was measured in tissues after incubation at 95^0C with thiobarbituric acid in aerobic conditions (pH-3.4). The pink colour produced by this reaction was measured spectrophotometrically at 532 nm to measure the MDA levels (Ohkawa et al., 1979)

3.5.4. Estimation of total protein

Protein estimation was carried out in earthworm tissue samples using modified Lowry's method using (Bio-Rad) DC protein assay kit (Bio-Rad, Hercules, California, USA) with BSA as standard. The blue colour produced by proteins with copper tartarate and Folin's reagent measured calorimetrically at 750 nm (Bradford, 1976).

3.6. Gene expression studies

The gene expression patterns of four free radical (ROS) scavenging enzymes such as Glutathione Peroxidase (GPX), Glutathione s-transferase (GST), Catalase (CAT), Superoxide dismutase (SOD) as well as Heat shock protein (HSP-70) were quantified in eight organophosphate pesticide (viz.,chlorpyriphos, quinalphos, dimethoate, triazophos, malathion, acephate, monocrotophos and dichlorovos) exposed *E. fetida* specimens (Three doses : 2 mg, 8 mg and 20 mg) to identify a possible biomarker gene. The total RNA from the exposed as well as non-treated earthworm (control) tissues was isolated using RNeasy mini kit (Qiagen, USA) according to the manufacturer's instructions. The quality of the isolated RNA was analyzed in 1% agarose gel and quantity by measuring the absorbance at 260/280 nm (NanoDrop 1000, Thermo Scientific, USA). The total mRNA was used as the template for cDNA synthesis using first strand cDNA synthesis kit (Thermo Fischer, USA). The concentration and purity of the synthesized cDNA was determined by measuring the absorbance at 260/280 nm. The synthesized cDNA was used as the template for the Reverse Trascriptase PCR. Forward and reverse primer sequences for the ROS genes Glutatione Peroxidase (GPx), Glutathione-S-transferase (GST), Catalase (CAT), Superoxide Dismutase (SOD) and Heat Shock Protein - 70 (HSP-70) were designed using PRIMER EXPRESS - 3.0.1 software (Applied Biosystems, USA) (Table - 1). The 18S rRNA gene which is already reported as a house keeping gene and internal standard in *E. fetida* was used as the endogenous control in the present experiment (Gong et al., 2012).

Sl. No	Gene Name	Forward Primer	Reverse Primer
1.	SOD	5'-CCGGGCCGAATTCAATC 3'	5'-GATCGTCCACCAGCTCATGTAC 3'
2.	CAT	5'-ACGCCGACGGAGAAGCT 3'	5'-TGCCTTGGTTGGTCTTGTGA 3'
3.	GPx	5'-TTCGCGGACTGGTAACAAAAC 3'	5'-CGATCAGCCTCTTCTGGTGAA 3'
4.	GST	5'-CCATCTTTGATCTGCTGGACAA 3'	5'-AGCCCATCCAGGGTGTGA 3'
4.	HSP 70	5'-CGCCAACGGCATCATGA 3'	5'-TCGCGACCAGTGCTCTTG 3'
6.	18SrRNA	5′-TTGATTACGTCCCTGCCCTTTG 3′	5′-GGTCCAATCCGAGGATCTCACTA 3′

Table - 1. Primers for RT-qPCR

Gene expression study using SYBR Green RT-qPCR was carried out in a Step One Real-Time PCR System (Applied Bio Systems, CA) based on the changes in fluorescence relative to the cyclic increase in the PCR products. The dye used was the SYBR Green, which emits fluorescence while binding to a double stranded DNA. The fluorescence value was recorded at the threshold cycle (CT). Prior to quantification, primer concentrations and other parameters were optimized. Real time PCR was carried out in 20 µl reaction volume containing 10 µl Power SYBR Green PCR Master Mix 2X (Invitrogen, USA) with the internal reference dye Rox, 1 µl forward and reverse primers (10 pmol), 2 µl cDNA and 6 µl sterile Millipore water. A thermal cycling programme with an initial denaturation at 95 ^{0}C for 10 min, 40 cycles of 95 ^{0}C for 15 s and 60 s at 60 ^{0}C, was used. The accuracy of the reactions was analyzed using melt curve analysis at the end of PCR programme. The final relative quantification was done based on the comparative CT method of $2^{\Delta\Delta}$CT as explained (Livak and Schmittgen, 2001).

The fold change in gene expression patterns in the tissues of pesticide exposed and non-exposed organisms were statistically analyzed through analysis of variance using SPSS version - 17 (SPSS Inc., Chicago, Illinois, U.S.A).

3.7. Histopathological analysis

The tissues for histological analysis were washed and cleaned in phosphate buffered saline (PBS) and stored in Bouin's solution till slide preparation. The slide preparation was outsourced and carried out at M/s. Thrissur Path Centre, Thrissur. The analysis was done in Leica microscope using Leica microsystems software at KFRI.

3.8. Statistical analysis

The data was expressed as mean ± SE from four replicates per treatment. Data was analyzed by one-way ANOVA followed by Dunnet's test for comparison between control and treatment groups. The level of significance was set at $P \leq 0.001$. Data of all the results in this study was obtained from at least three independent experiments in a similar pattern.

RESULTS

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4. RESULTS

The presence of xenobiotics in the environment always poses a risk for living organisms. The extent of risk depends on the toxicity and the damage of intoxication is related to specific organ alterations and clinical symptoms. Earthworms perform many essential and beneficial functions in soil ecosystems, and their ability to perform these functions can be inhibited upon exposure to harmful substances. Earthworms are useful model organisms because many aspects of their response to environmental contaminations can be assessed and connected to environmental outcomes, including their avoidance behaviour, growth rate, gene expression levels, enzyme activity levels, reproduction patterns and mortality. One of the methods to quantify the exposure to environmental contaminants and its potential impact on living organisms, is the monitoring the impact by the use of biomarkers. The present study has coupled different levels – molecular and biochemical - of parameters in order to define an efficient biomarker for OP toxicity.

4.1. Avoidance behaviour assay

All the studied eight organophosphate pesticides were tested for identifying the avoidance behaviour pattern of *E. fetida*. The pesticides include, acephate, chlorpyriphos, dichlorvos, dimethoate, malathion, monocrotophos, quinalphos and triazophos.

In acephate, the avoidance behaviour was significant from 4 mg/kg dose. Thereafter an increase in the response was noted (Figure - 4.1A). In chlorpyriphos, the response was significant from 1 mg/kg, however, at 2 mg/kg dose, the response was not statistically significant (Figure - 4.1B). In both the cases, a dose dependent increase in the avoidance response was shown by earthworms and more than 80 % avoidance was noted in the highest dose of 20 mg/kg. In dichlorvos exposure, a dose dependent response was observed and significant avoidance was recorded from 2 mg/kg exposure and maximum response was at 20 mg/kg (Figure - 4.2A). The response was entirely different in dimethoate exposure as the earthworms had shown an attraction in the first dose (1 mg/kg), however significant avoidance was noted from 2 mg/kg and a dose dependent increase was seen thereafter (Figure - 4.2B). In Malathion and monocrotophos exposures, the earthworms displayed a similar pattern of dose dependent avoidance behaviour. In malathion, significant response was detected from 2 mg/kg exposure and upto 80 % of the organisms had shown the avoidance in the highest dose of 20 mg/kg (Figure - 4.3A). In case of monocrotophos exposure, statistically significant avoidance response was evident from 4 mg/kg and further a dose dependent effect was noticed (Figure -

4.3B). As similar to dimethoate, attraction in the initial doses (1mg/kg), and statistically significant avoidance from 8 mg/kg exposure onwards were recorded for quinalphos (Figure - 4.4A). Triazophos had shown significant avoidance response from 4 mg/kg exposure and a dose dependent response was seen till the highest dose of 20 mg/kg (Figure - 4.4B).

4.2. Gene expression analysis - Reverse transcriptase PCR (RT-PCR) for characterization of free radical (ROS) scavenging enzymes

The relative fold level increase in the antioxidant gene expressions of free radical scavenging enzymes such as glutathione peroxidase (GPx), glutathione –S– transferase (GST), superoxide dismutase (SOD), catalase (CAT) as well as Heat Shock Protein (HSP 70) in *E. fetida* during four different doses (0, 2, 8 & 20 mg/kg) for eight organophosphate pesticides (acephate, chlorpyriphos, dichlorvos, dimethoate, malathion, monocrotophos, quinalphos and triazophos) exposures are shown in Figure - 4.5 to 4.8 respectively.

In the case of acephate exposure, an upregulation in the gene expression levels of GPx, SOD and CAT were seen in all the three doses of exposure at varying degrees. More than 12-fold increase in expression was resulted in case of GPx, however the other two genes had shown a statistically significant increase in the gene expression, upto 4-fold. In case of GST only the highest dose of exposure (20 mg/kg) made a significant change. The expression levels of HSP-70 gene had shown an upregulation in 2 & 20 mg/kg exposures, while the increase was not significant at 8 mg/kg exposure (Figure – 4.5A). In chlorpyriphos exposure, an upregulation in the gene expression levels of GPx, and HSP-70 were seen for all the exposures in a dose dependent manner. The GST gene expression level, was upregualted in 2 & 8 mg/kg exposure however was not significant in 20 mg/kg exposure. The catalase expression levels were found to be significant in 8 and 20 mg/kg exposure. No significant change in the expression levels of SOD was observed in any of the experimental doses of chlorpyriphos (Figure – 4.5B).

The dichlorvos exposure (2, 8 and 20 mg/kg) significantly upregulated all the five gene expression levels irrespective of exposure doses. Up to 19 fold increase was noted in GPx levels. Even though the response was varying, all the genes were showing significant responses (Figure – 4.6A).

In dimethoate exposure, the expression levels of GPx, GST and HSP-70 were down-regulated significantly compared to control. The highest down-regulation was noted in the lowest dose of 2 mg/kg in case of all three genes and an increased expression was noted with increasing dose. However, SOD and

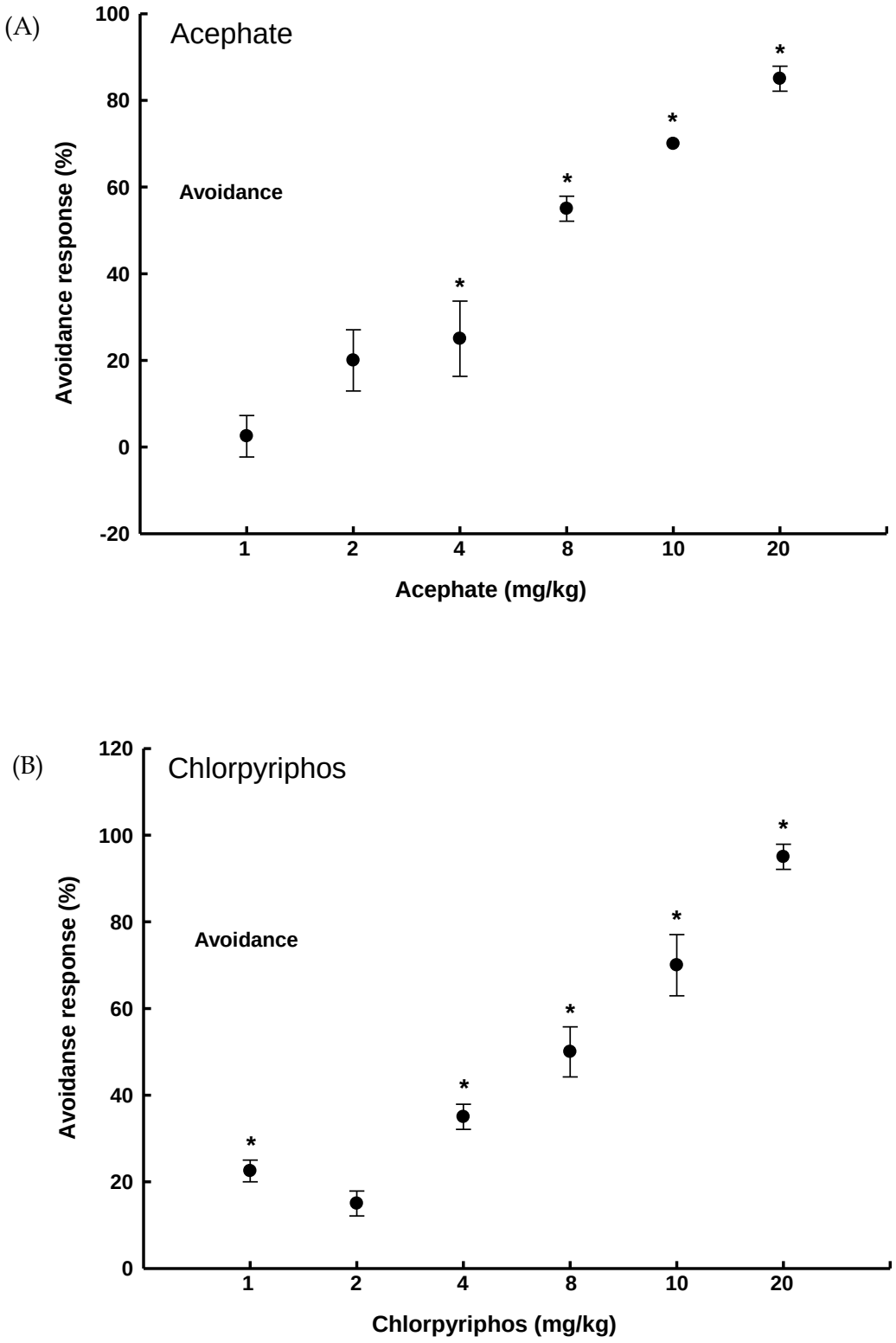
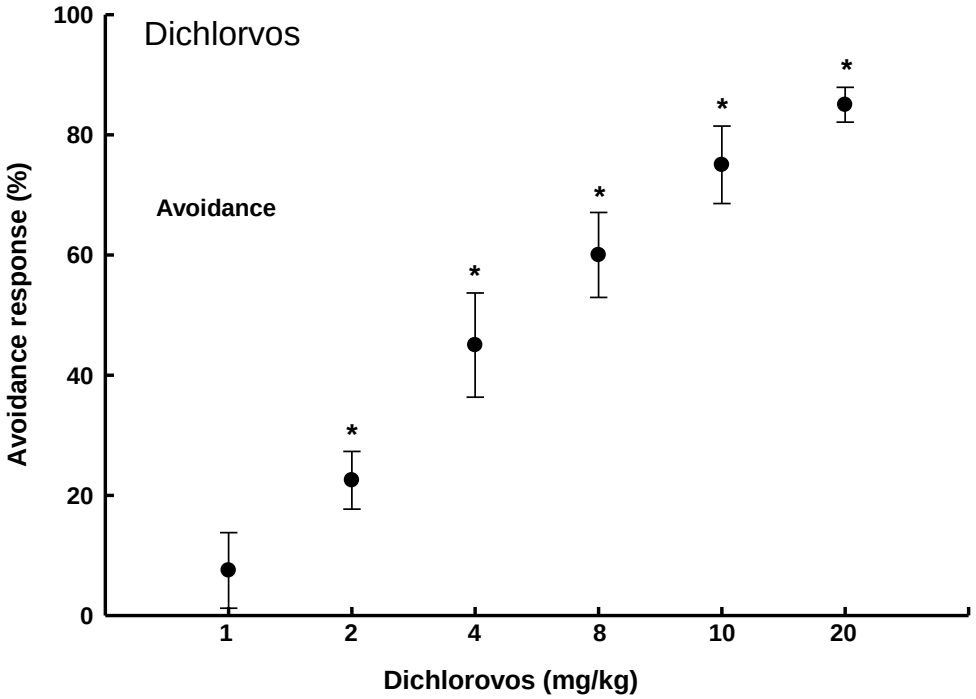


Figure – 4.1 : Dose dependent (0, 1, 2, 4, 8, 10 and 20 mg/kg) avoidance behaviour of *E. fetida* in (A) acephate and (B) chlorpyriphos exposed soils. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.

