TOXICANT INDUCED ANTIOXIDANT ACTIVITY IN *OREOCHROMIS MOSSAMBICUS* (Peters)

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By

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KOCHI – 682 016

MARCH 2010

Certificate

This is to certify that the thesis entitled **"TOXICANT INDUCED ANTIOXIDANT ACTIVITY IN *OREOCHROMIS MOSSAMBICUS* (Peters)"** is an authentic record of the research work carried out by Sri. Kesavan. K, under my scientific supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Marine Biology of Cochin University of Science and Technology and that no part of the thesis has been presented before for the award of any other degree, diploma or associateship in any University.

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Declaration

I hereby declare that the thesis entitled **"TOXICANT INDUCED ANTIOXIDANT ACTIVITY IN *OREOCHROMIS MOSSAMBICUS* (Peters)"** is a genuine record of the research work done by me under the scientific guidance and supervision of **Prof. Dr. N. Ravindranatha Menon,** Hon. Director and Emeritus Professor, Centre for Integrated Management of Coastal Zones, School of Marine Sciences, Cochin University of Science and Technology, Kochi and that this has not previously formed the basis for the award of any degree, diploma or associateship in any University.

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PREFACE

The presence of a xenobiotic compound in an aquatic ecosystem does not, by itself, indicate injurious effects. Connections must be established between external levels of exposure, internal levels of tissue contamination and early adverse effects. Many of the hydrophobic organic compounds and their metabolites, which contaminate aquatic ecosystem, have yet to be identified and their impact on aquatic life has yet to be determined. Therefore, the exposure, fate and effects of chemical contaminants or pollutants in the aquatic ecosystem have been extensively studied by environmental toxicologists.

Deleterious effects on populations are often difficult to detect in feral organisms since many of these effects tend to manifest only after prolonged cycles of ontogeny. Often the damages become conceivable very late in the ecosystem, thus rendering remedial actions pointless. In an environmental context, biomarkers offer promise as sensitive indicators demonstrating that toxicants have entered organisms, have been distributed in tissues, and are eliciting a toxic effect at critical targets. In this respect, it is also interesting to study the development and application of sensitive laboratory bioassays, based upon the responses of biomarkers. Bioassays offer many advantages for comparing the relative toxicity of specific chemicals or specific effluents.

Histopathological techniques are a rapid, sensitive, reliable and comparatively inexpensive tool for the assessment of stress responses to xenobiotics. Cytological and histopathological alterations provide a direct record of trace effect. Evaluation of histopathological manifestations provides insight into the degree of stress, susceptibility and adaptive capability of the stressed organism. The route that the toxicant takes

during its metabolisation often dictates the choice of organs for examining the effect of xenobiotics. In this context, gill, liver and the kidney of teleost are the best suited organ system to analyse both the biochemical and histopathological aberrations due to xenobiotic stress.

Fish are of special concern because of the properties of aquatic environment and its relationship with native organisms. An aquatic environment is characterized by marked spatial and temporal heterogeneity of its physicochemical parameters and processes. *Oreochromis mossambicus* (Peters), the cichlid species known by the common name tilapia, forms the experimental animal in the present investigation because of its year round availability, adaptability to varied situations and sensitivity to toxicant exposures. Antioxidant defense studies on oxidative stress in fish open a number of research lines aimed at providing greater knowledge of fish physiology and toxicology. In addition, such studies would provide more precise information concerning the response of antioxidant defenses in different species under various circumstances as well as on the regulatory mechanisms of this response. Such future studies will, no doubt, benefit aspects related to fish farming and aquaculture practices.

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Abbreviations & Symbols used in the thesis

%	percentage
&	and
/	per
µg	microgram
µl	microlitre
µM	micro molar
CAT	catalase
CDNB	1-chloro-2,4-dinitrobenzene
cm	centimeter
Cu	copper
e.g	example
EDTA	ethylene diamine tetra acetic acid
Fig.	Figure.
g	gram
GSH	glutathione (reduced)
HCl	hydrochloric acid
hrs.	hours
i.e	that is
I.U	International Units
kg	kilogram
l	litre
L^{-1}	per litre
M	molar
mal. green	malachite green

MDA	malondialdehyde
mg	milligram
min	minute
mm	millimeter
mM	millimolar
N	normal
nm	nanometer
^{o}C	degree Celsius
p^{k}	ionistaion constant
ppb	parts per billion
ppm	parts per million
rpm	revolutions per minute
ROMs	reactive oxygen metabolites
SOD	superoxide dismutase
TBA	thiobarbituric acid
TCA	trichloro acetic acid
U	units
USFDA	United States Food and Drugs Administration
UV	ultraviolet
Vit. C	Vitamin C
>	greater than
<	less than

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Chapter -1

INTRODUCTION

Pollution of the environment is a topic of national and international concern in recent years. Aquatic ecosystems are being contaminated rapidly by xenobiotics of variegated nature. The impact of aquatic pollution is so alarming that it has attracted even politicians and administrators apart from scientists and technocrats.

Sincere efforts are seldom made in developing countries to evolve methods for pollution monitoring and control, in parallel with the rapid advancements in industry, spreading of urban settlements and technological gallops. Methods for effective abatement of pollution are necessary and proper implementation of legislation and enforcement of existing regulations are highly essential considering the deleterious effects likely to occur to the healthy aquatic system.

Freshwater inland ecosystems like rivers, ponds and lakes and brackish water zones are receptacles for contaminants from agriculture, aquaculture, industry and human settlements. Both inland and coastal ecosystems are fragile in nature. These ecosystems are cardinal abodes of aquatic organisms of commercial, aesthetic and ecological importance. The brackish waters and rivers serve as nursery grounds for finfishes and shellfishes. The materials put into inland water bodies are subjected alterations due to various natural forces and processes resulting in the slow build up of contaminants demanding constant monitoring and surveillance. This is necessary for establishing an early warning system and to provide reliable data for environmental management.

Among aquatic vertebrates, fishes as sentinels of pollution studies are gaining importance. Fishes are poikilotherms and are capable of providing an imprint of physicochemical factors and anthropogenic materials in their structural, functional and behavioral patterns. An assessment of the impact of aquatic pollution would be possible only when the deleterious effects are documented employing sentinel species. Fish has been largely used to evaluate the quality of aquatic systems as bioindicators of environmental pollutants. Chronic exposure of fish to xenobiotics leads to biochemical disturbances (Gul *et al.*, 2004) apart from other connected reactions. Many of the toxic contaminants at sublethal levels mean an aquatic ecosystem can result noticeable effects on the physiology of fishes.

The quantity of pollutants in the environment can no longer be accepted as indices of contamination. Bioassays are recognized as a comprehensive approach to the assessment of aquatic pollution. The biological responses occurring in an organism, when measured in terms of the concentration of the test compound form the basis of bioassays.

The effects of chemical toxicants in living organisms can be studied by biomarkers which in a biological system reflect the defined toxicity mechanisms such as overproduction of reactive oxygen species (ROS) and oxidative stress. The effects of exposure to sublethal levels of pollutants to fish can be measured in terms of biochemical, physiological or histological responses (Mondon, *et al*., 2001). Changes in age and species distribution in a stock of fish population are general indicators of water pollution, but there are also responses specific to a single contaminant or a group of contaminants (Svobodova, *et al*., 1997).

A major aspect of investigation on the toxicology of various toxicants on animals is to find out the *modus operandi* of detoxification. It is

understood that detoxification paths at molecular level in tissues are also involved in detoxification. Fish has the capacity to regulate the uptake of heavy metals. This regulation takes place at various levels in different tissues. Study of histochemistry gives an insight into the mechanisms involved in detoxification. Biochemical changes accompanying detoxification can be detected at early stages by biochemical analysis. The biomarkers found in tissues might be useful warning signals of contamination in respect of the nature of the contaminant. The present study deals with the biochemical markers of oxidative stress in the fish *Oreochromis mossambicus*, in response to exposure to a heavy metal (copper), an organophosphorous pesticide (Metacid-50) and an industrial dye (Malachite green).

Biochemical markers or biomarkers are measurable responses to the exposure of an organism to xenobiotics. They usually respond to the mechanisms of toxic activity and not to the presence of specific xenobiotic and therefore may react to a group of either similar or very heterogenous xenobiotics. Biochemical markers detect the type of toxicity; in some of them, the magnitude of their response correlates with the level of pollution. Monitoring of the biomarkers in living organisms including fish is a validated approach and serves as early warning of adverse changes and damage resulting from chemical exposure (Van der Oost, *et al*., 2003).

Organisms when exposed to xenobiotics exert cytotoxic responses via production of reactive oxygen species (ROS) of superoxides, hydroperoxides, hydrogen peroxide and hydroxide. Oxygen radicals are formed as intermediates of cytosolic enzymes, electron transfer systems and by activated cells of the immune system. These highly reactive compounds damage most biomolecules like lipids, proteins and DNA, damage to the latter being responsible for genotoxic damage. Efficacy of

cellular antioxidant defense mechanisms determine the magnitude of oxidative stress resulting from reactive or more accurately activated oxygen species. Significant damages are an indication of failure of the antioxidant machinery. The cellular antioxidant defense system includes various water and fat soluble free radical scavengers of low molecular weight and several prophylactic inducible enzymes including superoxide dismutase, converting the superoxide radical to hydrogen peroxide, catalase reducing the hydrogen peroxide to water and oxygen and glutathione peroxidase concerned with the reduction of hydroperoxides and hydrogen peroxide. Of the two glutathione peroxidases, one is selenium dependent destroying several hydroperoxides including hydrogen peroxide and the other is selenium independent active with different organic hydroperoxides. Glutathione-S-transferanse family, possessing detoxifying properties towards lipid hydroperoxides generated by organic pollutants, provides a specific biomarker for contamination with organic pollutants like pesticides. All the enzymes concerned with the handling of reactive oxygen intermediates are to be located in all cell types even though their specific site of release may differ. Superoxide dismutase is of two types, one type containing Cu/Zn is secreted in cytoplasm and the other type carrying Mn is found in mitochondria. Catalase is always present in peroxisomes. The glutathione peroxidase and glutathione S-transferase systems are common components of cytoplasm of all types of cells. These inducible enzymes serve a vital role in protecting the cell from oxidative stress and are useful indicators of contaminant stress in aquatic organisms like fishes.

Organochlorine pesticides are almost completely replaced by organophosphorus compounds. Organophosphorus pesticides or singly organophosphates are esters of phosphoric, phosphinic or phosphonic

acid. Metacid 50 is the organophosphate used in the present study. Heavy metals and pesticides have become components of chemical wastes polluting natural aquatic communities, many of which are hazardous because of their ability to adversely affect organisms even at very low concentrations. Moreover they accumulate in food chain and kill non target organisms thereby disrupting the delicate balance of the ecosystem. The dye used in the present study is malachite green which is a triphenyl methane compound, extensively used as a therapeutic agent in aquaculture either singly or in combination with other dyes or formalin (Lanzing, 1965). The dye is also used in industries like coir products, textile etc. The present study was also aimed at elucidating the attenuation of toxic responses to different test compounds via treatment with an exogenous antioxidant. Vitamin C was the exogenous agent applied to alleviate the burden of toxicants.

Vitamin C (ascorbic acid) functions as an agent of biological reduction, hydroxylation of many enzyme systems and is an indispensable nutrient for fish as they cannot synthesize this nutrient.

Vitamin C cannot be synthesized by fishes due to the lack of enzyme L-gulonolactone oxidase (EC.1.1.3.8) (Dabrowski, 1990). Furthermore ascorbic acid seems to play an important role in the metabolism of α tocopherol (α-T). Several studies (Niki, 1987 a,b., Packer et.al, 1979) have demonstrated the ability of ascorbic acid to reduce the α tocopheroxyl radicals and thereby regenerate them to α- tocopherol (recycling of vitamin E ., Shiau and Lin, 2006).

Following the definition by Hinton and Lauren (1990), biomarkers can be defined as any contaminant-induced physiological and /or biochemical changes in a not-too-sensitive organism, which leads to the formation of

altered structure (a lesion) in the cells tissues or organs. It is a well known fact that any physiological and biochemical alteration, if only severe enough and/or protected will eventually result in structural modification and vice versa (Braunbeck, 1998). Contaminant-induced oxidative stress, being a condition capable of eliciting biochemical alterations, it is highly likely that histological modifications also occur. As such, fish diseases and pathologies, with a broad range of etiologies, are used as indicators of environmental stress since they provide a definite biological end-point of historical exposure. Additionally, histological biomarkers provide powerful tools to detect and characterize the biological end points of toxicant exposure (Stentiford *et al.*, 2003). In the present investigation, histopathological changes in test tissues in response to the test toxicants were also assessed.

Chapter -2

REVIEW OF LITERATURE

Aerobic life is inevitably linked with oxygen-dependent oxidative processes with the concurrent danger of cellular damage by reactive oxygen species (ROS). The term "oxidative stress" was introduced in biological sciences to denote a disturbance in the pro oxidant-antioxidant balance in favour of the former (Sies, 1985)

Metabolism of xenobiotic pollutants may lead to the additional generation of reactive oxygen species. Activation of ambient oxygen that occurs during the oxidation of xenobiotics may result in its release from the enzymes thereby disrupting cellular structures and functions. Furthermore, reduced moieties of pollutants or their metabolites react with oxygen, producing superoxide. Reactive oxygen species thus represent a common side product of xenobiotic metabolism and many pollutants actually exert part of their toxicity through the formation of reactive oxygen (Lackner, 1998).

Sources of reactive oxygen species in aquatic environment are as diverse as the synthetic materials disposed directly or indirectly into it although certain natural factors also contribute to it. Depending on the specific reactions leading to the activation of molecular oxygen, different concentrations ranging from hypoxia to hyperoxia favour reative oxygen species production (Jones, 1985., Ritola *et al*.,2002., Lushchak & Bagnyukova, 2006). Hypoxia may induce a "reductive stress" in cells leading to reactive oxygen species formation when oxygen concentration

increases. Redox cycles are one of the most important sources for superoxide radicals in tissues and many pollutants exert part of the toxicity via this mechanism (Kappus, 1987). Important reductase systems involved in redox cycling are described by Livingstone *et al*., (1990). But the very reduction machinery itself may be a source for superoxide and hydrogen peroxide (Bainy *et al*., 1996). The sight specific oxidative damage of some of the susceptible amino acids of proteins is now regarded as the major cause of metabolic dysfunction during pathogenesis (Bandyopadhyay *et al*., 1999).

Different types of xenobiotics including polycyclic aromatic hydrocarbons (PAHs), pesticides and other synthetic chemicals and heavy metals are capable of triggering oxidative stress (Lemaire & Livingstone, 1997., Lemaire *et al*., 1994., Washburn and Di Giulio, 1989., Radi & Matkovics, 1988., Halliwell & Gutteridge, 1999., Lopez-Torres *et al*., 1993., Gutteridge,1995). Current knowledge on various processes leading to contaminant – stimulated reactive oxygen species production and resulting oxidative damage is summarized by Livingstone (2003). A background on the general aspect of pro oxidant antioxidant and oxidative damage processes in animals and a review of the information known for aquatic organisms in relation to pollution and the use of pro oxidant chemicals in aquaculture are provided in the article. Information on damages due to chemical bioaccumulation in fishes and warning of undesirable changes in them is available in literature (Van der Oost *et al*., 2003). The cytotoxic reactive oxygen species are continuously produced by contaminant-stimulated phagocytes and excessive reactive oxygen species have shown their role in the development of local tissue damage in freshwater fish (Ahmad *et al*., 2000, 2003., Fatima *et al*., 2000).

Heavy metals, pesticides and many other organic chemicals comprise a most harmful group of pollutants in aquatic habitat. Aquatic

ecosystem is almost incapable of degrading such foreign compounds though some organisms possess limited capabilities for metabolizing them. Therefore anthropogenic organic chemicals released into fresh water and marine environments tend to accumulate them and cause long term effects (Kinne, 1984).