(A)



(B)

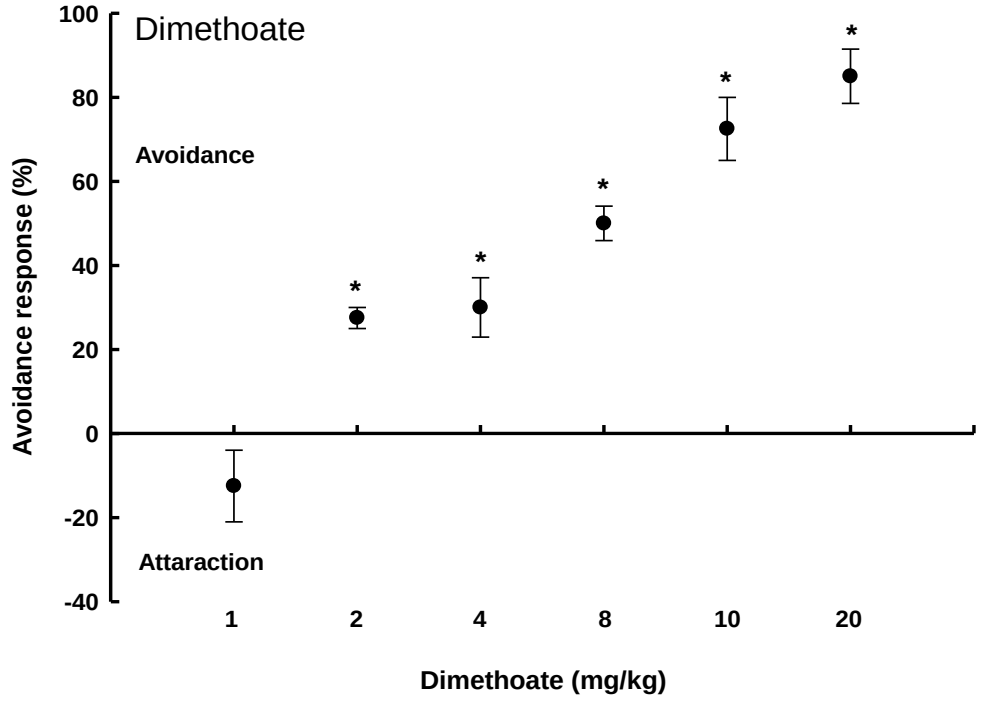


Figure – 4.2 : Dose dependent (0, 1, 2, 4, 8, 10 and 20 mg/kg) avoidance behaviour of *E. fetida* in (A) dichlorovos and (B) dimethoate exposed soils. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.

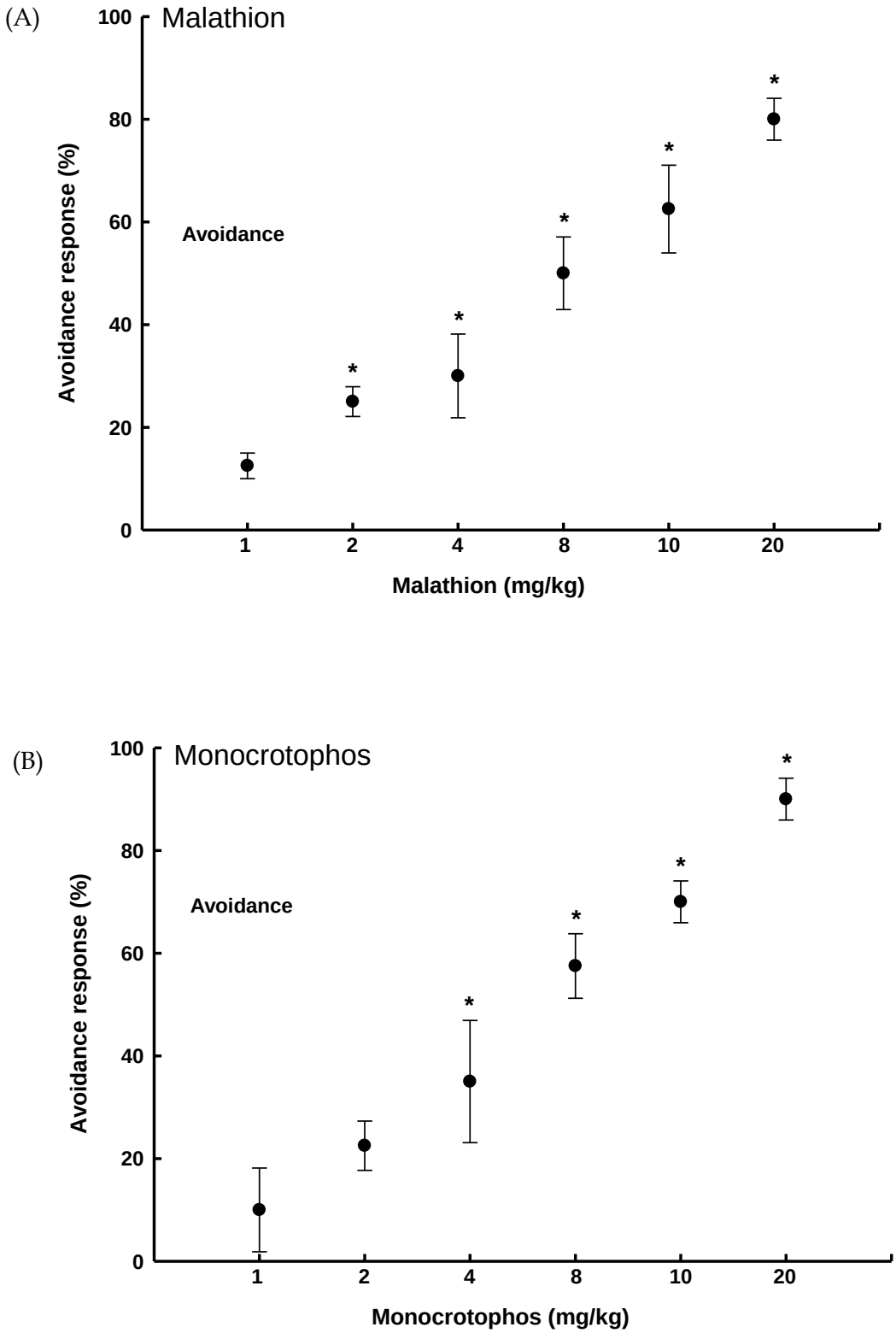
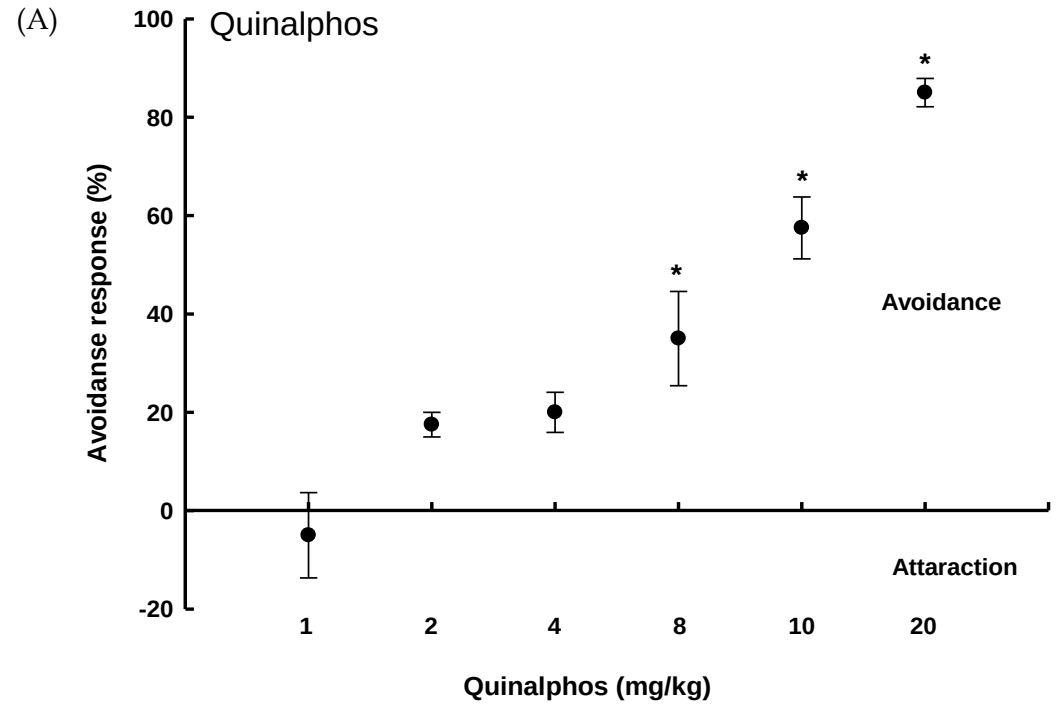


Figure – 4.3 : Dose dependent (0, 1, 2, 4, 8, 10 and 20 mg/kg) avoidance behaviour of *E. fetida* in (A) malathion and (B) monocrotophos exposed soils. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.



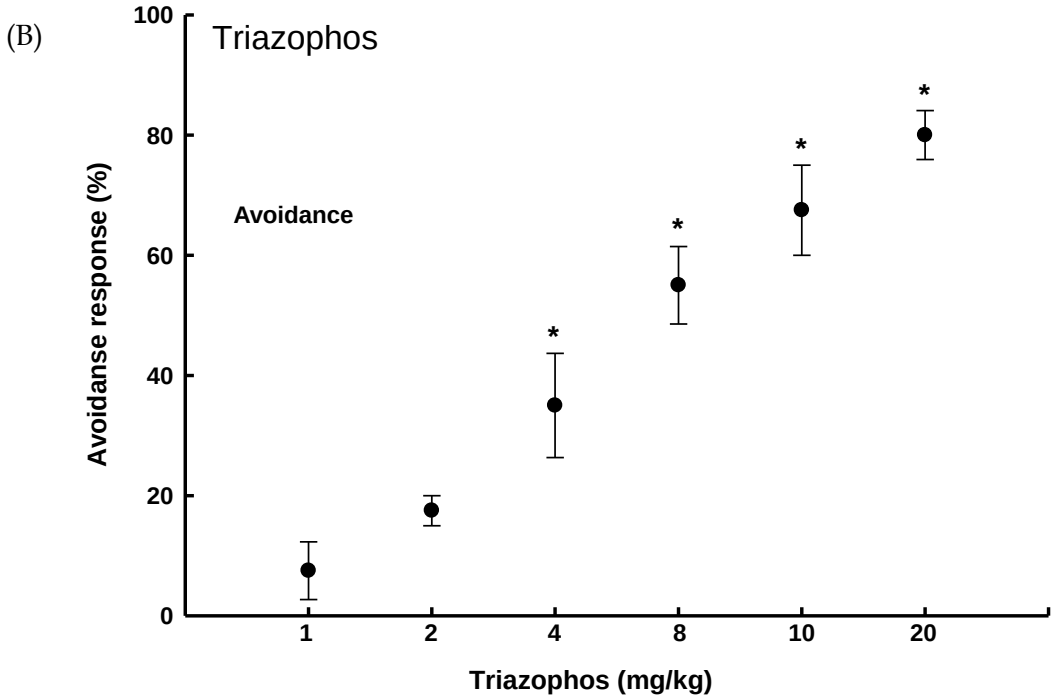


Figure – 4.4 : Dose dependent (0, 1, 2, 4, 8, 10 and 20 mg/kg) avoidance behaviour of *E. fetida* in (A) quinalphos and (B) triazophos exposed soils. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.

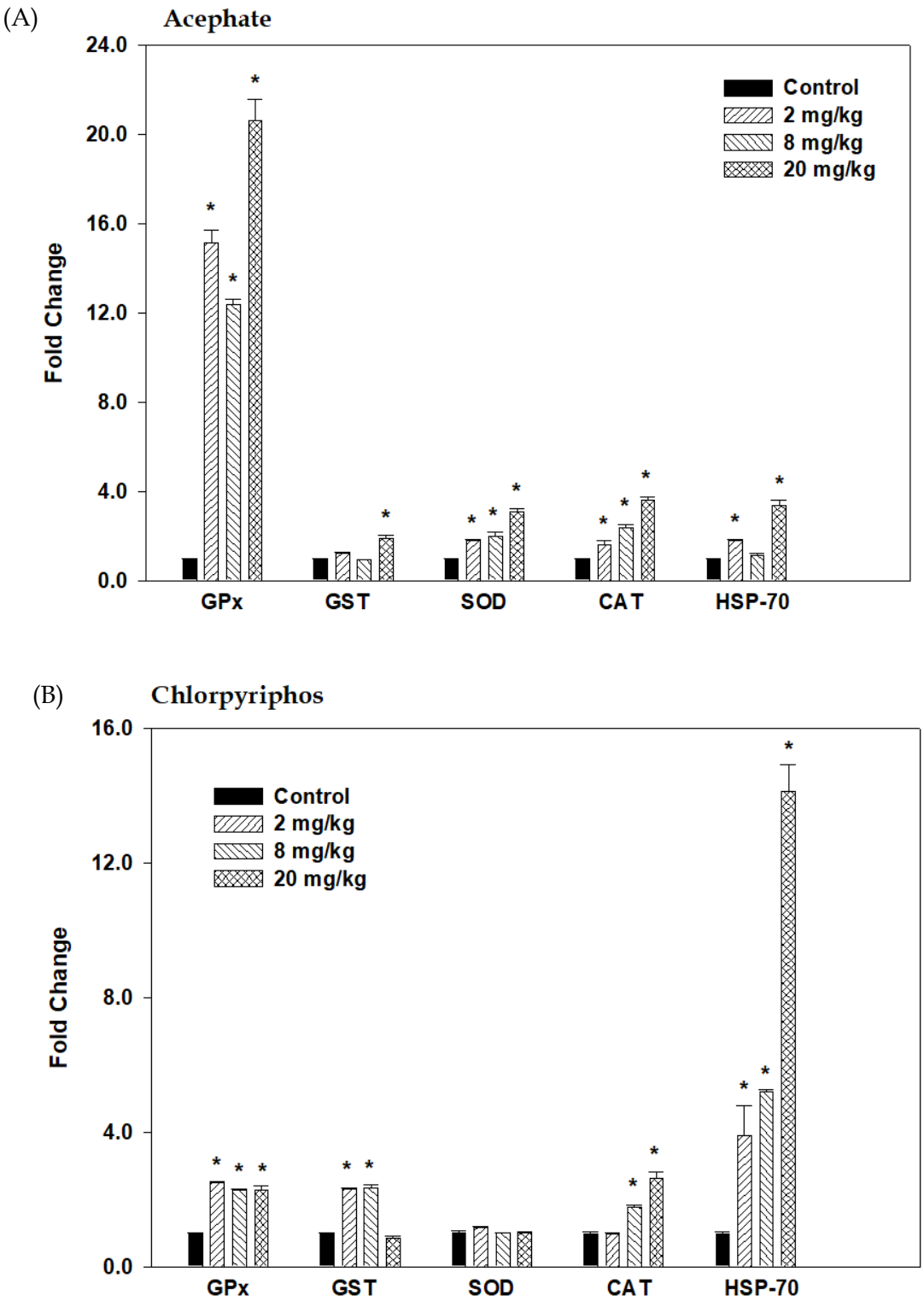


Figure – 4.5 :Dose dependent (0, 2, 8 & 20 mg/kg) effect of (A) acephate and (B) chlorpytiphos on the gene expression levels of oxidative stress related genes (GPx-glutathione peroxidase, GST – glutathione –s-transferase, SOD – super oxide dismutase, CAT – catalase and HSP-70 – heat shock protein – 70) in the test organism *Eisenia fetida*. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.

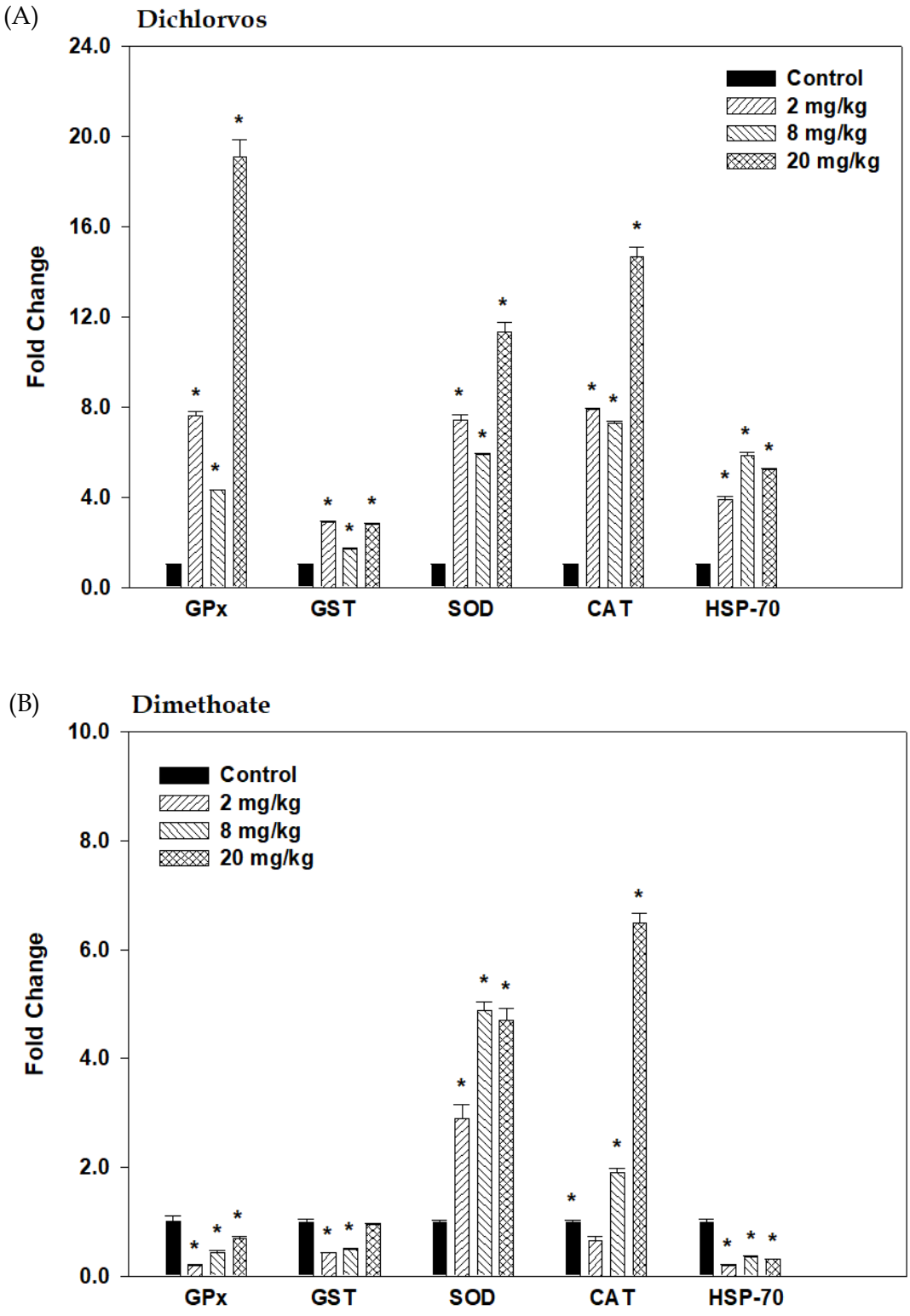


Figure – 4.6 :Dose dependent (0, 2, 8 & 20 mg/kg) effect of (A) dichlorvos and (B) dimethoate on the gene expression levels of oxidative stress related genes (GPx-glutathione peroxidase, GST – glutathione –s-transferase, SOD – super oxide dismutase, CAT – catalase and HSP-70 – heat shock protein – 70) in the test organism *Eisenia fetida*. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.

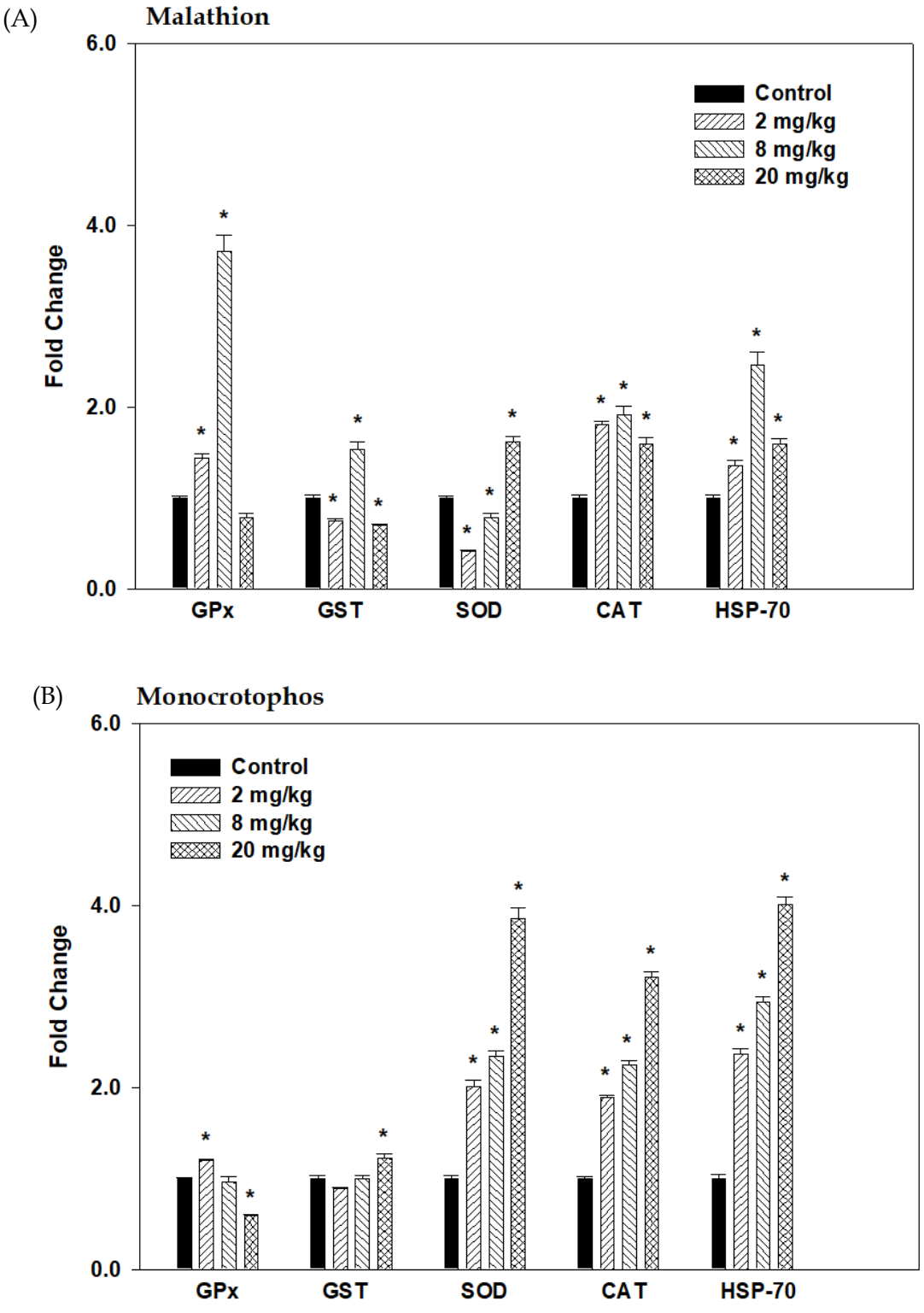


Figure – 4.7 : Dose dependent (0, 2, 8 & 20 mg/kg) effect of (A) malathion and (B) monocrotophos on the gene expression levels of oxidative stress related genes (GPx-glutathione peroxidase, GST – glutathione –s-transferase, SOD – super oxide dismutase, CAT – catalase and HSP-70 – heat shock protein – 70) in the test organism *Eisenia fetida*. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.

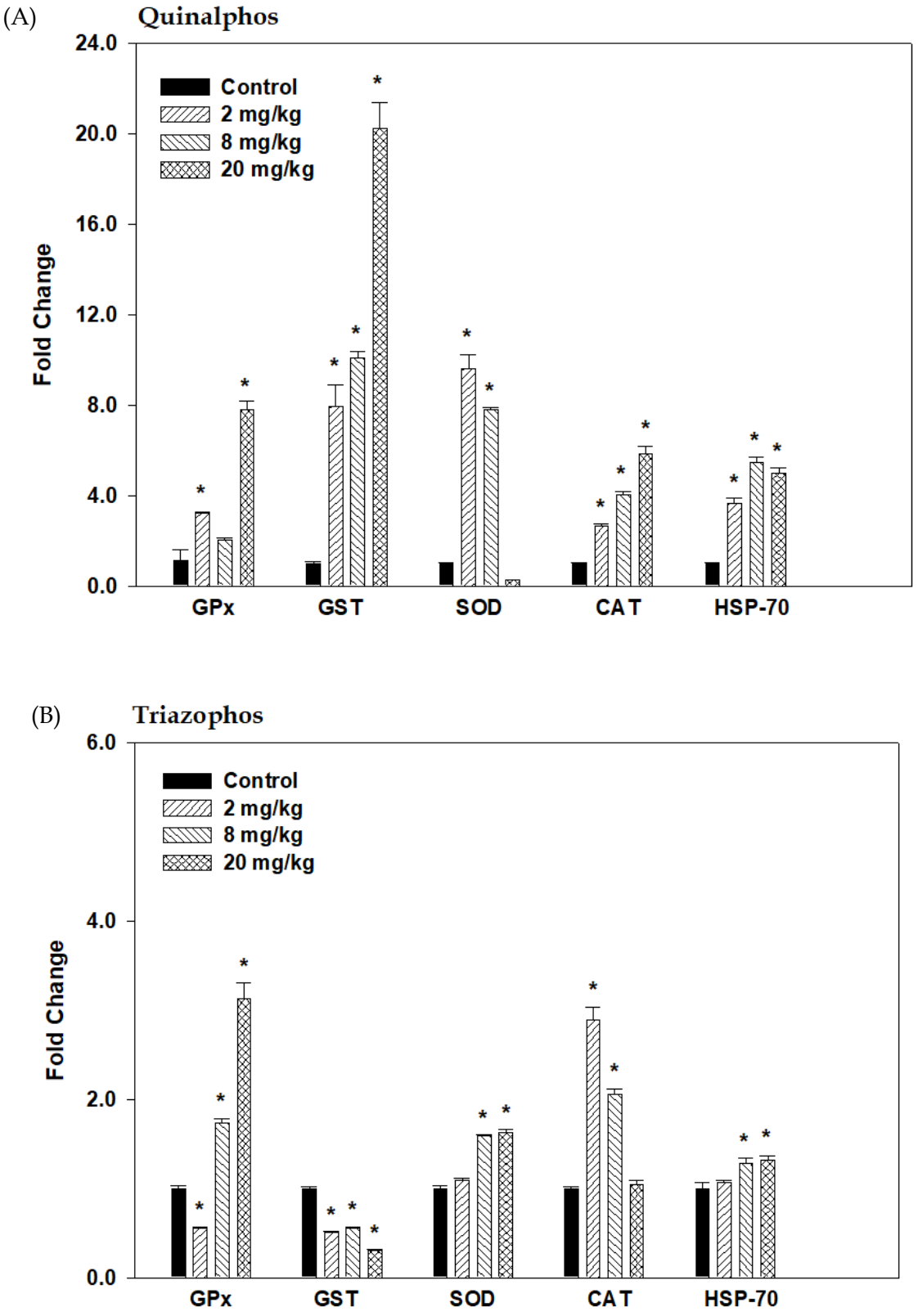


Figure – 4.8 :Dose dependent (0, 2, 8 & 20 mg/kg) effect of (A) quinalphos and (B) triazophos on the gene expression levels of oxidative stress related genes (GPx-glutathione peroxidase, GST – glutathione –s-transferase, SOD – super oxide dismutase, CAT – catalase and HSP-70 – heat shock protein – 70) in the test organism *Eisenia fetida*. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.

catalase had shown significant increase in expression levels with increase in dose (Figure - 4.6B).

Malathion induced an upregulation of gene expression in catalase and HSP-70 in all doses of exposure, however no dose dependent pattern was noted. The GPx levels were found to be significantly upregulated in initial doses of exposure and a down regulation was seen in 20 mg/kg exposure even though it was not significant. GST levels were found to be down-regulated in 2 and 20 mg/kg however had shown an upregulation in 8 mg/kg exposures. SOD levels were downregulated in 2 and 8 mg/kg doses and up-regulated significantly in 20 mg/kg exposure (Figure - 4.7A). Under various exposures of monocrotophos, a significant dose dependent increase in gene expression of SOD, catalase & HSP-70 was evident. However, GPx and GST had shown varying degrees of responses (Figure - 4.7B). In the case of quinalphos exposure (Figure – 4.8A), an increasing trend in gene expression pattern with a corresponding increase in the pesticide concentration was observed except for SOD which showed a reverse trend. Under different exposures of triazophos, an upregulation of the antioxidant genes like GPx, SOD and HSP while down regulation of GST and CAT genes was revealed (Figure – 4.8B).

4.3. Biochemical analysis

The different biochemical variables assessed for finding suitable biomarker for OP toxicity includes, lipid peroxidation, reduced glutathione, acetylcholine esterase and total protein during exposure. The indices were assessed in a dose dependent manner and the responses were recorded, which is being compared with avoidance behaviour responses and expression pattern of oxidative stress related genes.

4.3.1. Total Protein

There was a decrease in the total protein content in the pesticide exposed tissue in varying concentration after an exposure period of 24 hrs (4.9A, 4.10A, 4.11A, 4.12A, 4.13A, 4.14A, 4.15A & 4.16A). Significant decrease in total protein content in all pesticide exposed organisms from the lowest dose (1 mg/kg) was revealed in all the experimental groups except quinalphos and triazophos exposed organisms (4.15A & 4.16A). The depletion levels were noted mainly in a dose dependent manner with increasing doses of exposure. Triazophos showed maximum depletion in the highest dose with 49 mg/g tissue, which is much less compared to the values of other experimental groups of same dose. The decrease in total protein indicates lowered metabolism and increased utilization of stored energy resources during pesticide exposures.

4.3.2. Reduced glutathione (GSH)

The evaluation of reduced glutathione in pesticide exposed organisms, revealed the role of glutathione redox mechanism in detoxification of these harmful chemicals. The reduced glutathione was quantitatively analysed in the test organism *E. fetida* after exposure to acephate, chlorpyriphos, dichlorovos, dimethoate, malathion, monocrotophos, quinalphos and triazophos at different doses for 24 hrs. The results were represented in figures 4.9B, 4.10B, 4.11B, 4.12B, 4.13B, 4.14B, 4.15B & 4.16B respectively. The results indicated the crucial role of glutathione in the detoxification of these chemicals. A dose dependent decrease in the reduced glutathione was seen in all the pesticides except in dichlorvos exposure (4.11B). Significant depletion of glutathione was visible in the lowest dose of exposure 1 mg/kg in acephate, dichlorvos, malathion and monocrotophos. In dichlorvos exposure, significant depletion was observed in 1 mg/kg while the depletion in other doses of exposures, was insignificant . The decrease in GSH levels clearly indicated the involvement of glutathione pathway in the detoxification process of these chemical agents in the organisms.

4.3.3. Acetylcholinesterase assay

Acetylcholinesterase (AChE) inhibition is one of the classical characteristic features of organophosphate poisoning. AChE inhibition was studied in *E. fetida* after 24 hr exposure of eight different OP pesticides. All OPs showed dose-dependent decrease in AChE activity (4.9C, 4.10C, 4.11C, 4.12C, 4.13C, 4.14C, 4.15C & 4.16C). In acephate, dichlorvos and malathion the inhibition was seen from the lowest doses of exposure 1 mg/kg (4.9C, 4.11C & 4.12C). This inhibition may be due to accumulation of acetylcholine in the synaptic terminals, which may lead in alteration of nerve impulse transmission.