The processes of bioconcentration, bioaccumulation, biomagnification and metabolisation are not sufficiently studied in fishes. In the dynamic sense bioaccumulation describes any accumulation of chemicals in biota resulting from uptake, storage and sequestration of contaminants. The part of bioaccumulation resulting from direct partitioning of contaminants between aqueous and biological phases is called bioconcentration. The uptake paths for bioconcentration are via the gills or other tissues in direct contact with the ambient aqueous environment. The part of bioaccumulation resulting from ingestion of contaminated food items is called biomagnification although the term is also applied for progressive increase in contaminant concentration at higher trophic levels. Biomagnification also involves complex partitioning processes within the intestinal tract. There are complex equilibration processes between bound and dissolved contaminants anywhere at the fish/water interface and within the fish (Streit, 1998). Differentiating between bioconcentration and biological magnification is to some extent artificial in fish experiments (Opperhuizen, 1991., Streit., 1979 b, 1992).

The effects of exposure to sublethal levels of pollutants can be measured in terms of biochemical, physiological or histological responses of the fish (Mondon *et al*., 2001). Biochemical markers have been used as a research tool in toxicology, ecotoxicology and pharmacology. Most of the studies have utilized *in vitro* assays whereas *in vivo* tests have been employed in research concerned with aquatic environment. A detailed review to this effect is given by Drastichova (2004). An extensive review on pro

oxidant and antioxidant mechanisms in aquatic organisms is presented by Winston and Di Giulio (1991).

Investigations on oxidative stress and enzymatic and non enzymatic antioxidants in fishes and aquatic invertebrates in response to environmental changes and exposure to varied types of pollutants in the laboratory had been studied extensively (Trendazo *et al*., 2006., Martinez- Alvarez *et al*., 2005., Wilhem-Filho *et al.*, 1993., Oakes *et al*.,2004., Hamed *et al*., 2003.,Shi *et al*., 2005., Pedrajas *et al.*,1995., Doyotte *et al.*,1997., Dautrempuits *et al*.,2003., Achuba and Osakwe,2003., Ritola *et al.,* 2002., Wilhem-Filho *et al*., 2001., Haddad, 2004).

The glutathione system and antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), which detoxify superoxide anion ($O_2^{\cdot-}$) and H_2O_2, act as a primary defense as endogenous physiological antioxidants. A second line of defense is established by antioxidants, which can only be provided by nutritional supplements (Sen, 1995). The beneficial antioxidant action of tocopherols and vitamin C generate radical byproducts that are scavenged by GSH or α – lipoic acid (Sen, 1995). The definition of dietary antioxidants includes their ability to substantially decrease the adverse effects of reactive oxygen species such as reactive oxygen and nitrogen species on physiological function (Blumberg, 2004).

Cellular defense systems such as antioxidative enzymes and non enzymatic scavengers provide only a limited capacity to protect cells against oxidative damage. Thus, xenobiotic challenge may over stress cellular protective mechanisms and provoke DNA strand breaks, protein oxidation, lipid peroxidation and generation of toxic aldehydes which may consequently result in cell death and / or initiation of carcinogenesis (Winston and Di Giulio, 1991).

Many disease processes of clinical interest and aging process involve oxidative stress in their underlying etiology. The production of reactive oxygen species is also prevalent in the world's oceans and oxidative stress is an important component of stress response in marine organisms exposed to a variety of insults because of changes in environmental conditions such as thermal stress, exposure to ultra violet radiation or exposure to pollution. As in the clinical setting reactive oxygen species are also important signal transduction molecules and mediators of damage in cellular processes such as apoptosis and cell necrosis in marine organisms (Lesser, 2005).

The concept of "biomarkers" has recently received considerable attention among environmental toxicologists as a new and potentially very powerful and informative tool for detecting and documenting exposure to and effects of environmental contamination. A biomarker is xenobiotically-induced variation in cellular or biochemical components or processes, stimulus or function that is measurable in a biological system. Such responses integrate the pharmacokinetic and toxicological interactions within organisms exposed to mixtures of contaminants (Shugart *et al*., 1992). Mammalian species are usually used as models to study oxidative stress and to clarify the mechanisms involved in cellular damage and response. However, fish are of special interest because of the properties of aquatic environment and its relationship with organisms (Winston and Di Giulio, 1991., Kelly *et al*., 1998 and Nikinmaa and Rees, 2005).

Heavy metals are well known environmental pollutants. Aquatic organisms can easily absorb the metals and accumulation may occur in a considerably high concentration. The accumulation and biomagnification of heavy metals in the tissues of animals have received considerable attention. The accumulation of heavy metals in the organs of various animals may be

speedily transferred from the surrounding environment and into the food chain (Adema *et al*., 1972). Among the aquatic animals, fishes are valuable bioindicators of contaminant effects. In general, heavy metals during the earliest contamination stage accumulate in the soft tissues of fish, but after prolonged exposure, they become accumulated in harder tissues. The basic mechanism of reactive oxygen species production and ultimate toxicity produced by metal ions may involve mechanisms that are common to redox cycling organic xenobiotics, as, for example, the quinones. Furthermore, various studies have suggested that the ability to generate reactive oxygen species by redox cycling quinones and related compounds may require metal ions such as iron and copper. Thus common molecular mechanisms may be involved in the production of ROS and the toxicities of various xenobiotics (Stoths and Bagchi, 1995).

Copper, a trace element, plays an important role in the cellular metabolism. It is a cofactor for a number of enzymes including cytochrome oxidase and is thus essential for all eukaryotic cells. The multifaceted physiological roles of copper, chemistry and biochemistry of different copper compounds of biological interest and its central role in the formation of reactive oxygen species are given by Theophanides and Anastassopoulou (2002). But a high concentration of this element in the organism is toxic. Increased copper concentration in aquatic environment resulting from domestic, urban and industrial waste disposal result in copper uptake by aquatic animals.

Copper sulphate ($CuSO_4$. $5H_2O$) is commonly applied to freshwater aquaculture ponds to control external parasites and bacterial diseases and as a fungicide. It also finds application as an algal control agent in culture systems (Reddy *et al.*, 2006). An excess of the compound may become toxic to fish.

In the past in vivo and in vitro studies on the bioaccumulation of copper have been carried out by several workers (Kaviraj, 1989, Deb and Santra, 1997., Grosell *et al*., 2004). The redox nature of copper is also what makes this element a potent toxicant. Tight regulation of organ and cellular copper concentrations has therefore evolved to ensure sufficient copper for essential processes while at the same time preventing toxicity. Aquatic organisms can take up copper directly from the water and elevated ambient copper concentrations can lead to excess copper accumulation in several tissues. (Grosell *et al*., 1997, 2004). Oxidative stress is generally accepted as one of the major effects of excessive cellular copper concentrations.

Although the toxicological effects of copper in fish are well documented, there are discrepancies and variability in reported results (Paris-Palacios *et al*., 2000., Varanka *et al*., 2000., Karan *et al.*, 1998., Zyadah and Abdel-Baky, 2000., Parvez *et al*., 2006)

Various workers have conducted assessment of toxicity of copper (in the form of copper sulphate) to *Oreochromis mossambicus*. Animals of different age group and locality recorded variations in median lethal concentration (LC_{50}). Experiments carried out by Wu *et al* (2003) in Taiwan have reported a 96 hr LC_{50} value of 230ppb for tilapia hatchlings. In a study conducted in India, specimens of tilapia weighing 12.4±1g recorded a LC_{50} value of 4.27ppm when copper was the toxicant (James and Sampath, 2003).

The trend of lipid peroxidation in the study was indicative of stimulation of antioxidant enzyme activity. An increase in hepatic protein content and enhancement of antioxidative enzyme activities indicating an adaptive mechanism as a result of exposure to copper was reported in the

liver of *Brachydanio rerio* (Paris- Placacios *et al*., 2000). According to Varanka *et al* (2001), exposure of *Cyprinus carpio* to sublethal levels of copper induced marked decrease in antioxidant enzyme activity in liver but the total superoxide dismutase(total SOD) exhibited a sharply increasing trend. A marked and progressively increasing trend in hepatic load of copper with respect to time of exposure was also indicated in the above investigation. After a 14 day period of exposure of carp to five concentrations of copper sulphate, enzyme activities increased considerably in serum and gills and pathological disturbances were evident in gills (Karan *et al*., 1998). While investigating the biomarkers of oxidative stress in *Wallago attu* due to environmental exposure to heavy metals including copper, Parvez *et al* (2006), observed discernible changes in antioxidant enzyme activities in liver, kidney and gills. According to them, oxidative stress may not be a primary factor causing disturbances of survival, growth and reproduction. It may only be contributing to several stressors that fish are likely to face within a polluted environment. Hansen et al (2006) performed an in-depth analysis of biochemical effects of copper exposure in various tissues of brown trout (Salmo trutta). Superoxide dismutase (SOD) and catalase (CAT) enzyme levels were affected in all tissues during the exposure. Levels of metallothionein (MT) and activity of SOD and CAT dealing with metal-induced oxidative stress appear to be regulated not only through gene expression, but also through post translational mechanisms. The authors concluded that the gills seem to be incapable of metallothionein production and the reduced glutathione (GSH) might be the defense molecule of choice in gills.

The mechanism of uptake of copper by fish via the gills is a complex process. The constituents of the external gill surface in fish have a p^K value of 3.6 and at environmentally relevant pH values(>5), the

constituents of gill epithelia will be fully ionized resulting in negatively charged gill surfaces and potential metal interaction sites (Reid and Mc Donald, 1991). The p^K refers to the condition where the anion concentration (A^-) is equal to the concentration of the conjugate (HA). Ionic and covalent indices of copper is higher than those of iron and zinc and therefore copper would have a tendency to bind to a more diverse selection of ligands and it could therefore be expected that the gill binding capacity of copper would be greater (Wepener *et al*., 2001). Other studies have shown that adsorption of copper in the gills of freshwater fish was extremely rapid, occurring within the first four hours of exposure (Grosell *et al*., 1997). According to this study, copper bioconcentration would only occur within the gill tissue once the affinity of copper binding sites on the gill surface has decreased due to saturation. Once inside the intracellular compartments of the gill, there is a greater variety of binding sites like glutathione, metallothioneins and ATP ases.

Oxidative stress responses in freshwater teleost *Esomus dandricus* in response to exposure to copper were so apparent that it can be considered as biomarkers of oxidative damage (Vutukuru *et al*., 2006). Increased lipid peroxidation in viscera concomitant with decrease in the activity of catalase and superoxide dismutase clearly demonstrated the oxidative stress. Direct effect of dietary copper on lipid peroxidation in the liver, kidney and intestine of *Salmo salar* was reported by Berntssen *et al* (2000). But increased lipid peroxidation was accompanied by a decrease in glutathione peroxidase activity.

Study of pesticide induced effects on various antioxidants in fish and other aquatic organisms can provide the information about the ecotoxicological consequences of pesticide use. Among the pesticides, herbicides are the most dangerous for aquatic contamination both of the

sea and of freshwater since they are used near the soil, and, in many instances, in the water for aquatic weed control.

India is nearly self sufficient in its pesticide requirement, with around 95% of needs being met by local production. The country manufactures more than 90,000metric tones of pesticide annually, ranking the first in Asia and 12th in the world. The Indian pesticide industry has a turnover of more than 4.5 billion US dollars and an annual growth rate of 2.5%. In India, insecticides account for 76% of total domestic market while herbicides and fungicides have a significantly higher share in the global market (Lal, 2007). Though the application of pesticides is based on their toxicity targeted pests, they directly cause ill effects on non target species. This is especially so in aquatic animals since pesticides eventually reach aquatic ecosystem in considerable amounts as run off from land, contaminated groundwater, bottom sediments, urban runoff and outputs from municipal water treatment and manufacturing plants (Lal, 2007). Persistence of methyl parathion (metacid) in carp rearing pond was studied by Sabharwal and Belsare (1986). The experimental inclusion of 0.05% of Metacid, degraded progressively in course of time in water but accumulated in flooded soil, aquatic plants and fish within one day. Thereafter, its degradation was observed in soil and plant, but it continued to accumulate in carp even after the third day.

Pesticide-induced oxidative stress has also been a focus of toxicological research during the last decade. Organisms have evolved a variety of responses that help to compensate the physiological impact of environmental contaminants. The antioxidant defense mechanism forms the crux of this whole system. There has been a increasing efforts to employ antioxidants as a biomarker of toxic responses.

Organophosphate (OP) based pesticides account for a major percentage of pesticides used in domestic, agricultural and industrial applications throughout the world (Asplin, 1994). They are highly popular because they are effective, non persistent and relatively less expensive. Due to their rapid breakdown in water accompanied by low environmental persistence, organophosphate pesticides have largely replaced the use of organochlorines in recent years (Li and Zhang, 2001). The main target of organophosphate pesticides is acetyl cholinesterase (AChE) which hydrolyzes acetyl choline (Ach) in cholinergic synapses and in neuromuscular junctions. Resulting accumulation of acetyl choline in the synapses induces hyperactivity in cholinergic pathways. Some organophosphate pesticides are bioactivated readily in vivo by oxidative desulfuration to their oxons, which are very potent anticholinesterase agents.

There are increasing evidences to show that organophosphates reach the freshwater environment at concentrations high enough to affect aquatic animals (Hai *et al*., 1997). In this regard, fish are particularly sensitive to organophosphorous compounds. Methyl parathion (MP) is one of several organophosphorous compounds developed to replace organochlorine compounds (Machado and Fanta, 2003). Methyl parathion is extensively applied as an insecticide in agriculture, food storage shelters and pest control progarammes due to its high activity against a broad spectrum of insect pest. It is also widely used in fish culture systems to eliminate aquatic larvae of predatory insects that threaten fish larvae (Silva *et al.*, 1993). Methyl parathion is a highly toxic insecticide ranked by USEPA as a class I toxicant.

As in the case of other xenobiotics organophosphates are also capable of inducing oxidative stress leading to inactivation of antioxidant enzymes and depletion of oxyradical scavengers (Videira *et al*., 2001). In addition to

the inhibition of acetyl cholinesterase activity (as is the case with many of the pesticides), it has been reported that organophosphate compounds induce oxidative stress (Hai *et al*., 1997., Mohammad *et al.*, 2004).

Exposure of *Brycon cephalus* for 96 hrs in 2 ppm of methyl parathion (Folisuper 600) contaminated water has recorded marked variation in antioxidant enzyme activity (Monteiro *et al.*, 2006). The exposure resulted in the induction of superoxide dismutase, catalase and glutathione S-transferase (GST) activity in liver, gill and muscles. Although these enzymes recorded increased activity with marked rise in lipid peroxidation in all tissues, the level of glutathione peroxidase (GPx) showed a decrease in activity in gill and muscle, however, the level of glutathione peroxidase in the liver remained unaltered indicating a null effect. Gills and white muscles of fish were found to be the most sensitive organs of *Brycon cephalus* with poor antioxidant potentials. The metabolism of methyl parathion occurs principally in liver (Dauterman, 1971., Garcia-Repetto *et al.*, 1977).

In the mosquito fish (*Gambusia affinis*) Kavitha and Rao (2006), reported changes in locomotory behaviour and variations in antioxidant enzyme activities as a result of exposure to the organophosphorous pesticide, monocrotophos. The antioxidant enzyme activities and levels of lipid peroxidation increased during the first eight days of exposure which gradually leveled off probably indicating a recovery from the initial onslaught. A positive correlation was found between the recovery pattern of acetyl cholinesterase and locomotor behaviour.

The use of non enzymatic antioxidants as biomarkers in fish is not well documented. A recent study in *Channa punctatus* carried out by showed that non enzymatic antioxidants including glutathione, vitamin C,

protein thiols and non protein thiols are capable of imparting protective effect against antioxidative stress induced by paraquat, an organophosphorous herbicide (Parvez and Raisuddin, 2006). The study validated the use of non enzymatic antioxidants as biomarkers of toxicity due to pesticides in fishes. Depletion of glutathione (GSH), a non enzymatic antioxidant always indicates oxidative stress in fish. Glutathione plays a cardinal role in protecting the cell from oxidative damage and for detoxification and excretion of xenobiotics. Exposure of *Channa punctatus* to sublethal concentrations of metacid 50(0.106ppb) and carbaryl(1.6ppm) resulted in significant changes in hepatic and nephric glutathione levels (Ghosh *et al*., 1993).