4.3.4. Lipid peroxidation (LPx)

Lipid peroxidation levels indicated the amount of free radicals formed after a xenobiotic exposure. The earthworms exposed to eight organophosphates for 24 hr were analysed for malondialdehyde (MDA) levels. The results of the present study indicated that exposure of OP pesticides causes' oxidative stress as shown by increased levels of malondialdehyde (MDA) (4.9D, 4.10D, 4.11D, 4.12D, 4.13D, 4.14D, 4.15D & 4.16D). During acephate exposure, significant lipid peroxidation was evident from 2 mg /kg onwardstill the highest dose. Similar trend was noted in dichlorvos, dimethoate, malathion, monocrotophos, quinalphos and triazophos. Irrespective of the exposed pesticides, there was an increase in MDA levels in pesticide exposed samples, however chlorpyriphos exposure showed a different trend. The exposure of 1 mg/kg chlorpyriphos itself resulted in increased LPx levels; however, there was a decrease in LPx levels at 2 mg/kg and

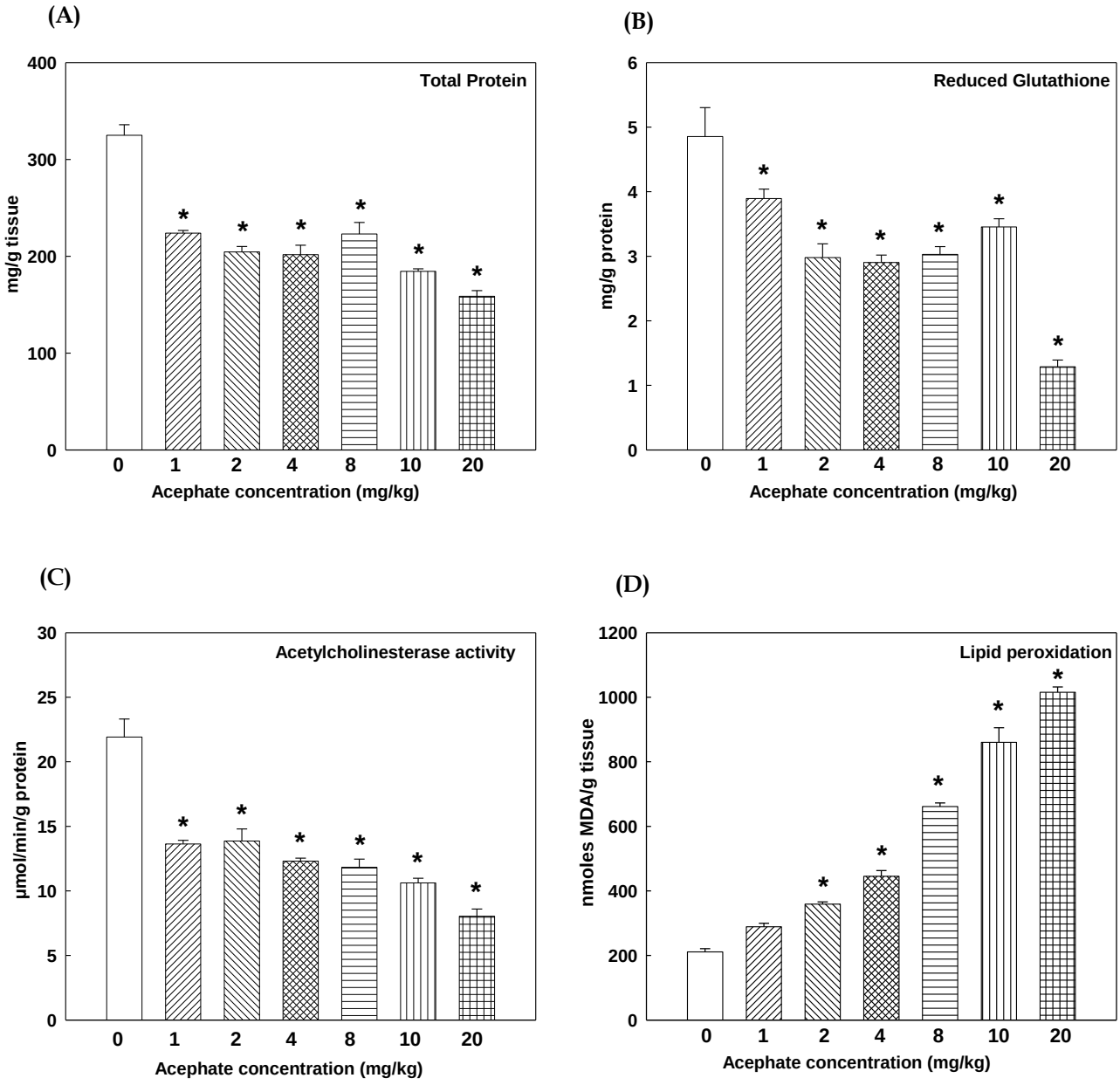


Figure – 4.9 : Dose dependent effect of acephate (0, 1, 2, 4, 8, 10 & 20 mg/kg) on the different biochemical variables in the test organism *Eisenia fetida*. (A) total protein, (B) reduced gluthathione (GSH), (C) acetylcholine esterase activity and (D) lipid peroxidation. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.

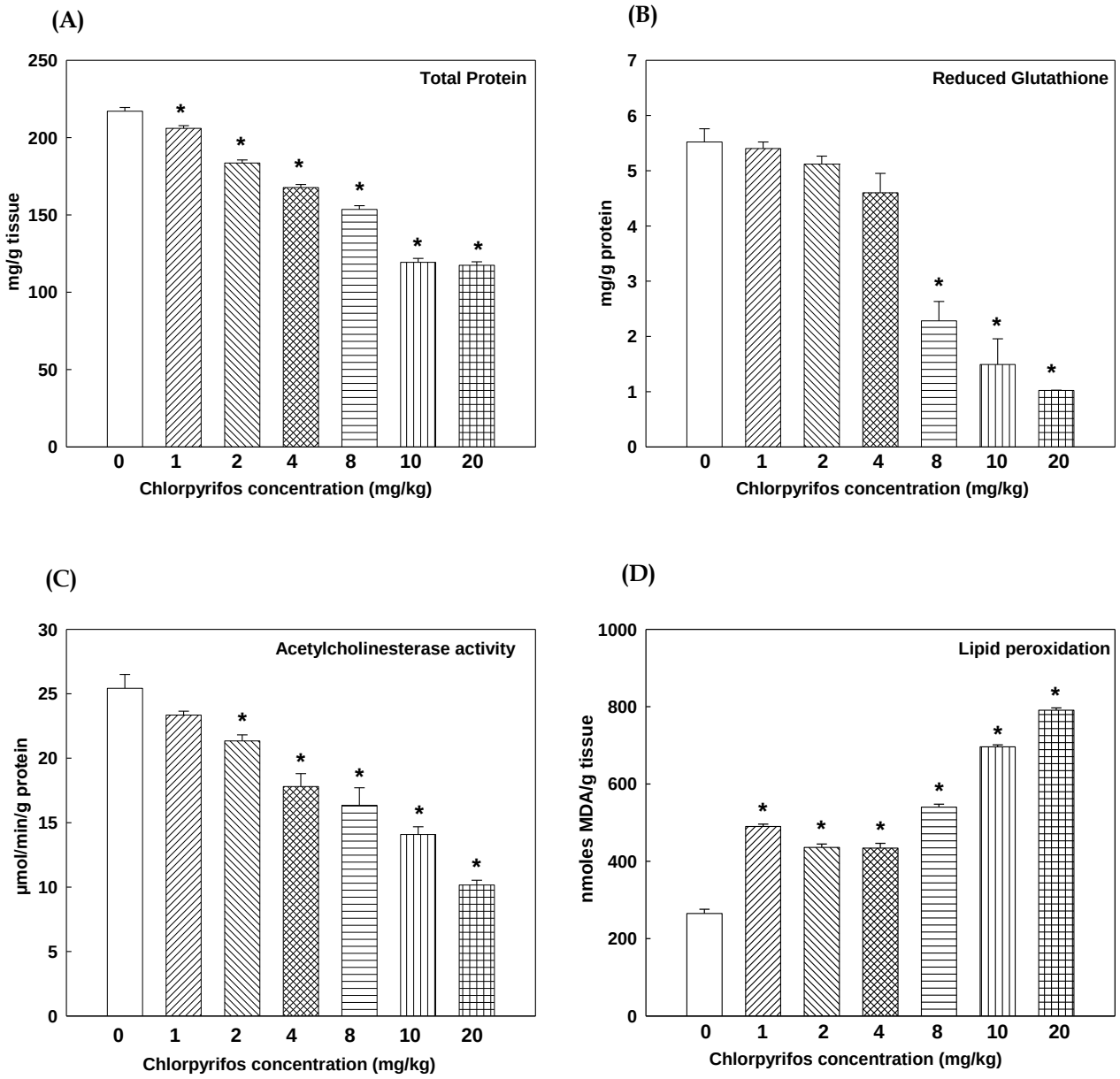


Figure – 4.10 : Dose dependent effect of chlorpyrifos (0, 1, 2, 4, 8, 10 & 20 mg/kg) on the different biochemical variables in the test organism *Eisenia fetida*. (a) total protein, (b) reduced gluthathione, (c) acetylcholine esterase activity and (d) lipid peroxidation. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.

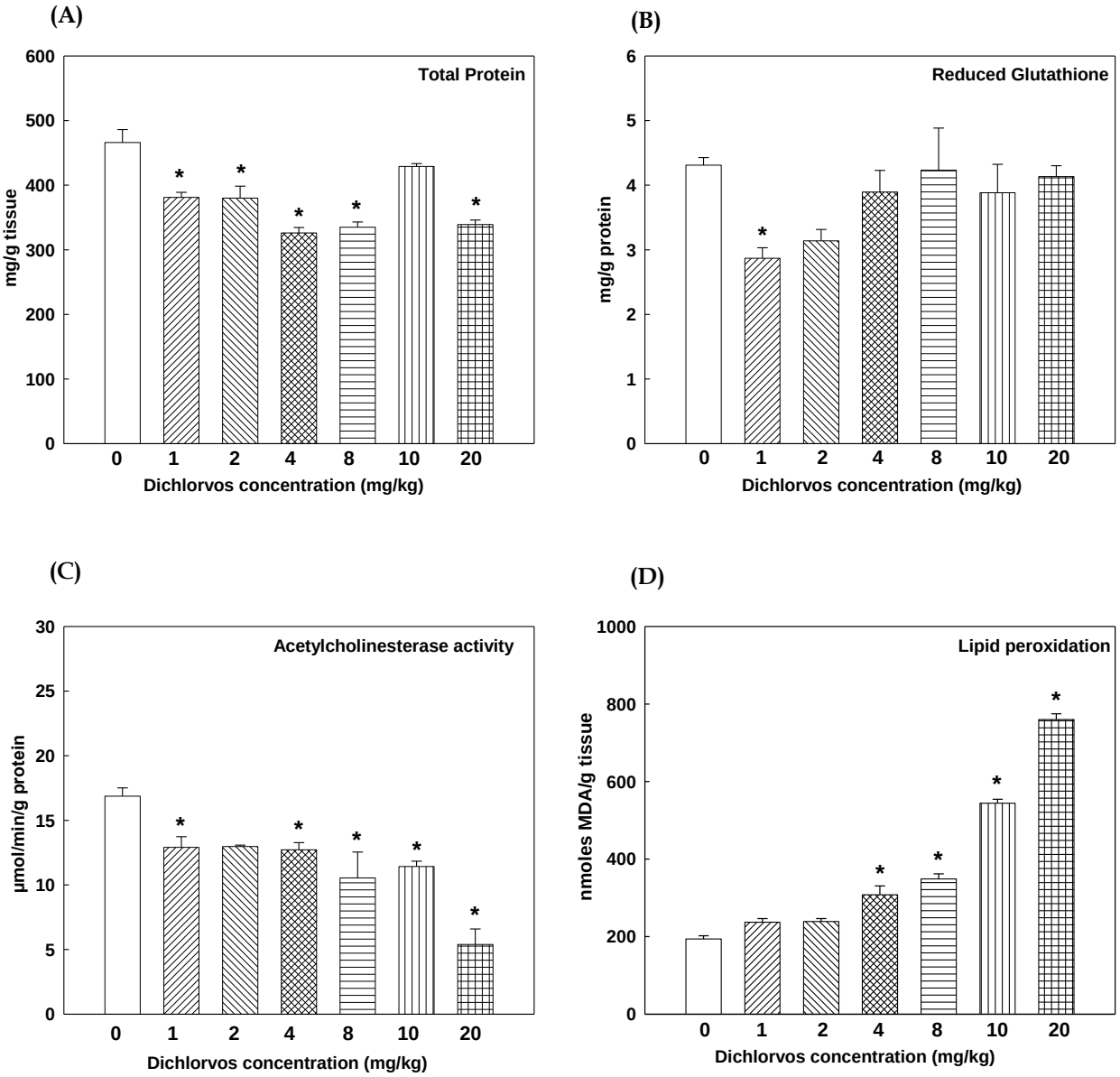


Figure – 4.11 : Dose dependent effect of dichlorvos (0, 1, 2, 4, 8, 10 & 20 mg/kg) on the different biochemical variables in the test organism *Eisenia fetida*. (A) total protein, (B) reduced gluthathione (GSH), (C) acetylcholine esterase activity and (D) lipid peroxidation. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.

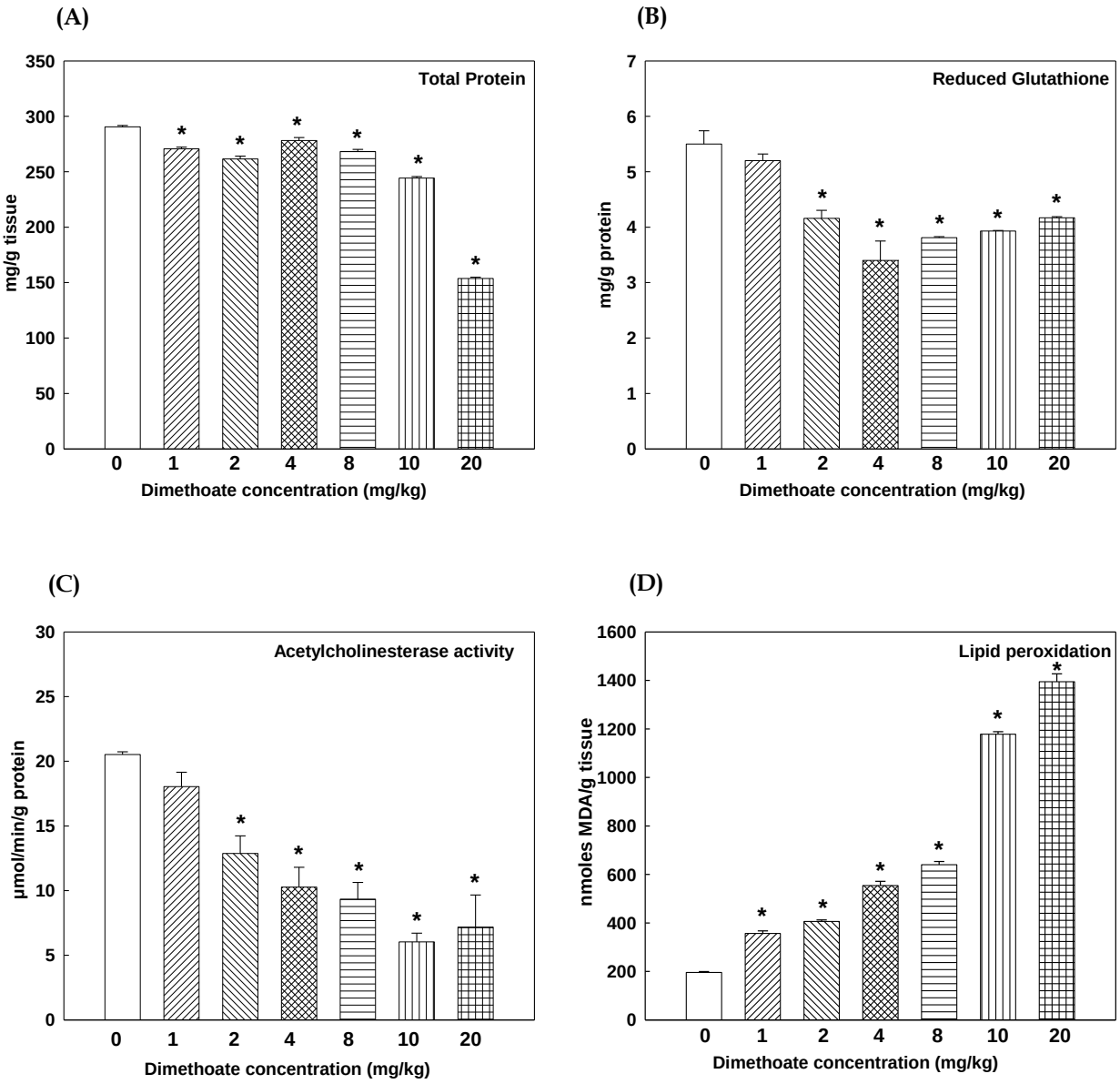


Figure – 4. 12 : Dose dependent effect of dimethoate (0, 1, 2, 4, 8, 10 & 20 mg/kg) on the different biochemical variables in the test organism *Eisenia fetida*. (A) total protein, (B) reduced gluthathione (GSH), (C) acetylcholine esterase activity and (D) lipid peroxidation. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.