Oruc and Usta (2006) evaluated the oxidative stress responses to sublethal levels of diazinone in *Cyprinus carpio*. This insecticide was found to elicit oxidative stress in gills, muscles, and kidney by increased levels of superoxide dismutase. They also observed the existence of a protective function of antioxidant enzymes against lipid peroxidation in the muscle tissue. Production of a tissue-specific adaptive response in carp to neutralize the oxidative stress due to pesticide exposure was found to exist.

An evaluation of the oxidative and neurotoxic effects of diazinon in the gills, kidney, muscles and alimentary tract of *Oreochromis niloticus* was carried out by Durmaz *et al* (2006). Sublethal diazinon concentrations (0.1, 1 and 2ppm) for 1, 7, 15 and 30 days, lead to decrease in acetyl cholinesterase activity in all tissues, with maximum inhibition in kidney. Diazinon induced increased the activity of suproxide dismutase in all the tissues, while kidney was the most affected organ. Tissue-specific alterations were observed in catalase and glutathione peroxidase (GPx) activities. The changes observed in the study were generally tissue specific and dose-dependent.

Parathion tends to accumulate in fish because of its high lipid solubility (the log octanol/water coefficient is 3.56 and the biocincentration factor is 355 for rainbow trout). In an attempt to characterize the toxicokinetics of parathion and paraoxon , Abbas *et al* (1996) found that the permeability of the gill epithelium of rainbow trout to parathion was very high and that the rate-limiting factor in its uptake was the delivery capacity of water to the absorption. Paraoxon was not detected after water and intra arterial administration of parathion showing its poor metabolism in fish. Significant branchial excretion of paraoxon was observed. The half life of parathion (56± 2.8hr compared with10.0±1.7 hr in rats) suggested that parathion may persist for a relatively long time in fish.

Malathion, an organophosphatic pesticide, is capable of inducing oxidative damage in *Sparus aurata* as evidenced by increased microsomal lipid peroxidation and the formation of new less active isoforms of superoxide dismutase (Pedrajas *et al*., 1995). Though there occurred an increase crease in microsomal thiobarbituric acid reactive substances (TBARS) after two days' exposure and it decreased below control levels after 7 days. This is explained by biotransformation of malathion or by the repair of the oxidative damage induced in membrane lipids.

Organophosphorous pesticides used to treat external parasitic infections of farmed fish are found to cause reactive oxygen species induction. For instance, dichlorvos, extensively used in Spain to treat copepod infestations in farmed fish induced oxidative disturbances in European eel (Pena-Llopis *et al*., 2003). The oxidation of glutathione (GSH) and inhibition of glutathione reductase(GR) indicated the presence of oxidative processes.

The neurotoxic effects of organophosphorous pesticides were traditionally thought to result from excessive accumulation of acetyl

choline and overstimulation of muscarinic receptors. However, research works carried out later identified the sequence of cellular events involved in organophosphorus induced toxicity mediated by NMDA (N-methyl D-aspartic acid) receptor. Such studies conclude that glutamate is the major excitoxin mediating neurotoxicity of organophosphorous pesticides (Solberg and Belcin, 1997). Although oxidative stress is present in the organophosphorous induced delayed neuropathy, several studies report the generation of reactive oxygen species and the onset of oxidative stress a few hours after organophosphorus intoxication. Therefore excessive oxidative stress might lead to sufficient brain and muscle necrosis, dysfunction of central nervous system and muscle tissues (Santos, *et al*., 2002) which results in respiratory distress and death of the organism.

Signs of intoxication such as muscular twitching combined with gyrating movements resulting from methyl parathion exposure were reported in *Anguilla anguilla* by Sancho *et al* (1997).

Organophosphorous pesticides can cause changes in ionic regulation in fishes. Chloride ions along with sodium and potassium play an important role in neuromuscular excitability, acid base balance and osmotic pressure of the body of teleosts (Singh *et al*., 2002). In *Heteropneustes fossilis*, Misra and Srivastava(1983) recorded hyperchloremia and hypochloremia at different time intervals of at different time intervals of acute exposure to malathion. Srivastava and Srivastava(1988) reported that malathion induced a dose-time biphasic response in blood chloride of the catfish *H.fossilis.* 1.46 ppm of malathion caused hypochloremia on the 15^{th}, 25^{th}, and 50^{th} day after exposure. Rao *et al* (1983) observed a decrease in the ionic levels of sodium, potassium and calcium in *Tilapia mossambica* . Srivastava *et al* (1997a, b) reported hypocalcemia in *H.fossilis* due to chlorpyriphos exposure.

Sublethal exposure of *Clarias batrachus to* the organophosphorous pesticide Rogor exerted an overdose effect (decrease) on the glycogen content of muscle tissue. A significant elevation in muscle lactate content, increased activity of lactate dehydrogenase and phosphorylase (a and b) were also observed in muscles (Begum & Vijayaraghavan, 1999). Such a condition occurs due to the pesticide-induced reduction in oxidative metabolism Exposure and recovery studies in eel (*Anguilla anguilla*) showed disturbances in energy metabolism as a result of fenitrothion exposure (Sancho *et al.*, 1997). Hyperglycemia, significant elevation in blood lactate levels and hypoproteinemia were evident in blood, gill and liver and were lowered to control values during the recovery period. The fish apparently shifted to anaerobic metabolism and proteolysis under sublethal stress of the pesticide. A 48 hr exposure of *Tilapia mossambica* to sublethal levels of methyl parathion leads to regulatory changes in aldolase and phosphorylase (Rao and Rao, 1983).

Reports of variations in hematology in fishes as a result of organophosphate insult are also available in literature. In *Cyprinus carpio*, Chandrasekara and Pathiratne (2005) demonstrated alterations in hematology and acetyl cholinesterase activity after and exposure to trichlorfon. A reduction of 67% beyond recovery was noticed in leucocyte count when exposed to 0.5 malachite green L^{-1} of trichlorfon for 24 hrs.

Malachite green, also called aniline green, basic green 4, diamond green B or Victoria green B is known by the IUPAC name : 4-[(4-dimethyl aminophenyl)-phynol-methyl]-N,N-dimethyl-aniline is a toxic chemical primarily used as dye. At low concentrations the dye is used as a topical antiseptic to treat parasitic fungal or bacterial infections in fish and fish eggs (Alderman, 1985., Meinelt *et al.*, 2001, 2003). It is widely used as an

efficient drug for treating saprolegniasis in fish hatcheries (Behrooz *et al.*, 2006., West, 2006). The compound also finds application in biological staining for microscopic analysis, bacterial staining, as an absorber in dye lasers, and in the detection of latent blood in criminalistics. It is also used as a food-coloring agent, food additive, a medical disinfectant and antihelminthic as well as dye in silk, wool, jute, leather, coir, cotton, paper and acrylic industries (Culp and Beland, 1996). The first reported use of malachite green was to control fungal infections in hatchery fish (Foster and Woodbury, 1936). Since then the compound has been used routinely in aquaculture as an antifungal and anti parasitic agent and as a general hatchery disinfectant.

Aquaculture industry has been using malachite green extensively as a topical treatment by bath or flush methods without paying any attention to the fact that topically applied therapeutants can also be absorbed systematically to produce significant internal effects. However, malachite green has now become a controversial compound due to the risks it poses to the consumers of treated fish (Alderman and Clifton-Hadley, 1993).

Though all the use of this dye has been banned in several countries and not approved by USFDA (Chang *et al*., 2001), it is still being used in many parts of the world due to its low cost, ready availability and efficacy(Schnik, 1988). Considerable amount of research is being devoted to work out the wide spectrum of biological effects it exerts on different animals. The US Food and Drug Administration have nominated malachite green as a priority chemical to test carcinogenicity (Culp and Beland, 1996). Although the use of malachite green has been prohibited for several years, non-compliant traces of its residues have still been detected in the monitoring schemes performed in European Union (EU). Finding residues of malachite green in aquaculture products has also been frequently reported in Rapid Alert System for Food and Feed (RASSF)

notification of the European Commission (Halme *et al*., 2006). Residues of malachite green have a notorious persistence in fish partly because of their poikilothermic nature that slows their metabolism compared to endothermic animals (Yndestad, 1993). In eel (*Anguilla anguilla*), Bergwerff *et al* (2004) reported no detectable residues of malachite green at their slaughter-ripe age, but any treatment following that life stage may give residues of the drug in market-ripe fish.

Toxicity of malachite green on teleosts has attracted the attention of numerous workers. Bills *et al* (1977) made a detailed study on LC_{50} values of malachite green on adults and fingerlings of various fish species and observed the effects of pH, temperature and exposure time on the toxicity of this dye. Srivastava *et al.,* (1995 a) observed that variations in the period of exposure bring about changes in LC_{50} values of malachite green in a fresh water catfish *Heteropneustes fossilis*. Several studies have shown that the dye is highly toxic to freshwater fish at chronic levels (Hormazabal *et al.,* 1992., Alderman and Clifton-Hadley, 1993). Carcinogenesis, mutagenesis, chromosomal fractures, teratogenecity and reduced fertility have also been reported in rainbow trout on treatment with malachite green (Bills *et al.*, 1977., Meyer and Jorgensen,1983., Gouranchat, 2000). Chromosomal aberration in eggs of malachite green treated freshwater fish has been reported by Worle (1995). This dye also acts as a respiratory enzyme poison (Werth and Boiteaux, 1967) and causes respiratory distress in rainbow trout (Ross *et al*., 1985) and *Oreochromis niloticus* (Omoregie *et al*., 1998).

Disturbances in carbohydrate metabolism and osmoregulation have been reported in catfish (*Heteropneustes fossilis*) after exposure to malachite green (Srivastava *et al*., 1995b). The dye causes hepatic and muscular glycogenolysis with concomitant hyperglycemia and hyperchloremia. The

dye has been found to increase sensitivity to hypoxia and impairs protein synthesis in fish (Svobodova *et al*., 1997). The compound has been shown to promote dissipation of the mitochondrial membrane potential, resulting in enhanced mitochondrial permeability and swelling followed by respiratory inhibition (Kowaltowski *et al.*, 1999). It causes decrease in plasma phosphorus and calcium levels in Tilapia (Yildiz and Pulatsu, 1999).

Decrease in hematocrit values and anaemic responses have been reported in rainbow trout and *Clarias gariepinus* (Tanck *et al.*, 1995., Musa and Omoregie., 1999). Decrease in monocyte count, hematocrit value and mean corpuscular volume and increase in mean corpuscular hemoglobin concentration have also been noticed after exposure to malachite green in fish(Svobodova *et al*., 1997).

Malachite green used to treat and prevent fungal and parasitic infections is reduced to leucomalachite green (L malachite green) and accumulate in the tissues of exposed fish (Doerge *et al*., 1998a). It is stored primarily in liver, kidney, muscles, skin and serum of fish. (Alderman and Clifton-Hadley, 1993., Machova *et al*., 1996., Rushing and Hansen, 1997., Doerge *et al.,* 1998a). Plakas *et al* (1996) analyzed uptake, tissue distribution and metabolism of malachite green. It was rapidly absorbed and concentrated in the tissue during water borne exposure. Alderman (1992) reported that malachite green is absorbed by fish and that all the fish tissues accumulated significant and fairly persistent residues of the dye.

Antioxidative vitamins have a number of biological activities such as immune stimulation, inhibition of nitrosamine formation and an alteration of metabolic activities of abnormalities including cancer. They can prevent genetic changes by inhibiting DNA damage induced by reactive oxygen

metabolites (ROMs). The major protective function of the vitamins against cancer is the scavenging of ROMs (Ray and Hussain, 2002).

Vitamin C (ascorbic acid) is an important water soluble antioxidant in biological fluids and an essential micronutrient required for normal metabolic functioning of the body. It acts as a biological reducing agent for hydrogen transport. It neutralizes ROMs and reduces oxidative DNA damage and genetic mutations (Frei, 1994). It acts as a co-antioxidant by regenerating α-tocopherol from the α-tocopheroxyl radical produced during scavenging of ROMs (Packer, 1997). Vitamin C can protect host cells against harmful oxidants released into the extracellular medium (Ray and Hussain, 2002).The free metal ion-independent protein oxidation in cells is exclusively prevented by ascorbic acid. Other scavengers of ROS including superoxide dismutase (SOD), catalase (CAT), glutathione, α-tocopherol, β-carotene etc are ineffective in protection against protein damage (Ghosh *et al*., 2003).

Since fish cannot synthesize vitamins at all or can only synthesize in insufficient quantity for normal development, growth and maintenance, they must be supplied in the diet. In comparison to terrestrial animals, the information available on vitamin requirements of aquatic species is scanty.

Vitamin C is an indispensable nutrient for fish, as they cannot synthesize this nutrient due to the lack of the enzyme L-gulonolactone oxidase (EC 1.1.3.8), therefore they depend on exogenous supply through dietary source (Shiau and Lin, 2006).

Dietary deficiency of vitamin and minerals leads to a number of pathological conditions in fishes. A detailed literature survey on this aspect was conducted by Lall and Lewis-McCrea (2007). Tilapia showed classical

vitamin C deficiency signs (reduced growth ratio, delayed wound healing, capillary fragility, hemorrhage, immunosuppression etc) when fed with a vitamin deficient diet in the absence of natural foods (Kumari and Sahoo, 2005., Shiau and Lin, 2006).

More than 25mg of ascorbic acid per kilogram diet is sufficient for juvenile and on-growing fish (NRC, 1993), while larval fish have a higher requirement (Dabrowski, 1992). Supplementation of dietary ascorbic acid is essential for a variety of physiological functions including increased resistance and wound healing (Halver, 2002). Tolerance to environmental stressors (Gapasin *et al*., 1998), as well as regulation of collagen synthesis also is known to demand ascorbic acid by organisms (Dabrowski, 1992). Consumption of oxidative products depletes antioxidant within the body such as vitamin C and E, selenium and carotenoids. These exogenous dietary antioxidants aid in preventing lipid peroxidation through their association with the cellular lipid membranes (Winston and Di Giulio, 1991., Halliwell and Gutteridge, 1996).

There are references in literature about the dual role the ascorbic acid. Buettener and Jurkiewicz (1996) concluded that vitamin C can serve as a donor antioxidant in free radical mediated oxidative processes. However as a reducing agent, it is also able to reduce redox-active metals such as copper and iron, thereby increasing the pro-oxidant chemistry of the metals. Thus ascorbic acid can serve both as a pro-oxidant and antioxidant. In general at low ascorbate concentrations, ascorbate is prone to be a pro-oxidant and at high concentrations ascorbate is prone to be an antioxidant. The author has elucidated the chemistry and mechanism of free radical protection by ascorbate in biological systems. It is proposed that ascorbate radical can function as a marker of free radical mediated oxidative stress. The ability of

ascorbic acid to stimulate the immune response besides its essentiality for growth when supplied at dietary levels higher than standard ones has been established in a number of animals including fish (Waghoo, 1994). The immunostimulatory effect ascorbic acid may be related to its antioxidant activity as a free radical scavenger, protecting cells from auto-oxidation and maintain their integrity for an optimal functioning of the immune system (Kumari and Sahoo, 2005). Considerable reduction in embryotoxicity to paraquat was found in *Xenopus laevis* as a result of inclusion of high doses (200 mg/l) of vitamin C (Vismara *et al*., 2001). The study strongly attributed the drastic reduction in oxidative damage to the administration of ascorbic acid.

Vitamin C is known to exert a powerful antioxidant effect on biological water soluble compartments and represent an outstanding antioxidant in plasma; it reacts directly with superoxide anion (O_2^-), hydroxyl radicals and lipid hydroperoxides (Jacob, 1995., Yu, 1994). Majority of in vivo studies showed a reduction in markers of oxidative DNA, lipid and protein damage after supplementation with vitamin C. Even in the presence of iron, vitamin C predominantly reduces in vivo oxidative damage, despite its well known pro-oxidant properties in vitro in buffer systems containing iron. Vitamin C protects against cell death triggered by various stimuli and a major property of this protection has been linked with its antioxidant ability (Valko *et al*., 2006).

Stress responses to xenobiotics can be assessed by histopathological techniques as they are sensitive, rapid and reliable tools. Structural changes and damages of tissues provide a direct record of stress effects. Stress-borne biochemical and subcellular dysfunctions tend to lead to cell damage. Chronically stressed organisms are often prone to structural and biochemical changes which would ultimately affect their physiology and well being.

Histopathological analyses of tissues could explain the stress effects expressed by damages occurring in organisms and also the extent of adaptability to changes.

Gills are the primary target and uptake site for many xenobiotics in water (Evans, 1987). Gill epithelial cells of rainbow trout have been identified as suitable cell models for toxicity screening purposes (Lilius *et al*., 1995). The uptake of water-borne copper in fish occurs primarily through the gills (Pelgrom *et al*., 1995). Adham *et al* (2000) have reported degenerative changes in the histology of gills, liver and kidney of *Clarias gariepinus* due to deprivation of vitamin C in the diet. A comprehensive review structural change in fish gill induced by heavy metals, pesticides, detergents, polycyclic aromatic hydrocarbons, ammonia, extremes of temperature and pH was given by Mallatt (1985). An account of etiological explanations for various sort of lesions were also included in this review. According to the work of Shaw and Handy (2006) dietary copper exposure induced structural alterations in the liver of *Oreochromis niloticus,* though no overt changes were noticed in gills. The liver returned to near normal condition after some days of suspension of dietary supply of copper. In a study, Fernandes *et al* (2007) observed changes in activities of antioxidant enzymes like superoxide dismutase, catalase and glutathione S transferase in gill and liver of *Liza saliens* and this was found to lead to histopathological abnormalities. The decrease in catalase activity in copper contaminated sites was attributed to osmotic stress caused by damaged gill epithelium. As a confirmatory measure of altered enzyme activities in response to sublethal copper exposure in gill and serum of carp, Karan *et al* (1998) reported morphological changes in gills. Recovery from the stress was also evident in gills after a few days of depuration. Sublethal

concentration of water borne copper leads to degenerative changes in gill, liver and kidney in *Lates calcarifer* (Krishnani *et al.*, 2003).

Gerundo *et al* (1991) have reported sinusoidal congestion, and focal necrosis in the liver of rainbow trout as a result of exposure to repeated doses of malachite green. Degenerative changes including cirrhosis in response to malachite green exposure were found to occur in the liver of *Heteropneustes fossilis* (Srivastava *et al*., 1998a). Exposure to this dye also caused severe damage to gills in rainbow trout (Gerundo *et al.,* 1991), and *H. fossilis* (Srivastava *et al*., 1998b). The dye caused hyperplasia of epithelial cells in the proximal convoluted tubules and shrinkage of glomeruli, forming gaps between capsule and tuft, necrotic changes like karyorrhexis, karyolysis, pyknosis and desquamation of epithelial cells and vacuolation in the kidney of *H. fossilis* (Srivastava *et al*., 1998b).

Different types of synthetic pesticides were found to be capable of producing deleterious effects in vital organs of fish. Altinok and Capkin (2007) studied the sublethal effects of endosulfan (organochlorine pesticide) and methiocarb (carbamate pesticide) in liver, kidney and gills of rainbow trout (*Oncorhynchus mykiss*). Histopathological changes were not evident in liver and kidney but the gills exhibited structural abnormalities due to the exposure to methiocarb. But endosulfan was capable of effecting structural changes in liver, kidney and gills. Exposure of *Cyprinus carpio* to acute level of deltamethrin (a synthetic pyrethroid) elicited damages to gill and kidney (Cengiz (2006). Progressive degenerative changes in the gills of *Oreochromis mossambicus* as a result of prolonged exposure to a constant level of chlorpyriphos (organophosphorous pesticide) in the culture medium was reported by Rao *et al* (2003). Formation of large subepithelial spaces on day 3, aneurism, lesions and erosion of lamellae on day 12 and excessive mucus secretion accompanied by extensive fusion of secondary

lamellae on day 18 were the major damages recorded by these authors. Similar observations like necrosis and exudation of mucus were noted in the gills of *Lepomis macrochirus* exposed to 0.05 ppm of malathion (Richmond and Dutta, 1989) and different concentrations of diazinon (Dutta *et al*., 1997). Duration dependent toxic responses to profenofos on gills of *Oreochromis mossambicus* were detailed by Rao *et al* (2003). Changes in the gill morphology of mosquito fish (*Gambusia affinis*) to malathion at different intervals (10, 20 and 30 days) as described by Cengiz and Unlu (2003) were essentially similar to those given by Rao *et al* (2003). Sublethal effects of endosulfan on the gill morphology of *Cyprinus carpio* was assessed by John and Jayabalan (1993). Toxic manifestations like curling and bulging of primary lamellae, hyperplasia and lamellar fusion were observed in the fish and moreover the damages were reported to be dependent both on duration of exposure as well as concentration of toxicant. Sakre and Al lail (2005) have reported severe histological lesions in the liver of *Clarias gariepinus* in response to exposure to synthetic pyrethroid, fenvalerate.

Chapter -3

LETHAL TOXICITY STUDIES

Contents

3.1 Introduction

Bioassay tests, also known as toxicity tests are the routine practices in pharmaceutical and toxicological investigations. The objective of such a test is to find out the concentration at which a test animal produces a selected response, usually pernicious in a population under controlled conditions of exposure. The adoption of a quantal response by having only two experimental alternatives dead or alive from which the relation between concentration and percentage effect can be defined is followed.

The concentration which creates a 50% live-death condition is defined as the median lethal concentration or LC 50. Death is usually used as a criterion of change in the 96 hour test while the extension of duration can be adopted for investigation of the various other related physiological,

biochemicals, behavioural or histological changes at concentrations considerably below the lethal concentration. The outcome of lethal toxicity studies on fishes by exposing them to copper sulphate and Metacid 50 are presented in the chapter.

3.2 Material and methods

3.2.1 Test animals

Oreochromis mossambicus (Peters), commonly known as tilapia was selected for the present study because of its availability throughout the year and suitability for toxicity testing. This teleost fish belongs to the Family Cichlidae in the Order Perciformes of the Subclass Actinopterygii. Tilapia, an exotic fish was introduced to the Indian inland waters in 1952 and is a good candidate for aquaculture. However its culture in India was later discouraged due to the inherent capacity of the species for prolific growth and reproduction thereby affecting the life of indigenous species suitable for aquaculture. The fish is widely distributed in freshwater ecosystems and also can tolerate wide fluctuations in salinity. It grows to a maximum size of 360mm (standard length). The species is an opportunistic omnivore consuming planktonic algae, aquatic insects and isopods. Small fishes are also consumed.

3.2.2 Laboratory conditioning of test animals

The specimens were collected from culture farms of the State Fisheries Station, Kerala State Agricultural University, Puthuveypu, Kochi and transported to the laboratory in well oxygenated polythene bags containing clean pond water.

Soon after arrival in the laboratory, they were released into freshwater in 1000 litre glass aquaria after proper acclimation. The glass aquaria were kept as holding tanks to maintain the bulk collection. The

polythene bags carrying the fishes were floated in the aquarium water for one hour. Then the fish are allowed to enter into the aquarium voluntarily by opening the bags. Vigorous aeration was provided in the tanks with natural photoperiod. The fishes were fed *ad libitum* with commercial pellet feed and minced clam meat. The walls of the holding tank were thoroughly cleaned periodically to avoid fungal growth. The excreta are siphoned off on a daily basis to prevent the build up of ammonia in the medium. Each batch of the bulk collection of fishes was conditioned in the holding tank water for 30 days before employing them for the experiments.

The tanks were kept in the wet laboratory, the temperature of the water was 26ºC±1ºC and the dissolved oxygen level was 7-7.5ppm. The pH of the water ranged from 6.9-7.3 and the hardness of the water was below detectable limits.

Individuals measuring 7.0cm±0.5cm in total length and weighing 14.0±3.2g were selected for the present study. A mixed population of one hundred individuals was used to exclude the possibilities of influence of sex of the individuals on the parameters studied. Healthy and vigorous individuals of uniform size selected from the bulk collection brought from the field were used for the experiments.

3.2.3 Toxicants

Three test compounds used for the study belonged to three categories *viz* a heavy metal, a pesticide and a dye. Copper was the heavy metal applied in the present study and the compound was copper sulphate pentahydrate ($CuSO_4.5H_2O$). The compound is used as a fungicide and algicide in aquaculture industry. The dye used here was malachite green in its zinc free oxalate form. Though banned in aquaculture, it is still

being used in the sector to treat ectoparasitic infestations in culture tanks. Metacid 50, the organophosphorous pesticide, selected for the study is widely used in agricultural fields.

3.2.3.1 Copper

Stock solution of copper was prepared by dissolving 3.96 g of analar grade copper sulphate in 1 litre of distilled water to obtain a concentration of 1000 ppm. A measured quantity of the stock solution is diluted with freshwater to obtain the desired concentration of the medium.

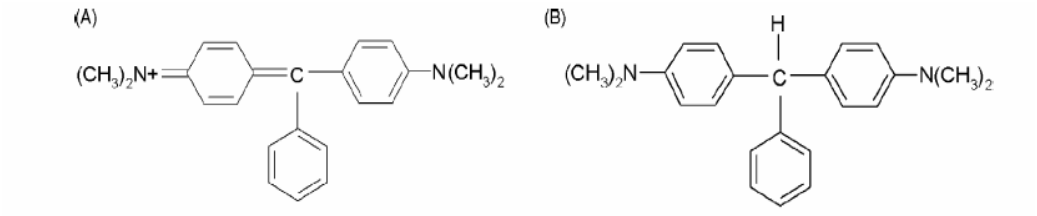
3.2.3.2 Metacid 50

Organophosphorous insecticide Metacid 50, was purchased from Bayer CropScience, India Ltd., Mumbai. The emulsifiable concentrate contained 50% w/w methyl parathion (o,o dimethyl o-(p-nitrophenyl) phosphorothionate) as its active ingredient with 10% w/w and 40% w/w of emulsifiers and solvents respectively. A suspension of this pesticide was prepared in water with acetone in 1:1 ratio and added to the test solution to get the desired concentration.

3.2.3.3 Malachite green

Aquatic grade malachite green oxalate (zinc free) was procured from Sencient India Ltd., Ahmedabad. The IUPAC name of the compound is Bis [4-[4-(dimethyl amino) benzhydrilidine] cyclohexa-2, 5-dien-1-ylidene] dimethyl ammonium] oxalate. Structural formula of the compound is given below indicating the original compound which is coloured and the colourless metabolite (leucomalachite green) formed inside the body of animals. The structural formula of the compound is given below:

(A) Green coloured original compound (B) Colourless metabolite (Leucomalachite green) formed in the body of animals due to enzymatic activity (after Sudova *et al*., 2007).



A 100 ppm stock solution of the compound (aquatic grade zinc free oxalate salt) was prepared by dissolving 0.1 g in 100 ml of distilled water. The required quantity is added to the experimental tank water and mixed well with a clean glass rod to get the desired concentration.

3.2.4 Studies on Lethal Toxicity

Experiments were carried out to assess the lethal responses of copper and Metacid 50 by the test organisms. Static renewal tests were adopted for both the test compounds (APHA1976, 1985., ASTM, 1980). Laboratory conditioned fishes of uniform size were selected to assess the medium lethal concentration of the two toxicants. Each experimental container was of 50 litre capacity, made of FRP and coated internally with teflon and provided with perpex lids. Ten fish each were accommodated in 50 litres of test solution. Duplicates and appropriate controls were run for each set of experiment. All the bioassays were carried out under laboratory conditions. The animals were not fed during the experiment and water was never aerated. The test medium was replenished totally every 24 hours. Care was taken to leave the animals with minimal disturbance.

3.2.4.1 Lethal Toxic Responses to Copper

The procedure adopted for the preparation of stock solution of the test compound (copper sulphate) is given under section 3.2.3.1. The experimental conditions and set up are explained in section 3.2.4 above.

The fishes were exposed to logarithmic series of concentrations of copper. Control set of ten animals were used for each concentration, kept in

water without the addition of copper sulphate. The specimens were inspected every 12 hours and were considered dead if no opercular beating was noticed and locomotory responses ceased even on mechanical stimulation. The dead animals were removed and the cumulative percentage mortality was recorded at every 12 hour. The median lethal concentration (LC 50) and its 95% confidence limits were calculated using probit analysis (Finney, 1971). The results were given in section 3.4.

The effect of exposure of *Oreochromis mossambicus* to copper concentration varying from 500 ppb to 2000 ppb is shown in table 3.1 and figure 3.1. It is evident from the results that mortality occurred at 500 ppb after 96 hour exposure and near 100 % mortality occurred in 1500 to 2000 ppb copper containing media. The 96 hour LC50 was calculated and was found to be 640 ppb.

3.2.4.2 Lethal Toxic responses to Metacid 50

The methods followed for preparing the stock solutions and test concentrations of the pesticide are explained under section **3.2.3.2**. The experimental conditions and methods adopted remain the same as given for lethal response studies on copper. The test concentration series were given in table 3.2.

In the case of Metacid 50, ten fish each was exposed to ten levels of Metacid 50 concentrations ranging between 1.24 ppb to 1.69 ppb. Lethality was gradual and 50% of the test organisms died in a concentration of 6.2 ppb. The toxicological statistics, log dose and probit regression are given in table 3.2 and graphical derivation of the LC 50 -96 hour is shown in fig 3.2.

3.2.4.3 Response to malachite green

Bioassays were not carried out with malachite green. As per the literature, 1ppm was found to be the maximum level of malachite green

applied for bath treatment to fishes to prevent or treat ectoparasitic or fungal attack (Mohapatra and Rengarajan, 1995., Srivastava *et al*., 1995a., Lanzing, 1965). The fishes were found to survive well when exposed to 100 ppb of the compound for more than 90 days. Test concentrations of 25 ppb and 12.5 ppb were fixed for sublethal studies as the aim of the exposure was to assess the biochemical and histological responses to minimal concentrations.

3.3 Results

Table 3.1. Determination 96 hour LC 50 (copper) in *Oreochromis mossambicus*

Conc. Of Copper (ppb)	No. of fishes died		No.of fish Used for Each trial	Mean % mortality	Log Conc. (x)	Probit Value (y)
	Trial 1	Trial2				
0	0	0	10	0	-	-
500	5	2	10	35	2.69897	4.615
1000	10	7	10	85	3.00000	6.036
1500	8	10	10	90	3.17609126	6.282
2000	10	10	10	100	3.30103	8.72

Probit analysis-Regression output

Constant=0.22125, Log LC_{50} = $\frac{\text{(Probit at 50\% mortality)} - \text{Const.}}{\text{X coefficient.}}$

Standard Error of y estimate = 0.87262

R^2 = 0.82524

No. of observations = 4

Degrees of freedom = 3

LC_{50} (probit) = antilog 0.806 = 0.640

Therefore LC_{50} = 640 ppb

X coefficient = 5.931

S.E. of coefficient = 1.92996

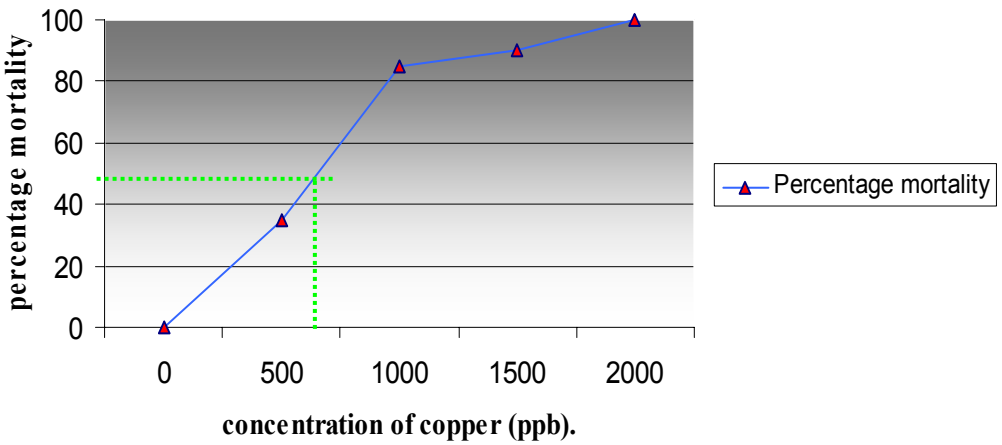


Fig. 3.1 Graphical derivation of 96 hour LC 50 for copper in *Oreochromis mossambicus*

Table 3.2 Toxicological Statistics for Metacid 50 in *Oreochromis mossambicus* Calculation of log dose/ probit regression line.