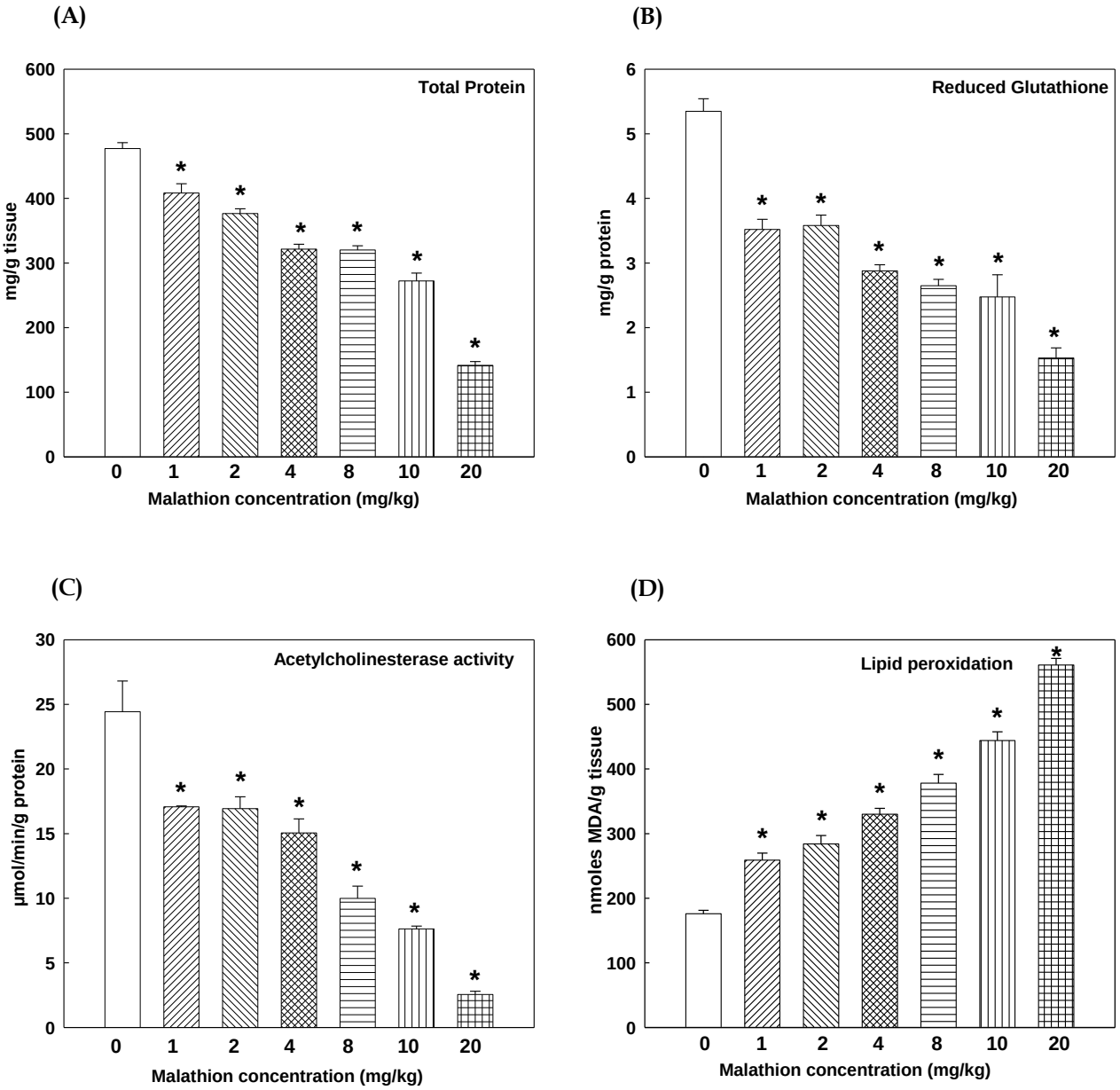


Figure – 4.13 : Dose dependent effect of malathion (0, 1, 2, 4, 8, 10 & 20 mg/kg) on the different biochemical variables in the test organism *Eisenia fetida*. (A) total protein, (B) reduced gluthathione (GSH), (C) acetylcholine esterase activity and (D) lipid peroxidation. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.

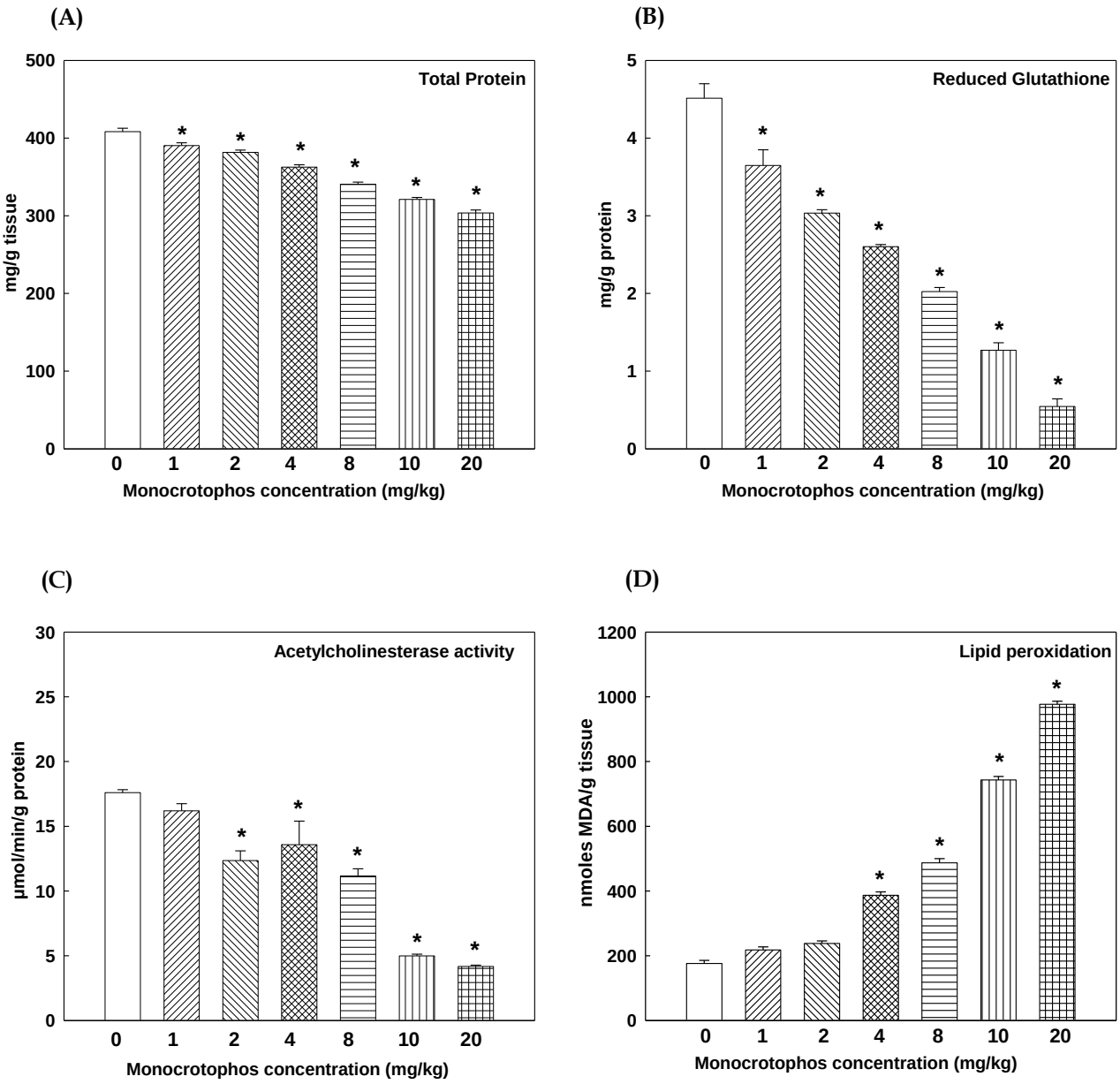


Figure – 4.14 : Dose dependent effect of monocrotophos (0, 1, 2, 4, 8, 10 & 20 mg/kg) on the different biochemical variables in the test organism *Eisenia fetida*. (A) total protein, (B) reduced gluthathione (GSH), (C) acetylcholine esterase activity and (D) lipid peroxidation. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.

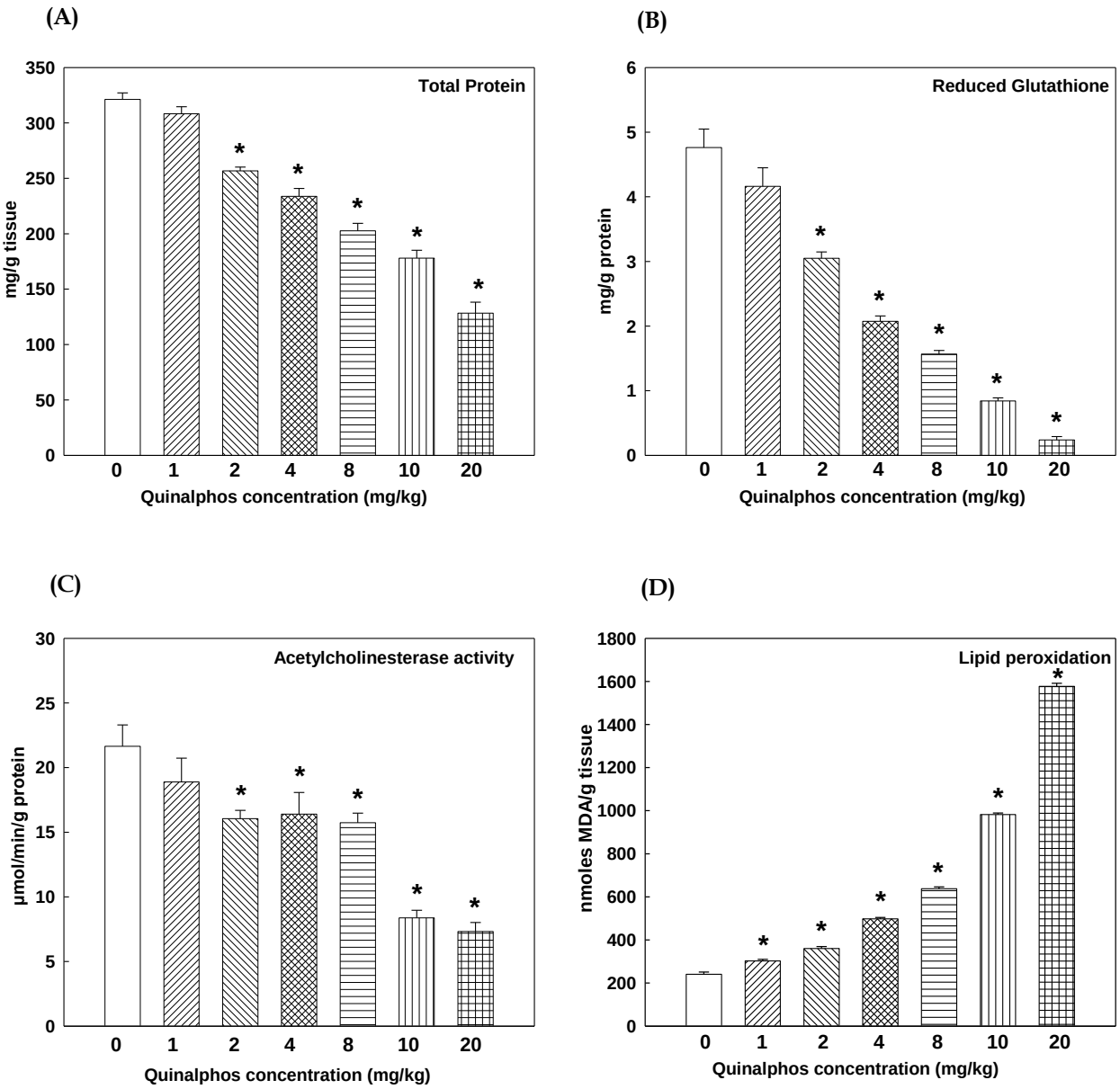


Figure – 4.15 : Dose dependent effect of quinalphos (0, 1, 2, 4, 8, 10 & 20 mg/kg) on the different biochemical variables in the test organism *Eisenia fetida*. (A) total protein, (B) reduced gluthathione (GSH), (C) acetylcholine esterase activity and (D) lipid peroxidation. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.

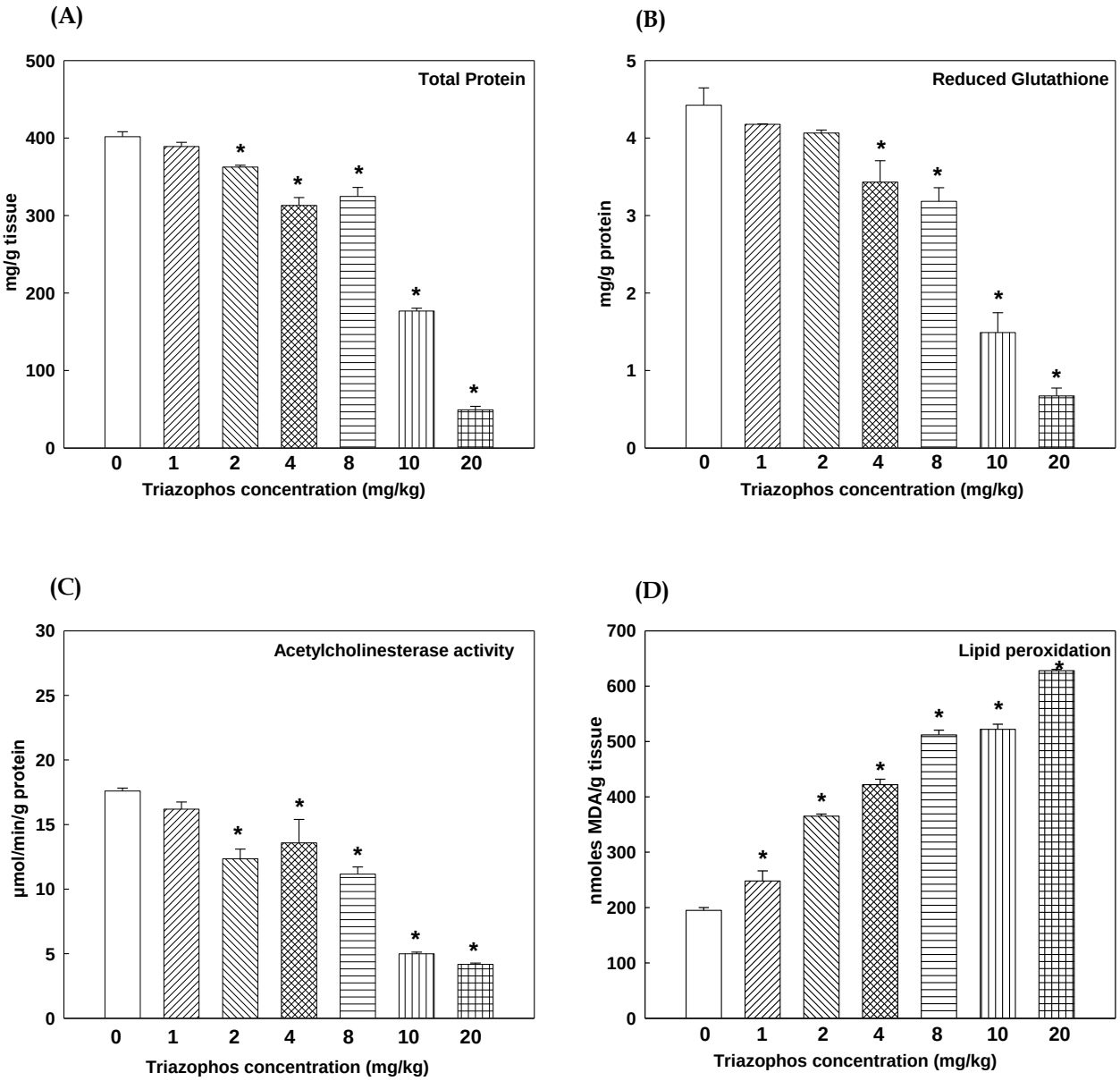


Figure – 4.16 : Dose dependent effect of triazophos (0, 1, 2, 4, 8, 10 & 20 mg/kg) on the different biochemical variables in the test organism *Eisenia fetida*. (A) total protein, (B) reduced gluthathione (GSH), (C) acetylcholine esterase activity and (D) lipid peroxidation. Values are mean ± SE of four replicates per treatment. * Significantly different from control group (0) at $p \leq 0.001$ by Dunnet's test.