Period : 96 hrs LC_{50}= 6.2ppb

Conc. (ppb)	No. of animals used	% of Mortality	Log Dose	Emp. probe	Exp. probe	Work probe	Wt. Coefft.	wt			
			i	yo	Y	y		w	wi	wy	y'
1.24	10	10	0.0934	3.72	3.36	3.75	0.208	2.69	0.25125	10.0875	3.36
2.42	10	20	0.3838	4.16	4.09	4.16	0.439	4.71	1.8077	19.5936	4.09
3.7	10	30	0.5682	4.48	4.55	4.47	0.581	5.81	3.30124	25.9707	4.55
4.91	10	40	0.6911	4.75	4.86	4.74	0.627	6.27	4.3332	29.7198	4.86
6.2	10	50	0.7924	5	5.11	5.01	0.634	6.37	5.04759	31.9137	5.11
8.31	10	60	0.9196	5.25	5.43	5.25	0.601	6.16	5.66474	32.34	5.43
10.6	10	70	1.0253	5.52	5.69	5.52	0.558	5.58	5.72117	30.8016	5.69
12.5	10	80	1.0969	5.84	5.87	5.83	0.503	5.32	5.83551	31.0156	5.88
14.7	10	90	1.1673	6.28	6.05	6.21	0.439	4.71	5.49798	29.2491	6.05
16.9	10	100	1.2279	7.6	6.2	6.65	0.37	4.39	5.39048	29.1935	6.2

Sw = 52.01 Swx = 42.851 Mean x = 0.8239

Swy = 269.936 Mean y = 3.1901

Swxx = 40.100 Swyy = 1433.100 Swxy = 234.414

Regression eqn. y=5.1901+2.5053(x-0.8239) b=2.5053

Variance:0.003255 If Y=5.0,x=0.7480 and Conc=0.0056

Fiducial limits ml, m2, with 95% confidence:

ml=0.7480-1.96*SQR (0.003255)=0.6362

M2=0.7480+1.96*SQR (0.003255)=0.8598

No. of samples=10 Degrees of freedom=8

Chi square=2.008 Chi for 5%= 15.5070

Chi for 95%=2.7330

Chi square value is below 95% critical value (2.7330) therefore agreement is too good and should be examined critically.

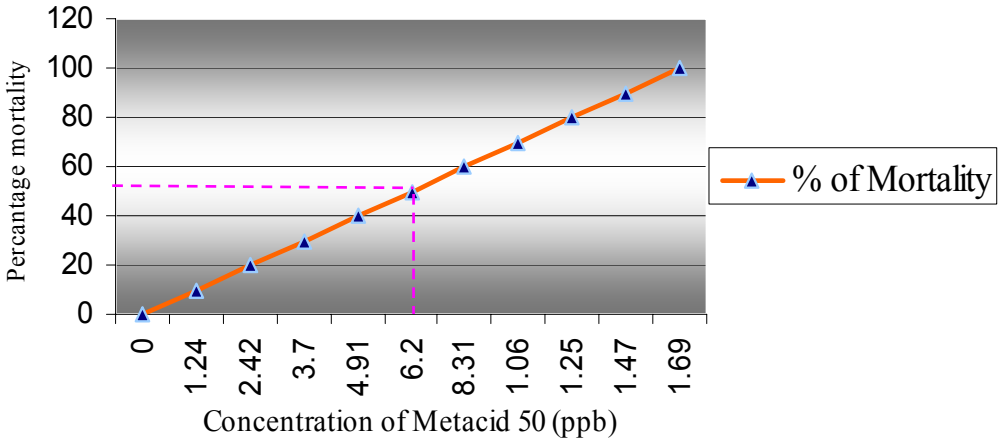


Fig. 3.2 Graphical derivation of LC 50 of Metacid 50 to *Oreochromis mossambicus*

3.4 Discussion

The present study on lethal toxicity of copper indicated that the 96 hour LC 50 value for fish of the experimental size group was 640 ppb.

The lethal toxicity of the metal to the same species (but of sizes different from those selected for the present assessment) was already established (Pelgrom *et al.*,1995., James and Sampath, 2003). According to Pelgrom *et al* (1995), the LC 50 for *Oreochromis mossambicus* of fingerling size was only 14.3 ppb and as per James and Sampath (2003) it was 4.6 ppm for individuals of size 8-9.5 cm.

During the experimental period, fishes were found to be reacting strongly even to slightest disturbance either mechanical or variation in light intensity. Such behavioural abnormalities included writhing with exhaustion and rapid swimming accompanied by increased opercular rate. The values obtained from the current study are clear indications of the fact that the LC 50 is greatly influenced by size of the experimental animal even though the influence of experimental situations like physico chemical parameters cannot be ruled out.

According to the toxicological statistics for Metacid 50, the 96 hour LC 50 is 6 ppb. The behavioural peculiarities resembled those that occurred during exposure to copper. The similarities in behavioural responses indicate that exposure to lethal levels of toxicant will result in certain death of *Oreochromis mossambicus* in natural conditions. However, in natural situations the animals will be in a position to swim away from the polluted area. This probably testifies the harmful effect of toxicant present at very low levels in chronically polluted areas. The study on sub lethal toxicity has shown that at concentrations considerably below LC50 levels each induce both biochemical and histopathological aberrations, which would ultimately affect the life and activity of these fishes.

Chapter -4

SUBLETHAL TOXICITY

Contents

4.1 Introduction

The study of biochemical responses in aquatic animals comprises an area of vigorous investigation within ecotoxicology for a number of reasons, including the need for highly sensitive biomarkers useful for biomonitoring in aquatic settings and the observations of elevated rates of structural changes in some aquatic species. Because of the role of reactive oxygen intermediates as cytotoxic mediators, the generation of free radicals and subsequent oxidative stress in aquatic animals like fish is of particular concern to environmental toxicologists.

An important aspect of free – radical mediated toxicity is that it is moderated in the host by several antioxidant cellular defense mechanisms

including enzymatic (i.e., catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase etc.) as well as non – enzymatic (e.g., vitamin C and E) systems. Antioxidants allow the organism to protect itself against xenobiotic oxidants. In addition, these enzymes enable reactive oxygen intermediate – producing cells and adjacent tissues to withstand the oxidative stress exerted by endogenously generated active oxygen species. Therefore, efficient elimination of reactive oxygen intermediates constitutes an essential component of the defense systems used by impacted species exposed to toxicants. While mammalian and bacterial antioxidants have been widely studied, less is known about these mechanisms in fish in general (Filho *et al*, 1992).

Sublethal toxic responses of organisms to a toxicant can be measured in terms of alterations or fluctuations in its vital physiological responses and rate functions. Changes in physiological activity and biochemical make up are interrelated. It is also known that altered biochemical machinery is a prelude to variation in physiology, serving as forecasting signals of toxicity. Induction of oxidative stress in the experimental fish by exposing them to copper, Metacid 50 and malachite green and estimating it by antioxidant enzyme assays is the mainstay of this chapter.

As a follow up of biochemical alterations, structural changes occurring in gill, liver and kidney were also studied. A broad outline of oxidative stress, antioxidant defense machinery and test tissues are given below.

4.1.1 Oxidative Stress and Antioxidant Defense Mechanisms

Oxidative stress refers to the cytological consequences of a mismatch between the production of free radicals and the ability of the cell to defend them. Oxidative stress can thus occur when the production of free radicals increases, scavenging of free radicals or repair of

oxidatively modified macromolecules decreases, or both. This imbalance results in a build – up of oxidatively modified molecules that can cause cellular dysfunction leading to cell death.

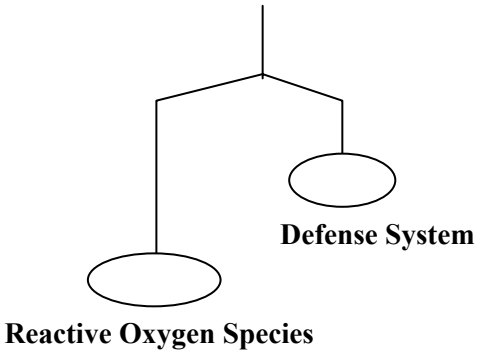


Fig. 4.1.1. Oxidative Stress Disturbed Balance in the Cell (After Gupta and Sharma, 1999)

Free radicals are normal product of cellular aerobic metabolism (Freeman and Crapo, 1982). Superoxide and hydroxyl species are the predominant cellular free radicals. Hydrogen peroxide and peroxynitrite, although not themselves are free radicals, contribute prominently to the cellular redox state. Together, these molecules are referred to as reactive oxygen species (ROS). The major sources of reactive oxygen species are mitochondrial oxidative metabolism, enzymatic reactions involving mixed function oxidation and auto-oxidation of small molecules. Mixed function reactions occur in the cytoplasm, plasma membrane and nuclear membrane, endoplasmic reticulum and peroxisomes. Superoxide is formed by leakage of high – energy electrons along the mitochondrial electron transport chain and by a variety of cytosolic and membrane – bound enzymes. Hydrogen peroxide also is produced along the electron transport chain as well as through auto-oxidation of small molecules and dismutation of superoxides by superoxide dismutase. Though not reactive itself, hydrogen peroxide in the presence of reduced metal, forms the highly reactive hydroxyl radicals.

$$H_2O_2 + O_2 \xrightarrow{Cu\ 2+ / Fe\ 2+} OH + OH^- + O_2$$

Peroxynitrite formed by the reaction of nitric oxide with oxygen is a highly reactive molecule that also breaks down to form hydroxyl radicals.

Reactive oxygen species can produce functional alterations in lipids, proteins and DNA. The incorporation of molecular oxygen into polyunsaturated fatty acids initiates a chain reaction in which reactive oxygen species including hydroxyl radicals, hydrogen peroxide, peroxyl and alkoxyl radicals are formed. Oxidative lipid damage, termed lipid peroxidation, produces a progressive loss of membrane fluidity, reduces membrane potential and increased permeability to ions such as calcium (Freeman and Crapo, 1982)

The Antioxidant Defense Mechanisms.

The antioxidant system protects tissues from the deleterious effects of free radicals. Free radicals are eliminated from the body following reactions with other free radicals or with antioxidants. Various antioxidant mechanisms are in operation leading to scavenging or detoxification of the reactive oxygen species. The antioxidant defenses are classified into three categories *viz* preventive or primary, diet derived or secondary and reparative or tertiary.

Primary Antioxidants

Antioxidant enzymes like superoxide dismutase, catalase glutathione peroxidase and glutathione reductase and non-enzymatic molecules like minerals and some proteins are included in this category. They are preventive in nature as they act by quelling of O_2, decomposition of hydrogen peroxide and sequestration of metal ions.

a) Superoxide Dismutase (SOD, E.C. 1.15.1.1)

Superoxide dismutase is a metalloprotein found in both prokaryotic and eukaryotic cells. The iron containing (Fe – SOD) and the manganese

containing (Mn – SOD) enzymes are characteristic of prokaryotes (Sheehan and Power, 1999). In eukaryotic cells, the predominant forms are the copper containing enzymes and the zinc containing enzymes located in the cytosol. The second type contains manganese and is found in mitochondrial matrix that contributes up to 60% of total tissue activity. The superoxide dismutase present in cytoplasm is manganese independent (Radi *et al*., 1985).

This enzyme catalyzes the dismutation of superoxide anion (a free radical) to hydrogen peroxide and oxygen (MacMillan-Crow *et al.*, 1998).

$$2O^{\cdot}_2 + 2H+ \longrightarrow H_2O_2+O_2$$

b) Catalase (CAT, E.C – 1.11.1.6)

This high molecular weight enzyme contains a haeme group (Fe (III) – protoporphyrin) attached to its active site. Located primarily in peroxisomes, the enzyme catalyzes the decomposition of hydrogen peroxide to water and oxygen.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Catalase guards the cell from oxidative damages due to hydrogen peroxide and hydroxyl radicals.

c) Glutathione Peroxidase (EC.1.11.1.9)

Glutathione peroxidases are selenoenzymes concerned with the reduction of hydroperoxides and hydrogen peroxide at the expense of glutathione. During this process hydrogen peroxide is reduced to water and organic hydroperoxides are reduced to alcohol.

$$2GSH + ROOH \longrightarrow GSSG + ROH + H_2O$$

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$$

Glutathione peroxidases in general exist in the cytosol and mitochondrial matrix. Glutathione peroxidases reside in the peroxisomes of fish liver cells (Orbea *et al*., 2000). Fish being more susceptible to oxidative damage have generally higher activities for glutathione peroxidase (Hassipieler *et al.*, 1994) than other enzymes involved in glutathione metabolism.

d) Glutathione Reductase (GR, E.C. 1.6.4.2)

Contained in cytosol and mitochondrial compartments; glutathione reductase catalyses the reduction of glutathione disulphide (oxidized glutathione) to glutathione (Schirmer and Kranth - Siegel, 1989).

$$GSSG + NADPH + H^{+} \longrightarrow 2GSH + NADP^{+}$$

e) Glutathione S Transferase (GST, E.C. 2.5.1.18)

It utilizes glutathione (GSH) in conjugation reactions with both exogenous and endogenous substrates. This group of enzymes is present in high amounts in liver cytosol and in lower amounts in other tissues. It catalyse the conjugation of electrophilic xenobiotic such as certain carcinogens to glutathione.

$R+GSH \longrightarrow R\text{-}S\text{-}G$ where R is an electrophilic xenobiotic.

Glutathione conjugates are more water soluble and are thought to be metabolized further by cleavage of the glutamate and glycine residues, followed by acetylation of the resulted free amino group of the cysteinyl residue to produce the final product, a mercapturic acid, which is then excreted (Habig *et al.*, 1974).

Secondary Antioxidants

This group includes glutathione, Vitamin C, uric acid, albumin, bili rubin, vitamin E, carotenoids, flavonoids and ubiquinol. The first two components are described as they only are considered in the present study.

Glutathione (GSH)

Glutathione is an oxyradical scavenging enzymatic antioxidant that comes under the second line of defense against oxidative damage of tissues. This tripeptide (gamma glutamyl cysteinyl glycine) is a multifunctional compound involved in various physiological and metabolic activities of fish. It takes part in thiol transfer reactions that protect the cell membrane and proteins. It is a cofactor for enzymatic reaction like conjugation of xenobiotics by glutathione S – transferase (Gallagher *et al.*, 1992). When glutathione scavenges a radical, a thiol radical is formed which undergoes dimerisation to oxidized glutathione disulfide or mixed disulfide with proteins. Both of these indicated an increased production of reactive oxygen species. Therefore the glutathione disulphide and mixed disulphides are reduced by glutathione reductase which restores the original glutathione level, at the expense of cellular NADPH. Glutathione is necessary for glutathione peroxidase activity which detoxifies organic and in organic peroxides and for glutathione S- transferases which are involved in the conjugation and excretion of xenobiotics.

Vitamin C

Vitamin C is not only a dietary requirement for fish ensuring optimal growth rates as well as collagen and hormone synthesis but it is also a powerful antioxidant in aqueous media and the major protective agent against oxidative damage is blood plasma. Ascorbic and deficiency may reduce the activity of xenobiotic metabolising enzymes (Andersson *et al*., 1988). Ascorbic and ameliorates copper and cadmium toxicity and is required for trace element homeostasis (Hilton, 1989; Thomas *et al*., 1982). It is released into the digestive tract, but reabsorbed almost quantitatively, thus conserving the animal's ascorbic acid pools. (Dabrowski, 1990).

Tertiary Antioxidants

These are a complex group of enzymes concerned with the repair of damaged DNA, damaged proteins, oxidized lipids and peroxides and also to impede chain propagation of peroxyl lipid radical. DNA repair enzymes, lipase, protease, transferase etc. are the examples.

4.1.2 Target Organs

Gill

The gills of a fish perform essential functions such as respiration, excretion and osmoregulation. In addition to respiratory epithelium, the secondary lamella consists of pillar cells (endothelial cells) and chloride cells. Gills are the primary sites of uptake of water borne pollutants in fish. The pillar cells may detoxify or bioactivate foreign compounds, via cytochrome P. 450 system (Gokosyr and Husoy, 1998). The gills are the principal sites for exchange of dissolved substances including metals. In teleosts, the gills consist of four arches at each side of the pharynx, which all bear a double row of gill filaments. The tips of the of adjacent arches touch, so that water has to flow between adjacent filaments. Each filament bears on its upper and lower surfaces, a row of closely spaced leaflets; the secondary lamellae. Secondary lamella can be likened to an envelope. Its two parallel faces are spaced apart and anchored by the pillar cells.

Morphologically and physiologically the basis of the gill function lies in (1) large surface area (2) counter current flows of water and blood and (3) small diffusion distances between water and blood. The large surface area and fine sieve like structure of gills make them particularly susceptible to continuous exposure to water borne noxious agents. Fish gill are primary markers for aquatic pollution. Therefore, functional impairment of gills caused by pollutants can significantly damage the

health of fish. For this reason, fish gills are considered to be the appropriate indicators of water pollution levels. (Cengiz and Unlu, 2003).

Liver

Liver was selected as another target organ. There are several reasons for selecting the liver as one of the sources for biomarkers. The liver of fish not only represents an organ central to numerous vital functions in basic metabolism but it is also a major site of accumulation, biotransformation and excretion of xenobiotic compounds (Triebskorn *et al.*, 1997). The bile produced within the hepatocytes is released into the proximal portion of the intestine and may serve as carrier of conjugated toxicants. Generally, large non-polar molecules and metabolites formed by hepatic biotransformation will be excreted via the hepatobiliary path of the liver. Teleost liver thus offers a suitable organ for biochemical and histological assessment of contamination.

Kidney

Kidneys in fish perform the important tasks of collecting undesired materials from various body parts and discharging them from the body. The renal function includes glomerular filtration to remove toxic waste products from the blood. In addition renal cells host several biotransformation enzymes. Kidney of a teleost fish receives the bulk of post-branchial blood flow.

4.2 Materials and methods

4.2.1 General protocol adopted for Sublethal Toxicity Studies.

Test animals measuring 7 cm in total length were subjected to the assessment of sublethal toxicity by exposing them to two selected sublethal concentrations of the test compounds. Ten healthy animals each were stocked in 50 litre of fresh water in FRP tanks. Duplicates were run

for each concentration. The medium was exchanged at every 24 hrs and the animals were fed soon after refilling with aerated fresh water. The medium was then charged with the required concentration of the toxicants and mixed well. The specimens were sampled every 96 hrs for antioxidant enzyme assays and lipid peroxidation studies and 7^{th} and 16^{th} day for histopathological studies. The total period of experiment was 16 day. The fishes were sacrificed by cervical dislocation by disrupting the contact between brain and spinal cord. The test organs were excised using a pair of sharp scissors, washed, blotted and weighed. The tissues for enzyme assays were kept cool by placing in ice bath.

The concentrations selected for different test materials and methods adopted for preparing the respective solutions are tabulated below:

Table 4.2.1. Test Solutions of Different Toxicants.

Test compound	Concentration	Method of Preparation
Copper (as $CuSO_4$. 5H2O)	1) 64 ppb($1/10^{th}$ of LC 50 value)	3.2 ml of 1000ppm stock solution was added to 50 l of water.
	2) 128 ppb (1/5 of LC 50 value)	5.4 ml of 1000 ppm stock solution was added to 50 l of water.
Metacid 50 (Commercial preparation)	1) 0.62 ppb ($1/10^{th}$ of LC 50 value) 2) 1.24 ppb ($1/5^{th}$ of LC 50 value)	3.1 ml and 6.2 ml of 10ppm stock solution were added to the medium to get the resultant concentrations of 0.62 ppb and 1.24 ppb respectively
Malachite green (as malachite green oxalate – zinc free)	1) 12.5 ppb 2) 25 ppb	6.25 ml and 12.5 ml of 100 ppm stock solutions were added to the medium to get the final concentrations of 12.5 ppb and 25 ppb respectively.

4.2.2 Assays of Lipid peroxidation, Antioxidant Enzymes and Estimation of Protein.

4.2.2.1 Assay of Lipid peroxidation.

Malondialdehyde (MDA) is one of the products of lipid peroxidation in tissues. This compound can react with thiobarbituric acid to yield a pink-coloured trimethine complex exhibiting an absorption maximum at 530 – 536 nm. The intensity of color is directly proportional to the concentration of malondialdehyde having a molar extinction co-efficient of 1.56×10^{5} / mol/ cm.

Peroxidation of membrane lipids was measured spectrophotometrically as thiobarbituric acid substances using the method of Niehaus and Samuleson (1968). The homogenates of liver, kidney and gill were prepared in 0.1 M Tris- Hcl buffer of pH 7.5 and were combined with TCA- TBA-HCl reagent (15% w/v, 0.375% w/v and 0.25 N respectively), mixed and boiled in a water bath for 15 minutes. It was then cooled and centrifuged at 2000 rpm. The absorbance of the coloured complex was read at 535 nm spectrophotometrically against a reagent blank. The rate of lipid peroxidation was expressed as nanomoles of malondialdehyde (MDA) formed per hour using a molar extinction coefficient of 1.56×10^{5} M^{-1} cm^{-1}.

4.2.2.2 Assay of Antioxidant Enzymes

a) **Assay for Catalase**

Catalase activity was determined by measuring the decomposition of hydrogen peroxide at 230 nm using the method of Chance and Maehly (1955). Briefly, the reaction mixture contained 0.01 M phosphate buffer, 30 µM hydrogen peroxide and the enzyme extract prepared by homogenizing the tissues in phosphate buffer (0.01M, pH 7.0) and centrifuging at 5000 rpm at 4° C. Specific activity was expressed as

international units (IU) per milligram protein. (1 IU = change in absorbance/min/extinction coefficient- 0.021).

Protein concentrations were measured by the method of Lowry *et al* (1951) using bovine serum albumin as standard. Samples were treated with Folin-phenol reagent and the absorbance was measured at 650 nm. The same method was followed for protein determinations wherever necessary.

b) Assay for Superoxide Dismutase

The enzyme in lysate was determined on the basis of the inhibition of the reduction of nitroblue tetrazolium (NBT) with NADH mediated by phenazine methosulphate (PMS) under basic conditions. (Kakkar *et al*., 1984). Tissue samples were homogenized in 0.33 M sucrose and differentially centrifuged under cold conditions to obtain the cytosol fraction. Before estimating the activity, initial purification was done by precipitating the protein from the supernatant with 90% ammonium sulphate and this fraction was dialyzed overnight against 0.0025 M Tris HCl buffer (pH 7.4). The supernatant served as the source of the enzyme. All measurement was performed in 0.052 M sodium pyrophosphate buffer of pH 7.6 at 28°C. The assay mixture contained 1.2 ml of sodium purophosphate buffer, 0.1 ml of 186 µM phenazine methosulphate, 0.3 ml of 300 µM nitriblue tetrazolium, 1.3 ml of distilled water and 0.1 ml of the enzyme source. After a lapse of 90 seconds at 30°C, the reaction was arrested by adding 1 ml of glacial acetic acid. The reaction mixture was then shaken vigorously with 4 ml of n-butanol. The upper butanol layer was removed. Absorbance of the chromogen in the butanol was measured at 560 nm against n-butanol blank. A system devoid of the enzyme concentration was kept as control. One unit of enzyme activity is defined as enzyme concentration required inhibiting the optical density at 560 nm

of chromogen production by 50 % in one minute under assay condition and expressed as specific activity in milliunits per milligram protein. As the assay was done for 90 seconds, the factor 2/3 was applied for calculating units.

c) Assay for Glutathione Peroxidase

The activity of glutathione peroxidase was analysed by a modified Mills' method 2 (Mills, 1959) as described by Hafeman *et al* (1974). Glutathione peroxidase degrades hydrogen peroxide in the presence of glutathione thereby depleting it. The remaining glutathione (GSH) is then measured by using 5, 5' - dithiobis 2-nitrobenzoic acid (DTNB). The incubation mixture at 37°C contained 80 mM sodium phosphate buffer (pH 7.0), 80 mM EDTA, 1mM sodium azide, 0.4 mM glutathione, 0.25 mM hydrogen peroxide and tissue homogenate. After 3 minutes, aliquots of this solution were removed and treated with metaphosphoric acid precipitation solution. The glutathione in the protein – free filtrate was then determined using 0.4 M disodium hydrogen phosphate and 1 mM DTNB in 1% trisodium citrate solution. The absorbance of this solution was recorded at 412 nm. A blank was carried out through the incubation simultaneously with the samples, since non- enzymatic glutathione oxidation by H_2O_2 occurs during incubation. One unit of glutathione peroxidase enzyme activity was defined as 1 µg of glutathione consumed per minute. The enzyme activity was expressed as microgram glutathione depleted per minute per milligram protein.

d) Glutathione S- Transferase Assay

The activity of glutathione S-transferase was measured through the conjugation of glutathione (GSH) with 1- chloro-2, 4-dinitrobenzene (CDNB) according to Habig *et al* (1974). The tissues were homogenized in 0.5 M phosphate buffer of pH 6.5. The assay mixture contained 200 µl

phosphate buffer, 20 µl CDNB and 730 µl distilled water in control tubes and 200 µl phosphate buffer, 20 µl CDNB and 680 µl distilled water in test sample tubes. The CDNB was dissolved in ethanol. Tubes were incubated at 37°C for 10 minutes. 50 µl of glutathione were added to each sets of tubes. After thorough mixing, 50 µl of tissue extract was added in test sample tubes. Increase in absorbance was noted at 340 nm for 5 minutes in a UV – visible spectrophotometer. One unit of transferase activity is defined as the amount of enzyme which catalyse the formation of 1 µ mole of thioether per minute per milligram protein using the extinction coefficient of 9.6 mM^{-1} cm^{-1}.

e) Assay for Glutathione Reductase

The decrease in absorbance of the assay mixture containing 0.12 ml of 0.5mM oxidized glutathione (GSSG), 0.12 ml of 0.1mM NADPH, 0.1 ml of pH 7.0) and 0.1 ml of enzyme extract of the tissues was read for 5 minutes at 340nm. The oxidized glutathione was replaced with distilled water for the controls. The enzyme activity was expressed as units per milligram protein per minute. (Zanetti, 1979).

4.2.2.3 Estimation of Endogenous Antioxidants

a) Estimation of Total Reduced Glutathione (GSH)

The total reduced glutathione (GSH) content of experimental tissues were determined at 305 nm as per the method of Patterson and Lazarow (1955). Tissue samples were weighed and homogenized in 0.5 M phosphate buffer. The incubation mixture contained the tissue extract, 50 µl alloxan, 50 µl phosphate buffer (pH 7.5) and 50 µl 0.5 N sodium hydroxide. After incubation for 5 minutes, the reaction was arrested by adding 50 µl of 1 N sodium hydroxide and the absorbance was measured. Controls were maintained with phosphate buffer instead of tissue extract. The values were expressed in milligram per 100 gram wet tissue.

4.2.3 Scheme Adopted for Histopathology

The test tissues (liver, kidney and gills) were carefully dissected out from freshly killed specimens, cleared off blood and extraneous matter and fixed by transferring them to 10% neutral buffered formalin (Short and Meyers, 2001). 4g of monobasic sodium phosphate and 6g of dibasic sodium phosphate were added to 100 ml. of 40% formalin and mixed well. 900 ml. of distilled water was added to this solution to make 1000 ml.of the fixative. The tissues were collected for fixation and processing during the 8^{th} and 16^{th} day of experiment. Tissues from the fishes unexposed to toxicants were processed to maintain as control. The liver was chopped into small pieces to ensure effective penetration of the fixative into the tissue. After 48 hours, the fixed samples of all test tissues were moved into different grades of alcohol for dehydration as per the following schedule.

1. 30% alcohol (10 minutes).
2. 50% alcohol (10 minutes).
3. 70% alcohol (10 minutes).

The tissues which are stored in fresh 70% alcohol were then treated with different grades of alcohol and subjected to further processing according to the following scheme.

1. 80% alcohol (10 minutes).
2. 90% alcohol (10 minutes).
3. 95% alcohol (10 minutes).
4. Absolute alcohol (10minutes and repeated twice).
5. Absolute alcohol and Xylene in a ratio of 1:1 (10minutes).
6. Acetone (2-3 minutes).

7. Xylene (10 minutes).
8. Xylene and Paraffin wax in a ratio of 3:1(10 minutes).
9. Xylene and Paraffin wax in a ratio of 1:1(10 minutes).
10. Xylene and Paraffin wax in a ratio of 1:3(10 minutes).
11. Pure wax (10 minutes and repeated twice).

The temperature of the wax was kept at 58-60º C.

Well-formed paraffin blocks with tissues embedded in it were cut into thin sections of 4-5µ in a microtome. The sliced tissues from the warm water bath were carefully transferred to clean glass slides and kept ready for staining after proper labeling.

Staining of the sectioned tissues (General H&E) was carried out after deparaffinisation and rehydration as indicated below.

1. 1.100% Xylene (5 minutes and repeated twice).
2. 95%, 80%, 70%, 50%, 30% Xylene respectively (5 minutes each).
3. Distilled water (5 minutes).
4. Meyer's Haematoxylin (11-15 minutes).
5. Running tap water (3 minutes).
6. Ammonia water (3-5 dips).
7. Running tap water (15 minutes).
8. Eosin (30 seconds for kidney, 1 minute for liver and 2 minutes for gills).
9. 95% alcohol (2 minutes and repeated once).
10. 100% alcohol (3 minutes and repeated once).
11. Xylene (3 minutes and repeated once).

The processed and stained tissues were mounted in permanent medium (DPX) under a glass cover slip and observed under Leika image analyser. Different types of lesions and anomalies were identified and

deviations from normal pattern of each tissue were quantified by calculating percentage damage per unit area (cm^2) and plotting against 8^{th} and 16^{th} day of exposure at high concentration of each toxicant. Four microphotographs, each developed from different area of a test tissue, was subjected to study the percentage alteration to exclude the chances of errors and the most representative picture was selected for deriving the conclusions.

Histological alterations were classified into five major categories (circulatory, proliferative, degenerative, inflammatory and structural), each possessing distinctive histological features and affecting specific areas of tissue related to function (Chen I *et al*, 2004). Degenerative alterations include general necrosis because they are considered to be a direct effect of toxicants, they are generally irreversible, and their persistence or progression may lead to a partial or total loss of organ function. Most proliferative (increased cell numbers) and structural (modified tissue or cellular architecture) alterations may or may not be reversible, depending on the severity and extent of the alteration. The circulatory (vascular congestion) change is easily reversible and may in fact be present only as a result of altered organism metabolic status rather than a direct effect of toxicant exposure.