4 mg/kg doses, which was followed by an increase from 8 mg/kg dose onwards (Figure – 4.10D). During all pesticide exposures, an increased levels of lipid peroxidation was observed with corresponding increase in thedose of respective pesticides. The results clearly indicated that OP pesticides generate ROS even at very low levels.

4.4. Histological analysis

The exposure effects of the eight studied organophosphates on the histology of the earthworm *E. fetida* after 24 hours are shown in Figures 4.17, 4.18, 4.19, 4.20, 4.21, 4.22, 4.23 & 4.24 respectively. Histologically, integument of earthworm is differentiated into cuticle, epidermis, (a single layer of cells) and a double layer of muscle fibers. The body wall is covered externally by a thin, pervious and flexible cuticle. The cuticle is supported by the underlying epidermis.

Histopathological analysis showed a slight muscle layer disorder as well as vacuole degeneration and inflammation of epidermis in all the treatments irrespective of the pesticides. Different degrees of hyperplasia and vacuole degeneration of epidermis were observed in higher doses of exposure. In certain regions of the skin, sensory cells present, some of which are photoreceptors, with a reticular cytoplasm containing a clear body, which seem to act as a lens. The short cells are found abundant on the prostomium and the first and last 2 (or) 3 segments others are small clusters of very tall, thin cells with minute sensory hairs.

In the control samples (4.17A, 4.18A, 4.19A, 4.20A, 4.21A, 4.22A, 4.23A & 4.24A), the chloragogenous layer, the muscle and epithelium maintained their normal structure. The epithelial tissue was vacuolated due to cytolysis as a result of OP exposure. There were many pyknotic cells in the chloragogenous and epithelial layers. It was observed that the muscle layer had lost its compactness and loss of nuclei in epithelial cells. In the pesticide exposed earthworms, the chloragogenous layer was damaged. The natural architecture of the muscles was disrupted. Besides being damaged, the epithelial tissues had prominently folds with glandular enlargements. The observed pathological changes in the intestines were characterized by villous atrophy, enteritis with cellular infiltration and formation of characteristic granulomas. Even though the extent of damages were different for different compounds and doses, pathological changes were mainly prominent in organisms exposed with organophosphates.

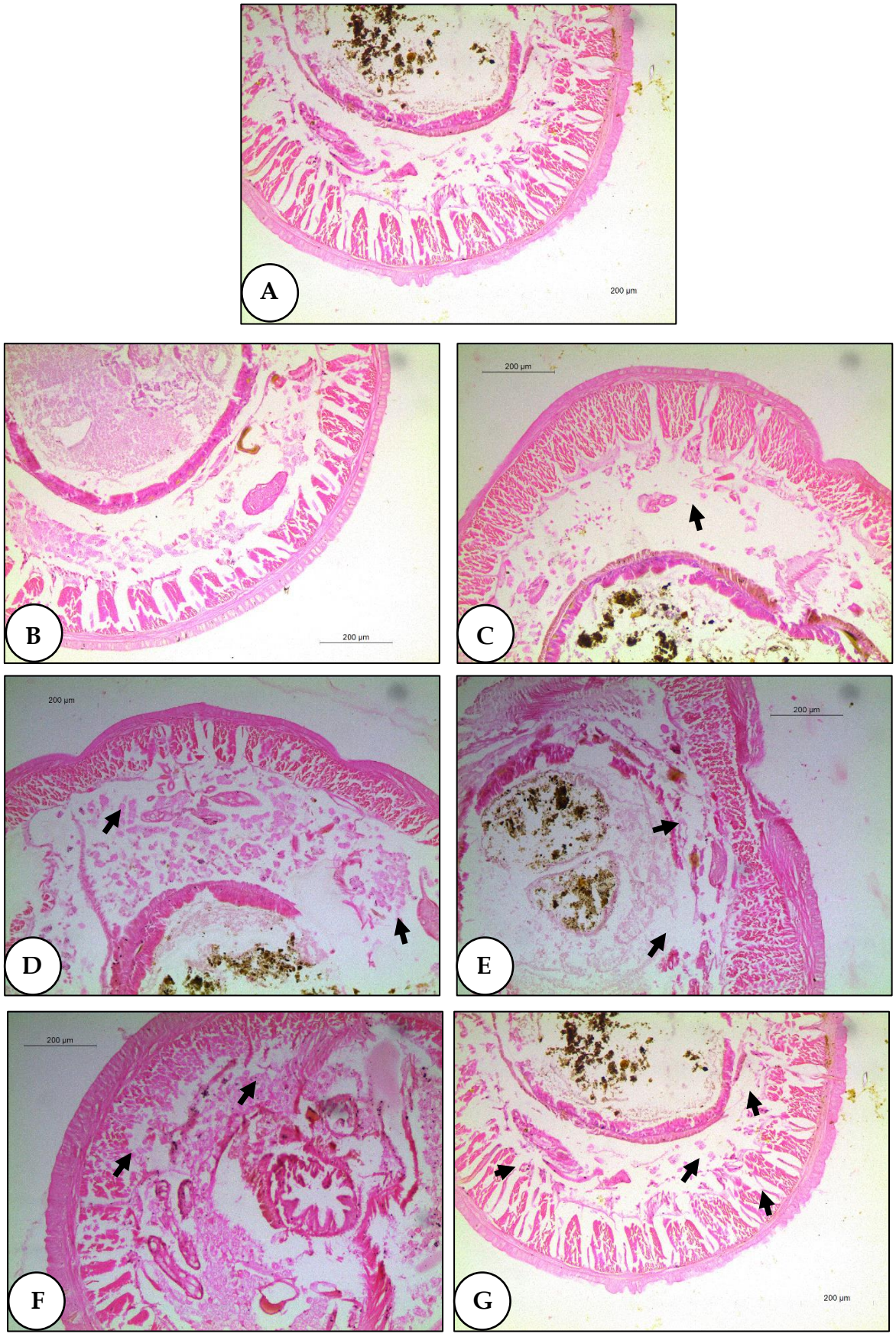


Figure – 4.17 : Representative slides from histological analysis of acephate exposed *E. fetida* tissue samples. (A) Control, (B) 1 mg /kg, (C) 2 mg/kg, (D) 4 mg/kg, (E) 8 mg/kg, (F) 10 mg/kg and (G) 20 mg/kg. Protein degradation, cell membrane damage, cellular infiltration and inflammation are marked with arrow marks.

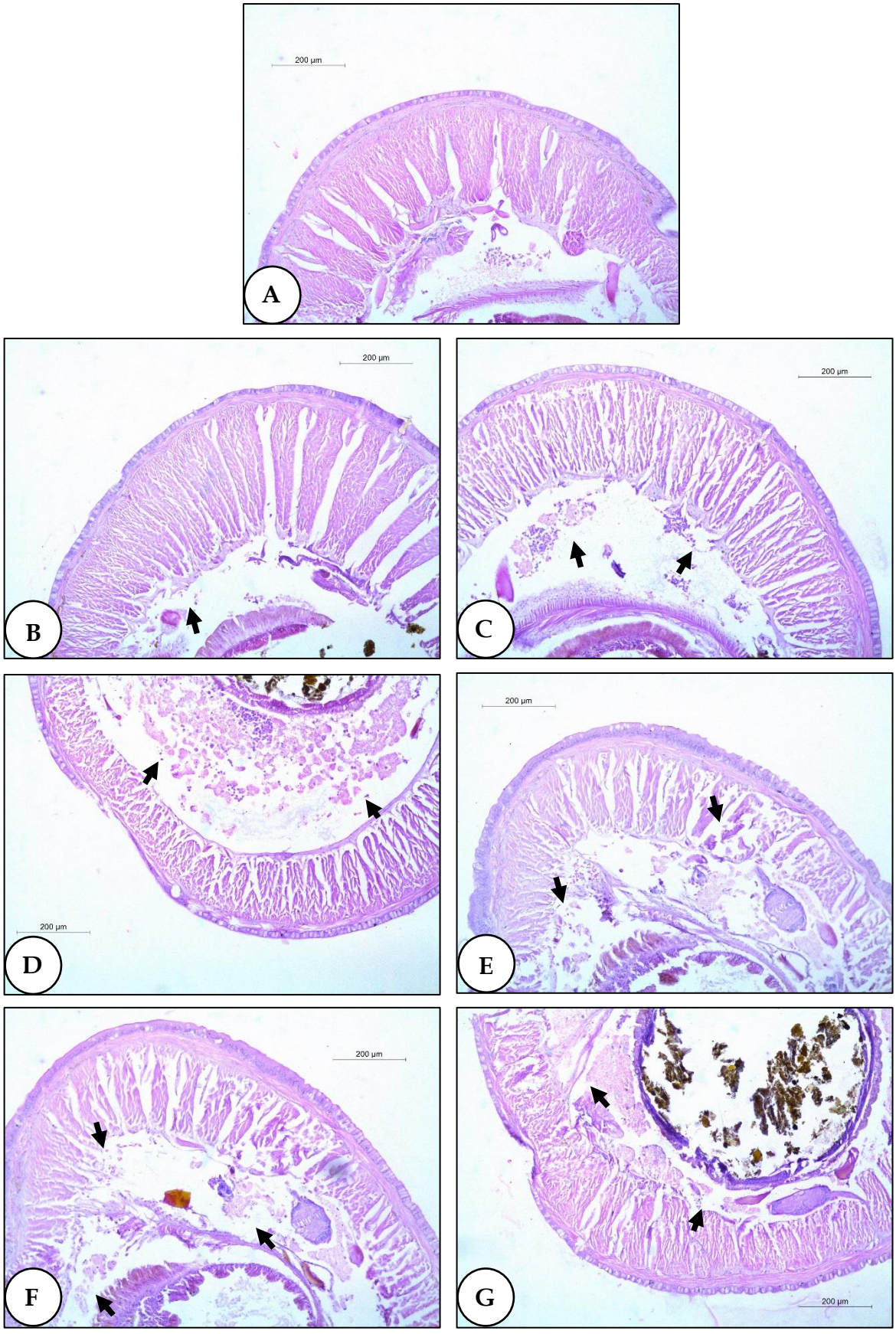


Figure – 4.18 : Representative slides from histological analysis of chlorpyriphos exposed *E. fetida* tissue samples. (A) Control, (B) 1 mg /kg, (C) 2 mg/kg, (D) 4 mg/kg, (E) 8 mg/kg, (F) 10 mg/kg and (G) 20 mg/kg. Protein degradation, cell membrane damage, cellular infiltration and inflammation are marked with arrow marks.

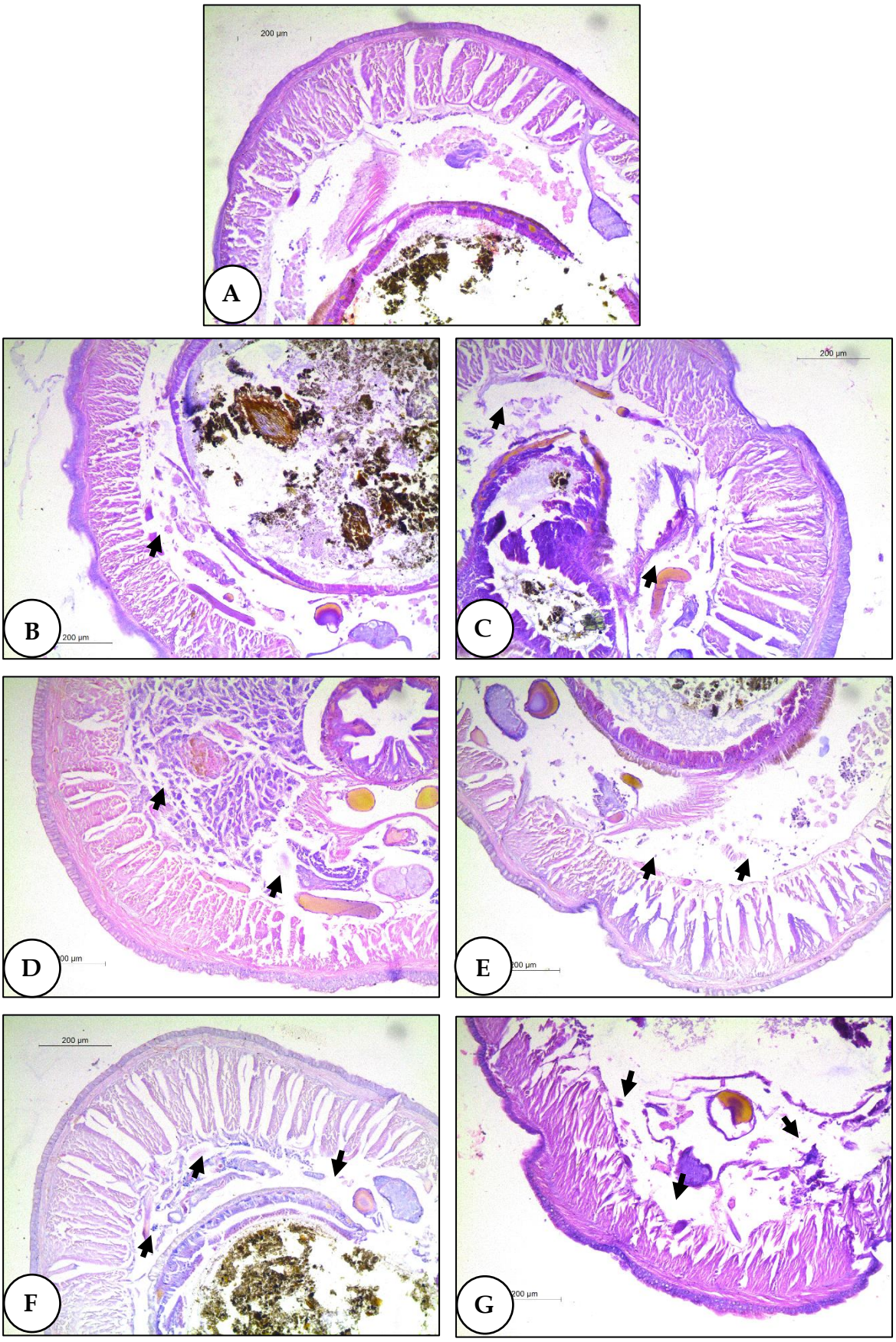


Figure – 4.19 : Representative slides from histological analysis of dichlorvos exposed *E. fetida* tissue samples. (A) Control, (B) 1 mg /kg, (C) 2 mg/kg, (D) 4 mg/kg, (E) 8 mg/kg, (F) 10 mg/kg and (G) 20 mg/kg. Protein degradation, cell membrane damage, cellular infiltration and inflammation are marked with arrow marks.