4.2.4 Statistical Analysis

For biochemical data, one-way ANOVA was used to compare variables among controls and treatments, and if significant (fixed at $P<0.05$) differences were found, these data were reanalyzed by post hoc test by multiple comparison using Dunnett's test (Dunnett *t*) to determine which individual groups were significantly different from control. For all type of analyses SPSS for Windows Version 15.0 was utilized and the charts were plotted using MS Excel of Windows Vista. All the data are presented as mean ± standard deviation (SD) of the mean of six observations.

4.3 Results

4.3.1 Effects of Exposure to Copper

All the antioxidant enzymes showed significant ($P<0.05$) variations in test tissues with concomitant increase in lipid peroxidation (deduced from increased level of malondialdehyde or more accurately, thiobarbituric acid reactive substances) after exposure to the two experimental concentrations of copper. The level of reduced glutathione enhanced significantly ($P< 0.05$) in all the test tissues when subjected to low concentration (64 ppb) but decreased after exposure to high concentration (128 ppb) when compared to that of control group.

The activity of catalase (a primary antioxidant) in liver has exhibited progressive and significant trend ($P<0.05$) after each sampling interval (4^{th}, 8^{th}, 12^{th} and 16^{th} day). The trend was similar to both the experimental concentrations of the toxicant, highest activity (18.51±0.38 I.U/mg protein) was recorded on 16^{th} day of treatment with high concentration (Table. 4.3.1.1. a & b, Fig. 4.3.1.1). When exposed to low concentration of copper, the activity of catalase in kidney decreased progressively during the experimental period. At high concentration, the enzyme behaved differently with a significant increase on 4^{th} and 8^{th} day but decreased afterwards, recording a sharp dip on 16^{th} day (Table. 4.3.1.2. a & b, Fig. 4.3.1.2). The activity of catalase increased significantly ($P<0.05$) in gills with a steady enhancement at low concentration with a slight decrease on 12^{th} and 16^{th} day of exposure at high concentration when compared to 4^{th} and 8^{th} day of sampling (Table. 4.3.1.1. a & b, Fig. 4.3.1.3).

The superoxide dismutase activity varied significantly in the tissues and concentrations at 0.05 level of significance (Table 4.3.1.2 a and b). In comparison with the control group, the activity levels of superoxide dismutase in liver were found to be increasing at low copper concentration

throughout the experimental period. But it revealed an activity less than that of control group on 4th and 8th day and approached almost a level equal to that of control on 12th and 16th day in high concentration. In the case of kidney, the activity level of this primary antioxidant enzyme has shown an exceptionally high activity on 8th day of exposure to 64 ppb of copper followed by an activity less than that of control fishes. The variation in activity at high concentration was less pronounced when compared to that of low concentration although a significant increase ($P<0.05$) was evident when compared to control animals. The gills showed an inconsistency in the activity of superoxide dismutase although the enhancement in activity at high concentration was significantly high when compared to control specimens and low concentration group ($P<0.05$) (Table 4.3.1.2 a & b, Fig. 4.3.1.4, 5 and 6).

The activity of glutathione peroxidase also displayed commendable variations on exposure to different concentrations of copper. Upon exposure to low concentration of copper, the three test organs have depicted an enhancement in activity levels. The increment was steady, reaching the maximum on 16th day. Nevertheless, at high concentration, the liver and gills showed a decreased activity approaching a minimal value on 16th day. In the case of kidney, however, the activity of glutathione peroxidase showed an elevated trend up to 12th day (Table 4.3.1.3 a & b, Fig. 4.3.1.7, 8 and 9).

Activity of glutathione S transferase which is an index of tissue vulnerability to metal toxicity showed concentration and time dependent elevation. Thus the activity was uniformly high in the case of fishes exposed to 128 ppb of copper, temporally and intoxicationally. Similar pattern was observed in the case of kidney and gills also, although the rate of activity and the range of rates varied. All the observations made were

statistically significant at 5% or 1% levels (Table 4.3.1.4 a & b, Fig. 4.3.1.10, 11 and 12).

The activity of glutathione reductase; an antioxidative enzyme concerned with the conversion of glutathione disulphide to reduced glutathione, has also shown an increased activity upon exposure to low concentration of copper in both liver and kidney. On a consideration of the activity values of control groups, gills were found to possess the highest level of this antioxidative enzyme and that of kidney the lowest. Though a steady increase was noted in liver for both the experimental concentrations, the kidney showed an increase in activity up to 12^{th} day and a dip on the 16^{th} day in the low concentration. At high concentration of copper, the activity enhancement was more intense but the decreasing trend has commenced early (on 12^{th} day) and reached a low on the 16^{th} day. The gills have shown an augmented activity initially (4^{th} day) recording a downward trend afterwards (Table 4.3.1.5 a & b, Fig. 4.3.1.13, 14 and 15).

The extent of lipid peroxidation as assessed by the rate of malondialdehyde formation was found to increase progressively with concentration and duration of exposure. The highest level was observed on 16^{th} day at high concentration in liver, kidney as well as in gills (Table 4.3.1.6 a & b, Fig. 4.3.1.16, 17 and 18).

The level of reduced glutathione was identical in all the tissues. There was an enhancement at low concentration of copper and a reduction at high concentration when compared to that of the control group. The gills were found to possess high amount of glutathione in comparison to the levels maintained in liver and kidney (Table 4.3.1.7 a & b, Fig 4.3.1.19, 20 and 21). Further, the boost up of glutathione at 64 ppb of copper and the radical declension at 128 ppb were independent of duration.

Table 4.3.1.1 a). Variation in Catalase activity in Tissues of *Oreochromis mossambicus* after exposure to copper.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	11.41± 0.0138	11.39± 0.0137	11.43± 0.0155	11.45± 0.0151
	64 ppb	11.39± 0.1183	12.412± 0.2072	13.64± 0.0000	14.71± 0.1510
	128 ppb	11.46± 0.0080	13.65± 0.2969	15.60± 0.3684	18.51± 0.3827
Kidney	Control	3.097± 0.0121	3.103± 0.0121	3.105± 0.0121	3.098± 0.0264
	64 ppb	2.785± 0.0259	2.418± 0.1835	1.790± 0.1502	1.700± 0.0576
	128 ppb	2.858± 0.1098	3.870± 0.4265	3.678± 0.2998	2.117± 0.0750
Gills	Control	10.37± 0.0297	10.43± 0.0306	10.36± 0.0306	10.37± 0.0306
	64 ppb	10.29± 0.2614	11.37± 0.3816	11.62± 0.5054	11.61± 0.3555
	128 ppb	13.32± 0.6127	13.44± 0.5983	12.30± 0.3729	12.40± 0.0763

Table 4.3.1.1 b). ANOVA for changes in catalase activity in relation to copper exposure

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	4406.862	2	2203.431	1574.459	.010
Duration	28.507	3	9.502	6.790	.024
Concentration	150.668	2	75.334	53.830	.010
Error	291.093	208	1.399		
Total	4877.130	215			

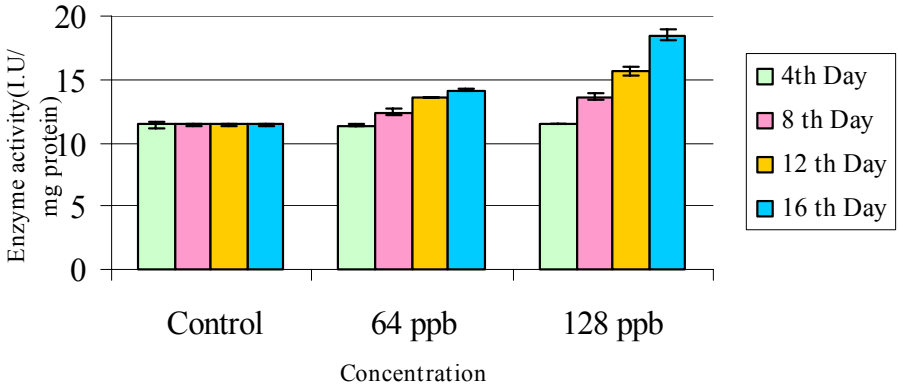


Fig. 4.3.1.1. Catalase activity in liver of *Oreochromis mossambicus* after exposure to copper

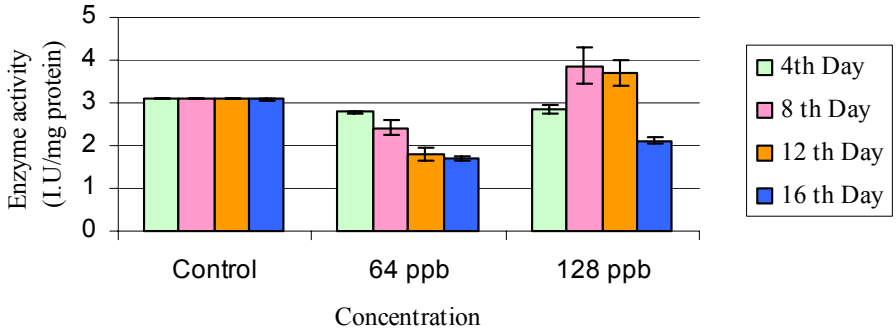


Fig. 4.3.1.2. Catalase activity in kidney of *Oreochromis mossambicus* after exposure to copper

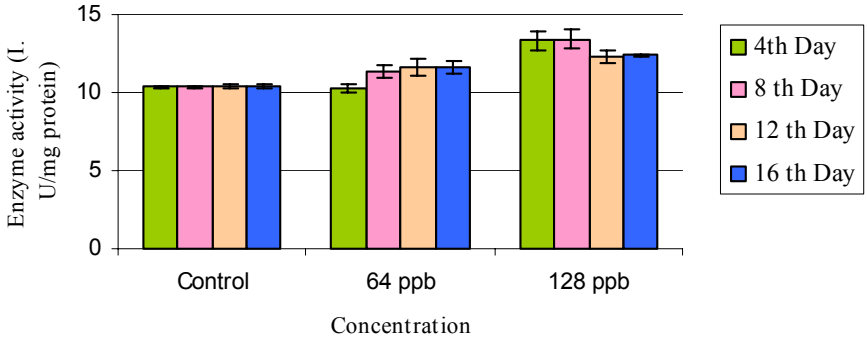


Fig. 4.3.1.3. Catalase activity in gills of *Oreochromis mossambicus* after exposure to copper

Table 4.3.1.2 a). Variation in Superoxide Dismutase activity in Tissues of *Oreochromis mossambicus* after exposure to copper.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	0.990± 0.0085	0.991± 0.0085	0.988± 0.0104	0.990± 0.0081
	64 ppb	0.971± 0.0434	1.473± 0.0524	1.744± 0.3676	3.415± 0.1852
	128 ppb	0.877± 0.0235	0.852± 0.1469	0.910± 0.1008	1.004± 0.1112
Kidney	Control	0.321± 0.0036	0.317± 0.2560	0.324± 0.1300	0.320± 0.0420
	64 ppb	0.383± 0.0206	1.621± 1.8970	0.319± 0.0054	0.311± 0.0069
	128 ppb	0.350± 0.0057	0.361± 0.0078	0.415± 0.0086	0.328± 0.0133
Gills	Control	1.047± 0.0280	1.044± 0.5200	1.049± 0.0921	1.048± 0.0016
	64 ppb	1.232± 0.0044	0.539± 0.2333	0.782± 0.0236	0.579± 0.0119
	128 ppb	1.249± 0.1050	1.504± 0.0535	1.282± 0.0835	1.651± 0.3911

Table 4.3.1.2 b). ANOVA for changes in superoxide dismutase activity in relation to copper exposure.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	26.631	2	13.316	42.253	.001
Duration	1.974	3	.658	2.088	.103
Concentration	4.007	2	2.003	6.357	.002
Error	65.548	208	.315		
Total	98.160	215			

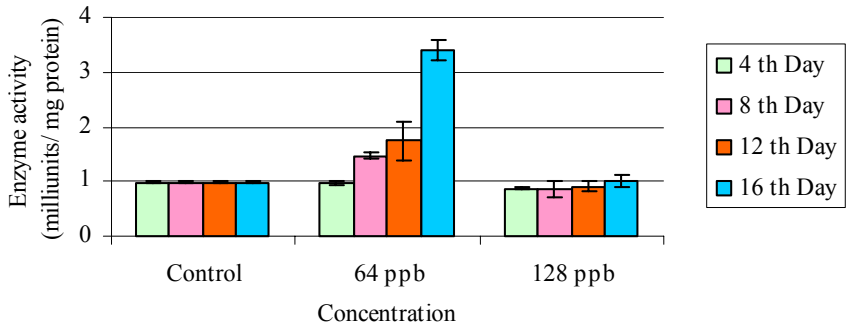


Fig. 4.3.1.4. Superoxide Dismutase activity in liver of *Oreochromis mossambicus* after exposure to copper

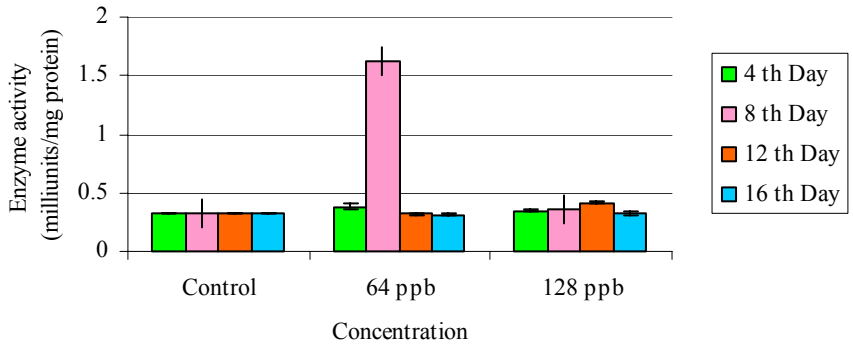


Fig. 4.3.1.5. Superoxide Dismutase activity in kidney of *Oreochromis mossambicus* after exposure to copper

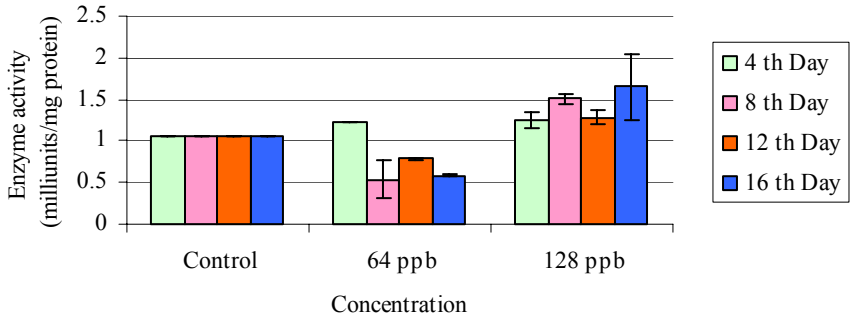


Fig. 4.3.1.6. Superoxide Dismutase activity in gills of *Oreochromis mossambicus* after exposure to copper

Table 4.3.1.3 a). Variation in Glutathione Peroxidase activity in Tissues of *Oreochromis mossambicus* after exposure to copper.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	8.779± 0.0040	8.883± 0.0210	8.880± 0.0010	8.881± 0.0061
	64 ppb	28.42± 0.3683	31.56± 0.2056	39.50± 0.6950	33.12± 0.8673
	128 ppb	22.49± 0.2873	20.36± 0.2172	14.62± 0.3720	11.52± 0.2785
Kidney	Control	5.330± 0.0001	5.829± 0.0400	5.830± 0.6100	5.831± 0.0110
	64 ppb	8.365± 0.3749	10.26± 0.3871	13.14± 0.7082	14.80± 1.4799
	128 ppb	9.31± 0.2181	11.57± 0.3665	13.35± 0.6243	10.74± 0.7128
Gills	Control	13.04± 0.1400	13.06± 0.0240	13.01± 0.0150	13.00.4± 0.0300
	64 ppb	22.90± 1.2364	31.56± 0.6321	38.81± 0.8524	46.04± 1.2124
	128 ppb	17.88± 0.4705	16.99± .05699	16.48± 0.3790	13.48± 0.6102