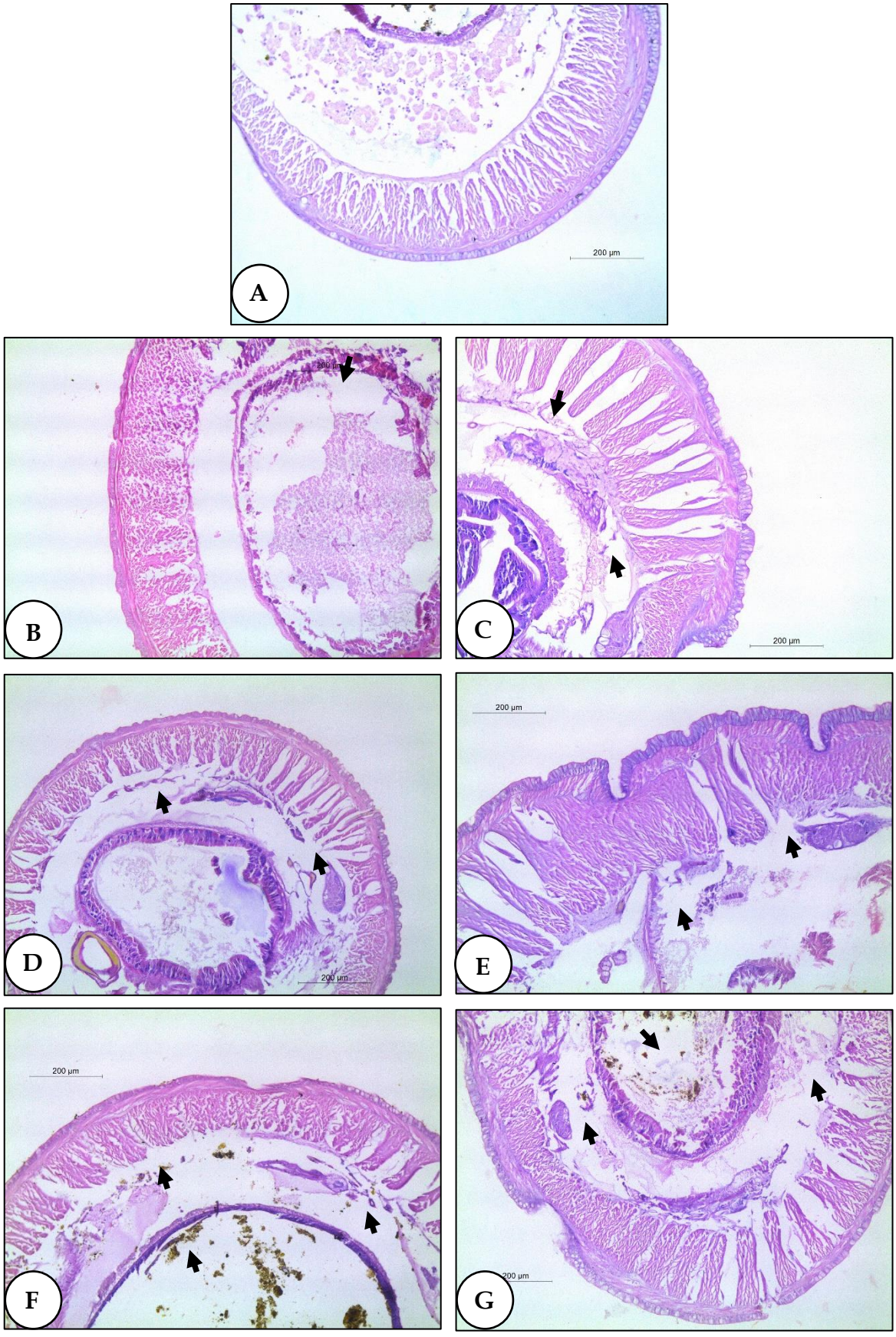


Figure – 4.20 : Representative slides from histological analysis of dimethoate exposed *E. fetida* tissue samples. (A) Control, (B) 1 mg /kg, (C) 2 mg/kg, (D) 4 mg/kg, (E) 8 mg/kg, (F) 10 mg/kg and (G) 20 mg/kg. Protein degradation, cell membrane damage, cellular infiltration and inflammation are marked with arrow marks.

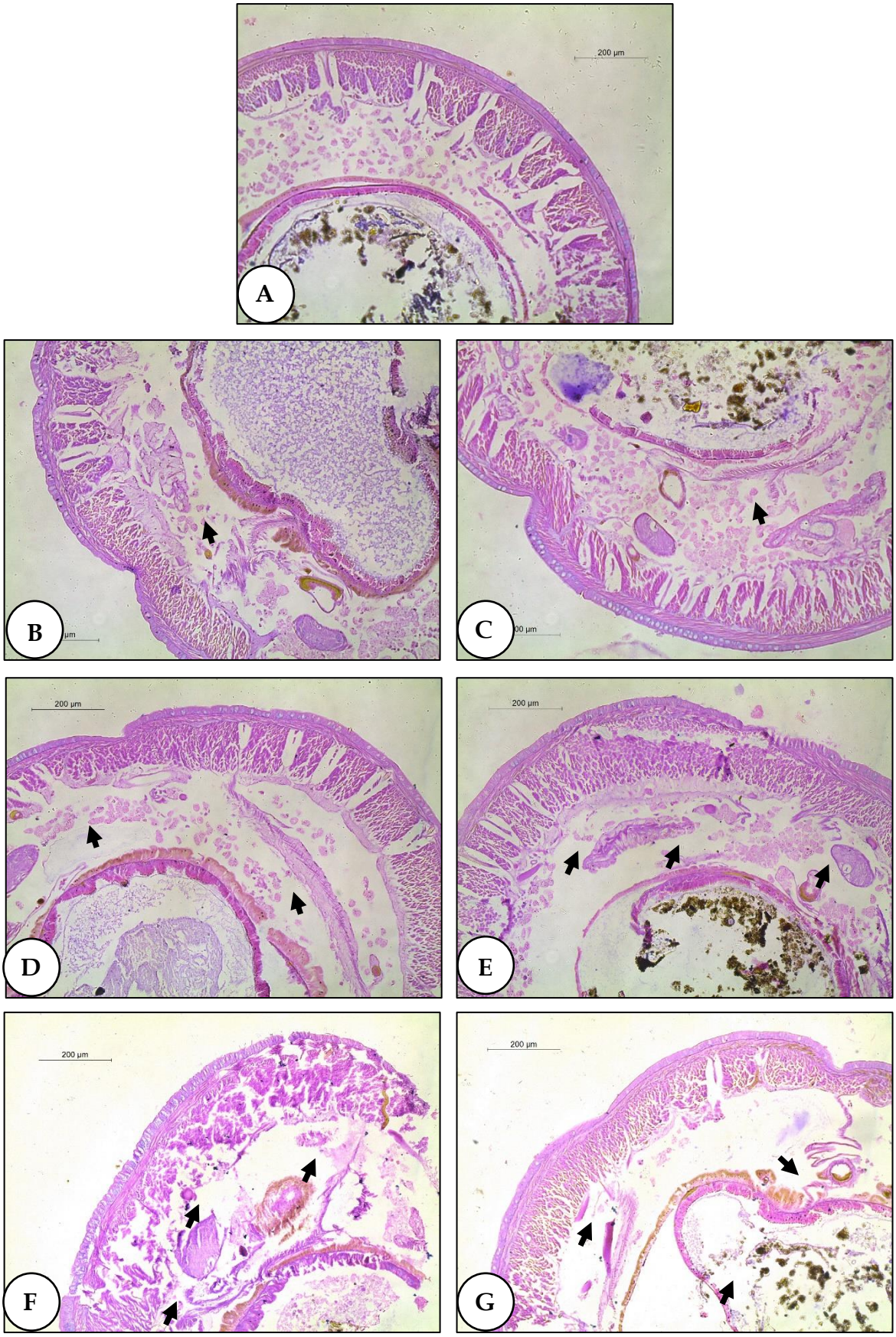


Figure – 4.21 : Representative slides from histological analysis of malathion exposed *E. fetida* tissue samples. (A) Control, (B) 1 mg /kg, (C) 2 mg/kg, (D) 4 mg/kg, (E) 8 mg/kg, (F) 10 mg/kg and (G) 20 mg/kg. Protein degradation, cell membrane damage, cellular infiltration and inflammation are marked with arrow marks.

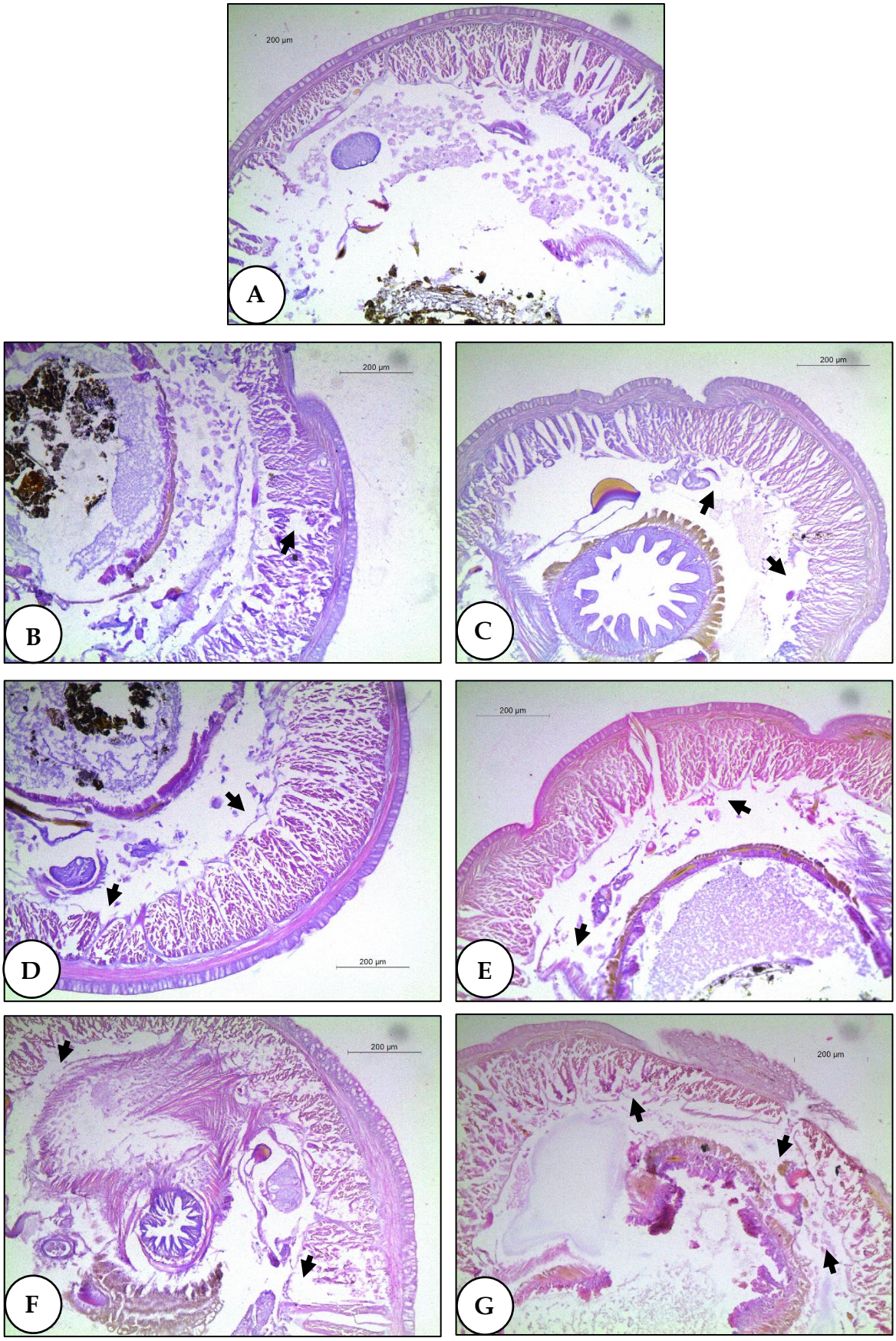


Figure – 4.22 : Representative slides from histological analysis of moocrotophos exposed *E. fetida* tissue samples. (A) Control, (B) 1 mg /kg, (C) 2 mg/kg, (D) 4 mg/kg, (E) 8 mg/kg, (F) 10 mg/kg and (G) 20 mg/kg. Protein degradation, cell membrane damage, cellular infiltration and inflammation are marked with arrow marks.

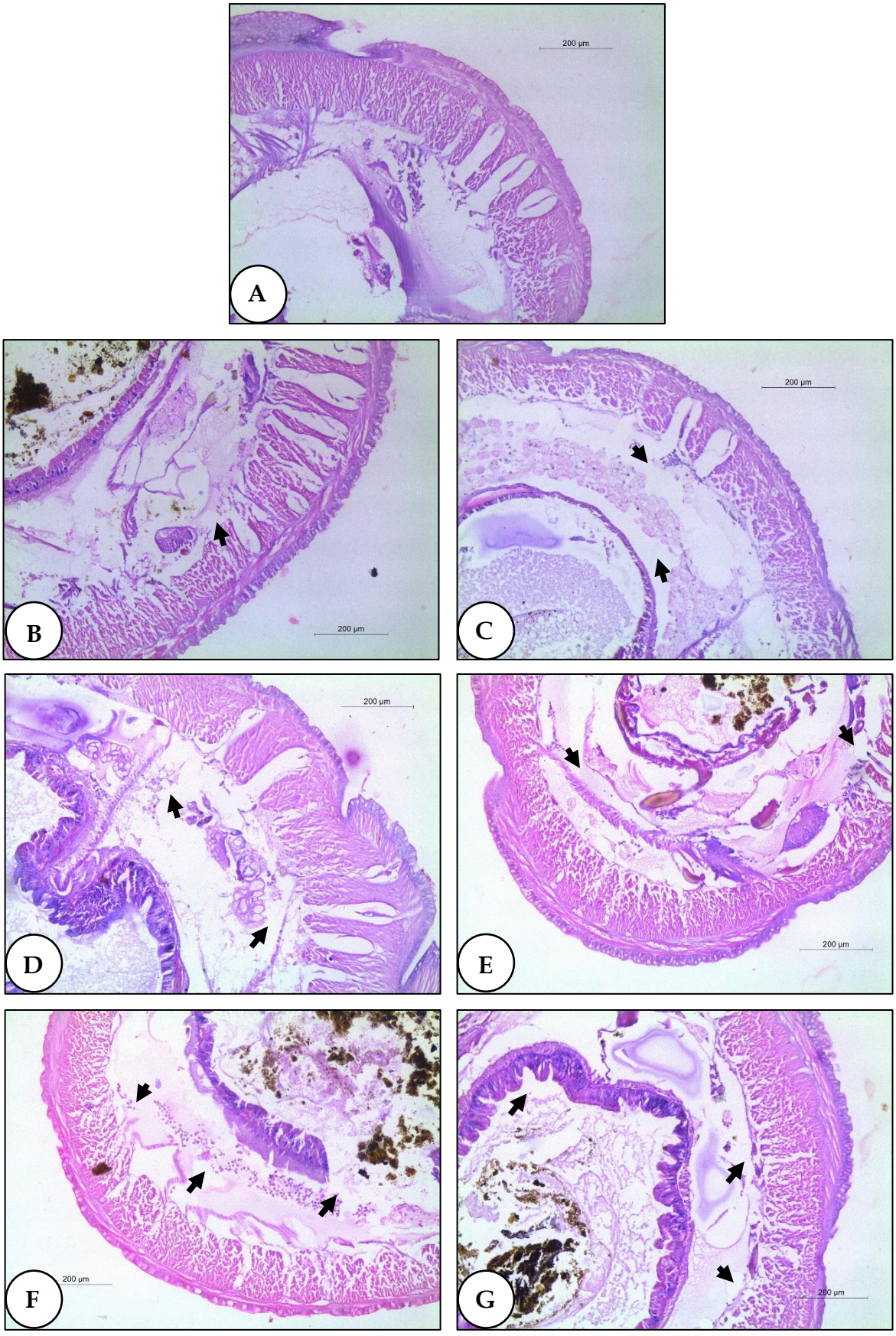


Figure – 4.23 : Representative slides from histological analysis of quinalphos exposed *E. fetida* tissue samples. (A) Control, (B) 1 mg /kg, (C) 2 mg/kg, (D) 4 mg/kg, (E) 8 mg/kg, (F) 10 mg/kg and (G) 20 mg/kg. Protein degradation, cell membrane damage, cellular infiltration and inflammation are marked with arrow marks.

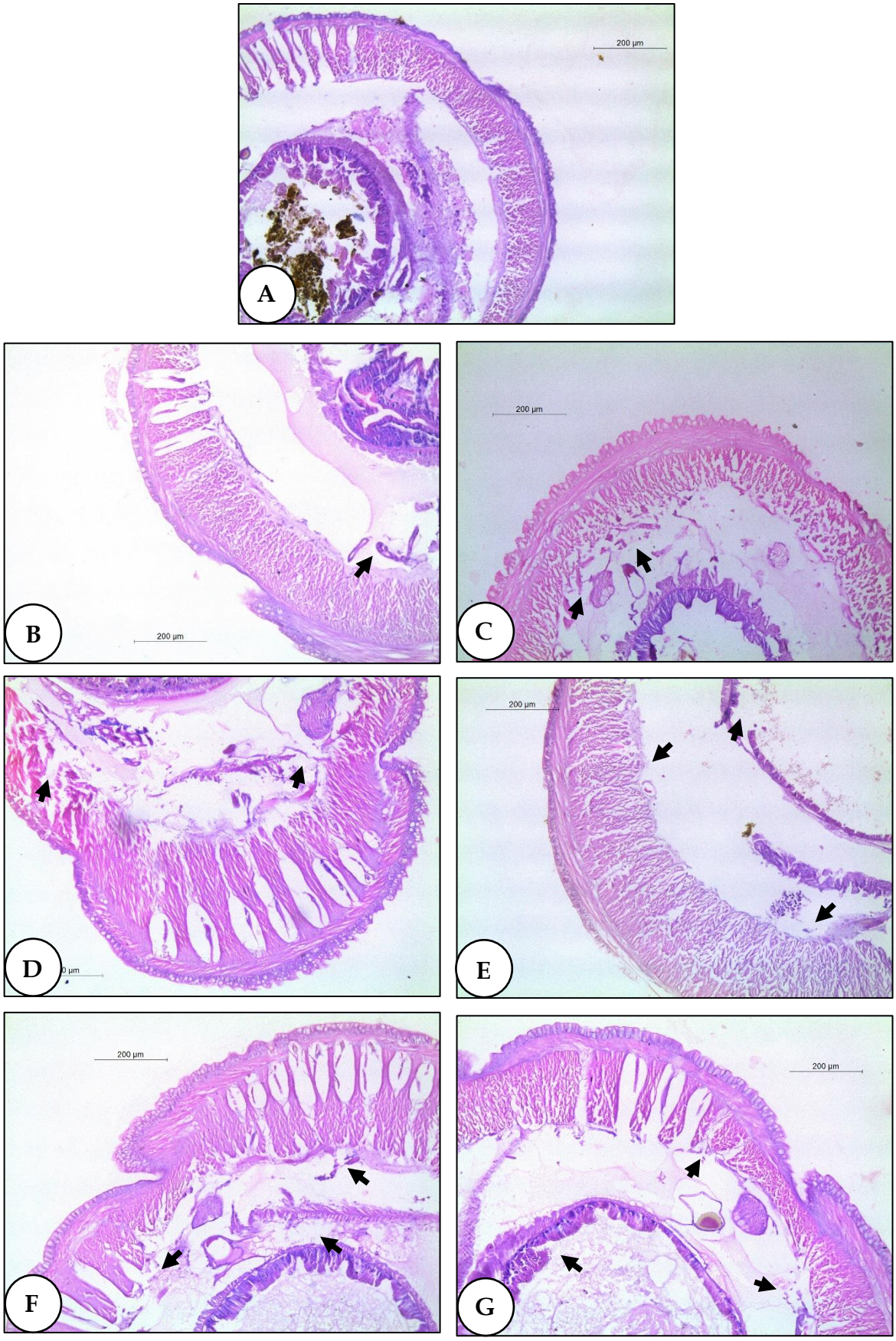


Figure – 4.24 : Representative slides from histological analysis of triazophos exposed *E. fetida* tissue samples. (A) Control, (B) 1 mg /kg, (C) 2 mg/kg, (D) 4 mg/kg, (E) 8 mg/kg, (F) 10 mg/kg and (G) 20 mg/kg. Protein degradation, cell membrane damage, cellular infiltration and inflammation are marked with arrow marks.

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DISSCUSSION AND CONCLUSION

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5. DISSCUSSION AND CONCLUSION

Earthworms are important biological engineers that have a tremendous potential in agro-ecosystems. Earthworms have attained renewed scientific attention in India and abroad because of their wide application in different fields such as bioindicators, vermicomposting etc.. Earthworms functions as flexible natural bio-reactors to harness the beneficial soil micro flora and eliminate pathogen, thus changing organic waste into valuable products such as vitamins, growth hormones, bio-pesticides, bio-fertilizers, antibiotics, enzymes and protein-rich worm biomass. These have been used for centuries for decomposing of wastes and improving soil nature. Earthworms can digest all municipal and industrial organic wastes including sewage sludge and convert them to useful biofertilizers. Their internal organs can function as a 'biofilter' and can 'detoxify' or 'purify' wastewater of industrial and municipal origin. Many chemical contaminants including heavy metals and organic pollutants in soil can bio-accumulate and bio-transform by earthworms and clean-up the contaminated lands for re-development.

Earthworms are important constituents of the soil system, mainly because of their complimentary effects on soil functions and structure (Paoletti, 1999; Jongmans et al., 2003). In addition, earthworms increase formation of organic matter layer in topsoil and leading to increase in soil fertility. Since involved in all these processes, earthworms are considered as excellent bioindicators of soil pollution (Cortet et al., 1999; Lanno et al., 2004). Many earthworm species (e.g., *Eisenia fetida* and *E. andrei*) are utilized in toxicity testing methods and as bioindicators for assessing environmental contamination (OECD, 1984). The term bioindicator, defines an organism that gives information on the environmental conditions of its habitat by its presence, absence, survival, reproduction rate, growth, or immobilization, etc (van Gestel and van Brummelen 1996). In addition, biomarkers can give strong indication of a cause–effect relationship between the occurrence of adverse effects at the individual level and the contaminant in the environmental media (Neuparth et al., 2005).

Earthworms are widely used as model organisms because many characteristics of their response to environmental distresses can be measured and linked to environmental consequences, including their mortality, growth rate, reproduction patterns, avoidance behaviour and enzyme activity levels (Yeardley et al., 1996). The earthworm avoidance test developed in 1996 was widely used (Yeardley et al., 1996) for rapid screening and assessment of soil function and impact of chemicals and contaminants. International Standards Organization (ISO) has established earthworm avoidance test guidelines (ISO, 2008) which are to be followed in earthworm related behaviour studies. A standard earthworm

avoidance method to test toxicity of contaminated soil was published by Environment Canada (Environment Canada, 2004). The avoidance tests are ideal for rapid screening of new chemicals or materials for soil applications because it require less experimental time and have higher sensitivity to chemicals and contaminants than other toxicity testing methods (Schaefer, 2004; Yeardley et al., 1996).

The present study evaluated the response of earthworm *E. fetida* - which is widely used as a model soil organism - against eight organophosphate pesticides; acephate, chlorpyriphos, dichlorvos, dimethoate, malathion, monocrotophos, quinalphos and triazophos. The organisms showed significant avoidance against all the chemicals in varying degrees. Responses were in tune with some earlier reports; *E. fetida* was able to detect and avoid low concentrations of the fungicide Mancozeb (Reinecke et al., 2002). According to Loureiro et al., (2005) carbendazim and benomyl caused strong behavioural effects on the species *E. andrei* at concentrations starting at about 1 mg/kg. In our study it was noted that among the pesticides tested, in chlorpyriphos the responses were significant from 1 mg/kg. In dichlorvos, dimethoate and malathion, significant avoidance was recorded at 2 mg/kg and for acephate, monocrotophos, quinalphos and triazophos, significant responses were recorded at 3 mg/kg. These results indicate the relevance of avoidance tests as a tool for differentiating the pesticide contamination at very low doses. Renoux et al. (2000) reported that earthworms stayed on the soil surface instead of burrowing into the TNT contaminated soil. This phenomenon was also observed in this study, as earthworms stayed on the soil of acute test treatments and can be inferred as a clear behavioural response. On contrary, there are also occasions where the test was not sensitive as evidenced by the endogeic lumbricid species *Aporrectodea caliginosa* which did not avoid toxic concentrations of organophosphate insecticides (Hodge et al., 2000).

The assessment of early transcription responses of *E. fetida* to the exposed pesticides at three different doses could reveal the alterations in the key biological processes owing to the toxic action of pesticides. The gene expression levels of major enzymes and stress proteins involved in oxidative stress pathway via., glutathione peroxidase (GPx), glutathione-s-transferase (GST), super oxide dismutase (SOD), catalase (CAT) and heat shock protein-70 (HSP-70) were assessed for transcription responses of the organism towards organophosphates. Though few transcriptomic studies were done to understand the effect of xenobiotics/pesticides in earthworms and other organisms at the gene expression levels (Novais et al., 2012a, b; Reichert and Menzel, 2005; Svendsen et al., 2008), through the present study we attempted to analyse the effect of

pesticides on oxidative stress pathway in order to identify a suitable biomarker which gives a sufficiently early response in pesticide exposure.

In general, there was an increase in the number of affected transcripts with increasing concentrations of exposure. Glutathione peroxidase has shown upto 20 fold upregulation in acephate exposure along with significant upregulation on other pesticides. Pesticide concentration had a role in extent of upregulation even though some alterations were seen. Glutathione peroxidase is involved in scavenging free radicals in biological systems. The upregulation of GPx under oxidative stress conditions has been reported earlier not only in pesticide exposure (Theodore et al., 2007; Jennifer and Manisha, 2016) but also in oxidative stress induced by other xenobiotics also (Zbynek et al., 2015., Homa et al., 2016., Maria et al., 2015; Mamangam and Vanisree, 2015; Zhifeng and Zhaojie, 2016). Analysis of GST transcripts showed significant upregulation, however not up to GPx levels. It was also reported that in general, chlorinated compounds appear to demonstrate a weak capacity to induce GST in invertebrates (Agra et al., 2009). However, in mammalian cells, chlorpyriphos and methyl parathion exposure has increased the transcripts of GSTA1 (Medina-Díaz et al., 2011). Similarly, transcription levels of other analyzed macromolecules also indicate the detrimental effect of OP exposure in the oxidative stress pathway. Catalase is one of the enzymes involved in cellular antioxidant defence systems against free radicals. Catalase is a peroxisomal hydroperoxidase that converts hydrogen peroxide to oxygen and water (Mosleh et al., 2007). Catalase transcription levels were found to increase up on OP exposure irrespective of the chemical involved thus directly indicating the involvement of antioxidant defence mechanism in OP toxicity.

OP's are reported to cause oxidative stress through formation of reactive oxygen species (ROS) (Abdulaziz et al., 2011; Vidyasagar et al., 2004). Major biomolecules such as lipids, proteins, and nucleic acids may be attacked by free radicals (ROS), and lipids are the most susceptible group among these. The oxidative damage of lipids causes the formation of malondialdehyde (MDA) as the end product of lipid peroxidation (Vidyasagar et al., 2004, Pujari and Jadkar, 2011). Malondialdehyde is an oxidised product of membrane lipid and its level indicates the levels of oxidative stress. Once formed, these free radicals start their own cascade of events thereby causing potentially harmful effects on biological molecules. OP pesticides are reported to cause peroxidative damage of the membranes and accumulation of peroxidative products in tissues and serum of rats. Rosalovsky et al., 2015 reported that erythrocytes of rats exposed to chlorpyrifos had higher levels of lipid hyperoxides indicating oxidative stress. Monocrotophos exposure caused increased levels of lipid peroxidation in rat

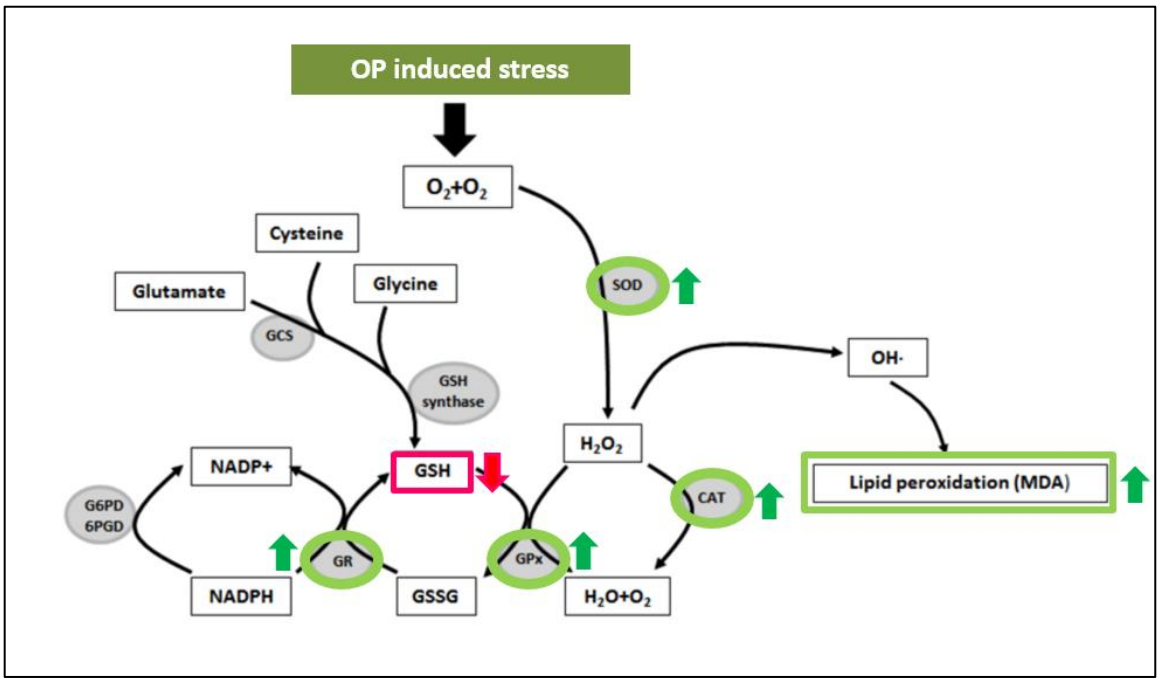
tissues by free radical mediated toxicity (Yaduvanshi et al., 2010). The oxidative stress by increased levels of ROS may have harmful effects on macromolecules like DNA and proteins.

The current study indicated that there was depletion in protein levels during OP exposure. During stress, organisms require higher amount to meet the energy demand. The decreased protein level may be due to their degeneration and possible utilization in several metabolic processes. Similar findings of decrease in total protein were reported in fresh water fish exposed to chlorpyrifos (Malla, 2012). Decreased total protein levels may be due to inhibition of RNA synthesis and disturbing protein metabolism as observed in fresh water cat fish (*Channa punctuates*) exposed to the pesticides malathion and carbofuran (Thoker, 2015). Mosleh et al., (2002) reported a decrease in total protein in both earthworms *E.fetida* and *L. terrstris,* when exposed to endosulphan and aldicrab pesticides. Decrease in total protein levels in *E. fetida* when exposed to insecticides phorate and cartap was reported recently (Sandeep et al., 2017). This could be due to increase in total free amino acid level owing to high proteolytic activity by protease and impaired incorporation of amino acids for protein synthesis.

Glutathione (GSH) is the major macromolecule involved in detoxification mechanisms and in many cellular redox reactions. We could observe a dose dependent decrease in GSH levels when exposed to OP pesticides. GSH being a reducing agent plays a vital role in detoxification and provides antioxidant protection in the cellular system. The antioxidant role of GSH is due to the presence of cysteine residue. Being water soluble, antioxidant GSH can directly oxidise a number of ROS to GSSG in the process. GSH is also involved in reduction of large hyperoxides before they attack unsaturated lipids or convert already formed hyperoxides to hydroxyl compounds. *E. fetida* when exposed to chlorpyrifos, there was an increase in GSH levels upto 4mg/kg dose after which the GSH levels were declined. This indicates the detoxification role of GSH. Similar results of decrease in GSH were observed in rat liver tissues, when exposed to chlorpyrifos (Varma and Srivastava, 2003). The observations were contradictory to the results obtained when *E. fetida* was exposed to contaminated slit sludge which contained high amounts of organic compounds and heavy metals. The levels of GSH were significantly increased than control indicating detoxification mechanism during oxidative stress (Mitana et al., 2016*).* Similar results of GSH reduction was observed during acute exposure of triazophos in wistar rats (Mohineesh et al., 2014). The levels of GSH decreased when *E. fetida* was exposed to mercuric chloride (Gudbrandsen, 2005). GSH counter balances free radical mediated damage by eliminating the compounds responsible for

lipid peroxidation or by increasing the NADPH activity that protects detoxification enzymes.

Acetylcholinesterase (AChE) inhibition is a classical indicator of organophosphate toxicity. The phosphate group of the organophosphorus compound attacks the hydroxyl group of serine at the active site during enzyme inhibition. The inhibition of AChE, the enzyme that terminates nerve impluses by hydrolysing acetyl choline to acetic acid and choline in neuromuscular junction and synapse leads to accumulation of acetyl choline and dysfunction of the nervous system leading to death (Massoulie et al., 1993). The current study indicated a dose dependent inhibition of AChE by all the OP compounds examined. Similar inhibition of AChE was reported in *E. fetida* when exposed to chlorpyrifos (Rao et al., 2003). Inhibition of acetylcholinesterase activity indicated by *in vitro* neurotoxic potentiality revealed competitive inhibition and altered K (m) values widely in a dose-dependent manner. OPs inhibit AChE and the re-establishment of the active enzyme is slow and depends upon elimination of the pesticides. Similar effects of inhibition of AChE were obtained when *E. andrei* was exposed to the pesticides endosulphan, malathion, temephos and pirimiphos-methyl (Sandra et al., 2013). The accumulation of acetyl choline also leads to muscarinic and nicotinic toxicity and causes a clinical syndrome named coiling in earthworms (Henson Ramasey et al., 2007). A schematic representation of the entire process assessed in the study is given below.



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