Table 4.3.1.3 b). ANOVA for changes in Glutathione Peroxidase activity in relation to copper exposure.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	5932.919	2	2966.460	104.404	.003
Duration	270.643	3	90.214	3.175	.025
Concentration	11237.245	2	5618.622	197.747	.001
Error	5909.934	208	28.413		
Total	23350.741	215			

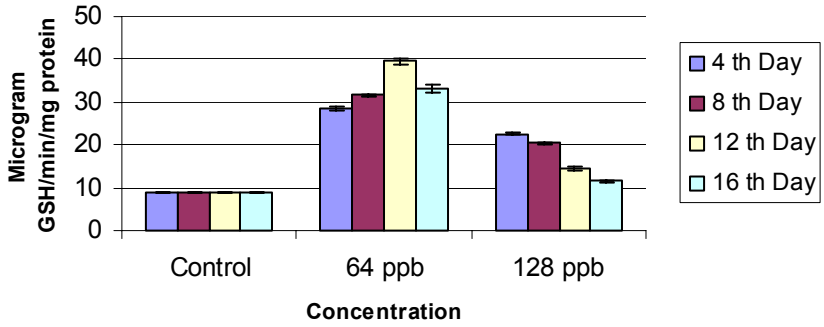


Fig. 4.3.1.7. Glutathione Peroxidase activity in liver after exposure to Copper

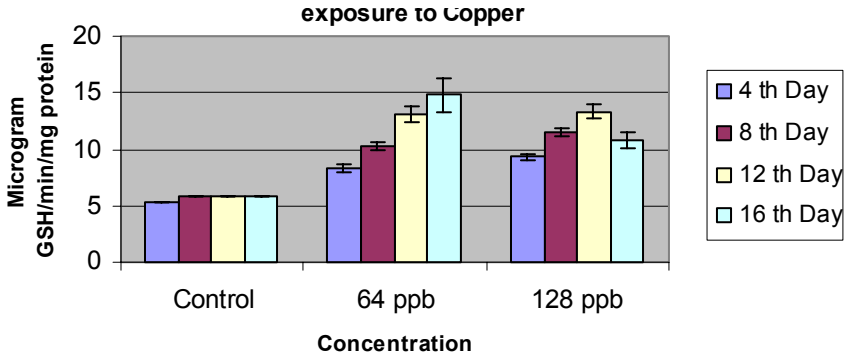


Fig. 4.3.1.8. Glutathione Peroxidase activity in kidney after exposure to Copper

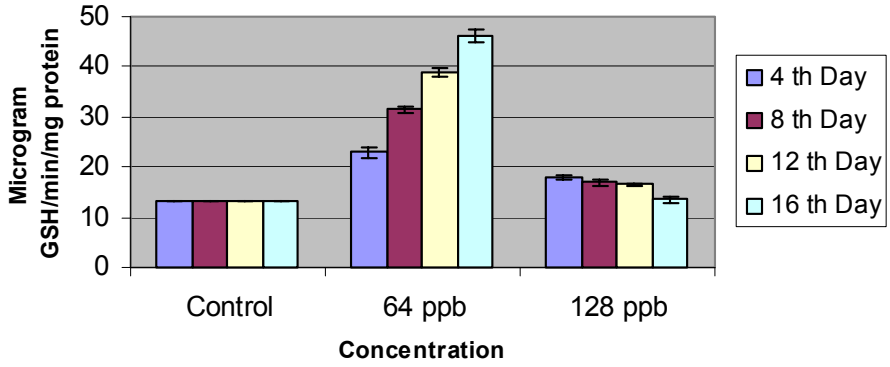


Fig. 4.3.1.9. Glutathione Peroxidase activity in gill after exposure to Copper

Table 4.3.1.4 a). Variation in Glutathione S Transferase activity in Tissues of *Oreochromis mossambicus* after exposure to copper.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	22.74± 0.0580	22.78± 0.0300	22.72± 0.0210	22.69± 0.1100
	64 ppb	28.11± 0.7105	32.66± 2.2222	40.76± 0.3240	43.67± 0.3670
	128 ppb	30.45± 0.6993	37.08± 1.2060	43.64± 0.6256	53.48± 0.6146
Kidney	Control	12.43± 0.0401	12.43± 0.0160	12.43± 0.0130	12.43± 0.3300
	64 ppb	12.79± 0.1987	17.41± 0.4272	22.69± 0.2840	29.83± 0.7815
	128 ppb	13.71± 0.1528	21.46± 0.4234	28.42± 0.5654	36.19± 0.9381
Gills	Control	14.96± 0.1700	14.96± 0.4700	14.96± 0.0310	14.96± 0.1500
	64 ppb	16.59± 0.3630	19.53± 0.3297	25.09± 0.2604	31.30± 0.1520
	128 ppb	17.41± 0.3095	22.93± 1.3272	29.93± 0.6176	34.73± 0.2103

Table 4.3.1.4 b). ANOVA for changes in Glutathione S transferase activity in relation to copper exposure.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	8273.328	2	4136.664	287.878	.031
Duration	4569.462	3	1523.154	105.999	.020
Concentration	7551.632	2	3775.816	262.766	.014
Error	2988.862	208	14.370		
Total	23383.284	215			

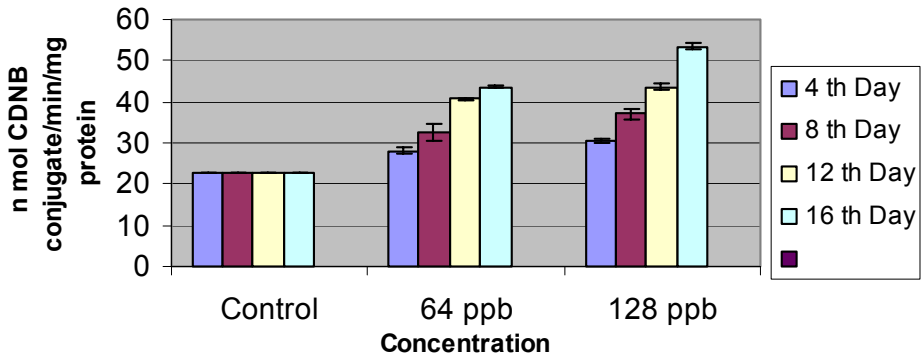


Fig. 4.3.1.10. Glutathione S Transferase activity in liver after exposure to Copper

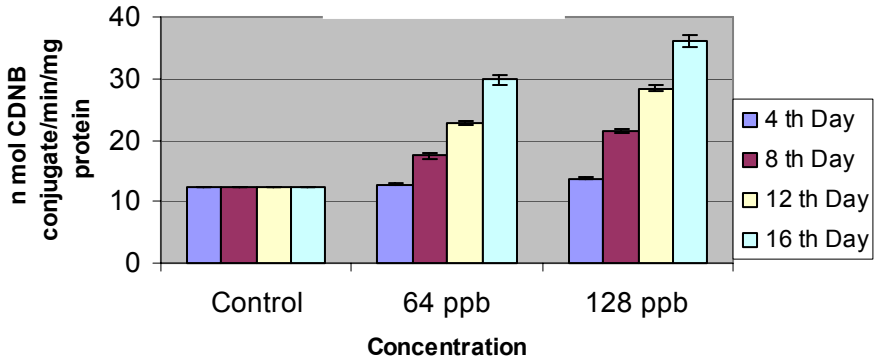


Fig. 4.3.1.11. Glutathione S Transferase activity in kidney after exposure to Copper

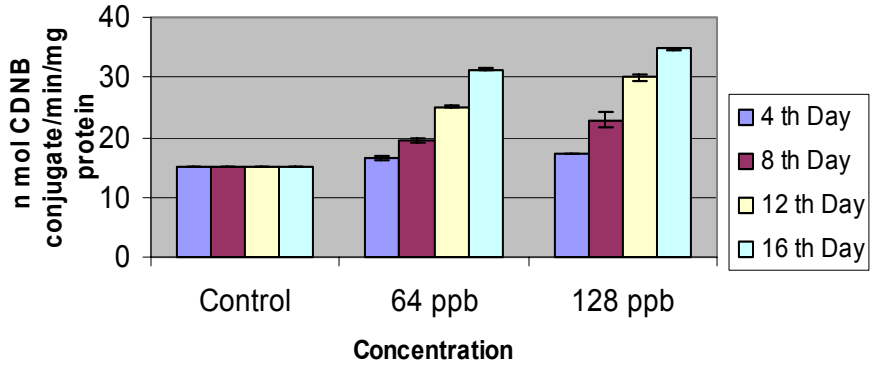


Fig. 4.3.1.12. Glutathione S Transferase activity in gill after exposure to Copper

Table 4.3.1.5 a). Variation in Glutathione Reductase activity in Tissues of *Oreochromis mossambicus* after exposure to copper.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	3.621± 0.0100	3.620± 0.0200	3.619± 0.0400	3.620± 0.1200
	64 ppb	6.213± 0.1715	7.298± 0.1338	8.298± 0.13167	10.15± 0.0750
	128 ppb	11.38± 0.3728	11.74± 0.6277	12.76± 0.2108	17.21± 0.1392
Kidney	Control	1.923± 0.0300	1.925± 0.0110	1.920± 0.0200	1.922± 0.0000
	64 ppb	2.682± 0.1465	5.316± 0.2489	7.455± 0.2156	3.495± 0.2046
	128 ppb	9.132± 0.1180	11.31± 0.3334	4.227± 0.2012	1.523± 0.1850
Gills	Control	2.647± 0.1300	2.650± 0.2000	2.649± 0.0600	2.646± 0.0200
	64 ppb	3.075± 0.0629	2.368± 0.2797	1.118± 0.0637	1.103± 0.0489
	128 ppb	1.963± 0.2615	2.163± 0.1184	2.180± 0.1170	1.015± 0.0472

Table 4.3.1.5 b). ANOVA for changes in Glutathione Reductase activity in relation to copper exposure.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	1402.906	2	701.453	111.269	.001
Duration	11.002	3	3.667	.582	.628
Concentration	670.946	2	335.473	53.215	.001
Error	1273.427	202	6.304		
Total	3358.281	209			

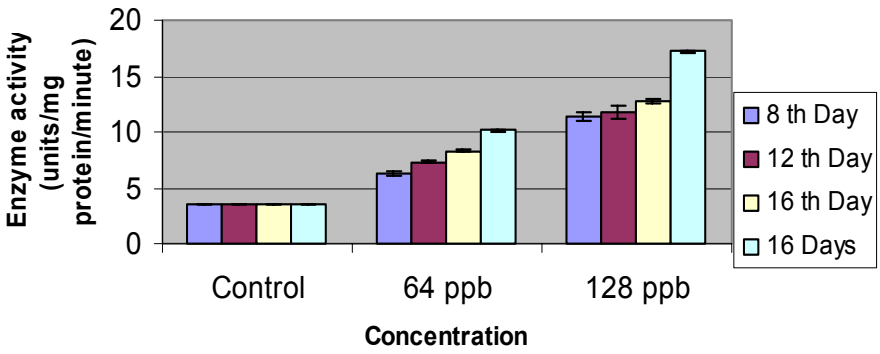


Fig. 4.3.1.13. Glutathione Reductase activity in liver after exposure to Copper

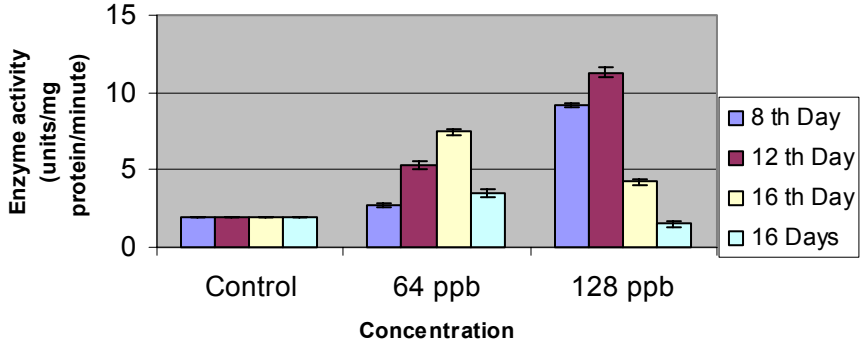


Fig. 4.3.1.14. Glutathione Reductase activity in kidney after exposure to Copper

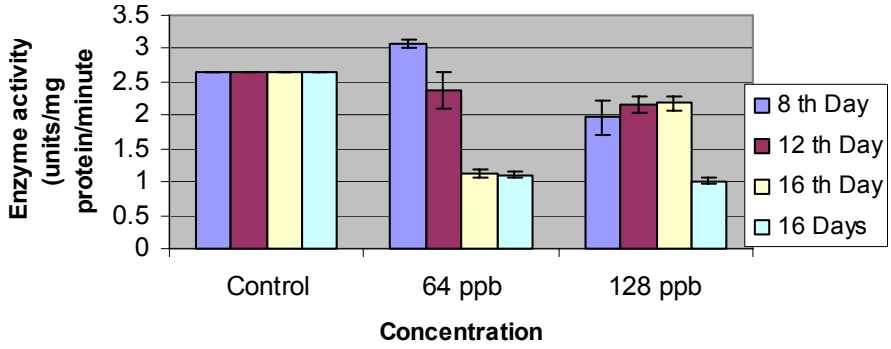


Fig. 4.3.1.15. Glutathione Reductase activity in gill after exposure to Copper

Table 4.3.1.6 a). Level of lipid peroxidation in Tissues of *Oreochromis mossambicus* after exposure to copper.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	0.725± 0.2100	0.727± 0.0700	0.726± 0.0300	0.723± 0.0900
	64 ppb	0.812± 0.0052	0.838± 0.0372	0.832± 0.0089	1.895± 0.1894
	128 ppb	2.294± 0.2176	2.568± 0.0880	3.145± 0.1931	7.609± 0.2317
Kidney	Control	0.357± 0.1800	0.360± 0.0500	0.361± 0.0410	0.359± 0.0400
	64 ppb	0.429± 0.0490	0.821± 0.0195	1.387± 0.1550	2.238± 0.1776
	128 ppb	1.232± 0.1886	2.342± 0.1362	6.305± 0.1893	9.227± 0.2884
Gills	Control	0.616± 0.0140	0.618± 0.2200	0.620± 0.1500	0.617± 0.0700
	64 ppb	0.722± 0.0042	0.926± 0.0205	3.588± 0.0847	3.790± 0.1072
	128 ppb	0.748± 0.0106	1.472± 0.2559	3.122± 0.1818	4.223± 0.1145

Table 4.3.1.6 b). ANOVA for changes in levels of lipid peroxidation in relation to copper exposure.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	4.797	2	2.399	1.455	.236
Duration	212.316	3	70.772	42.921	.025
Concentration	368.772	2	184.386	111.824	.021
Error	342.971	208	1.649		
Total	928.856	215			

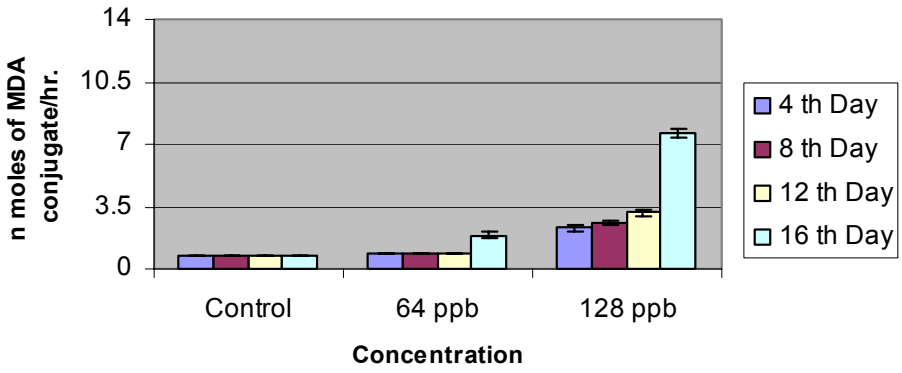


Fig. 4.3.1.16. Lipid Peroxidation in liver after exposure to copper

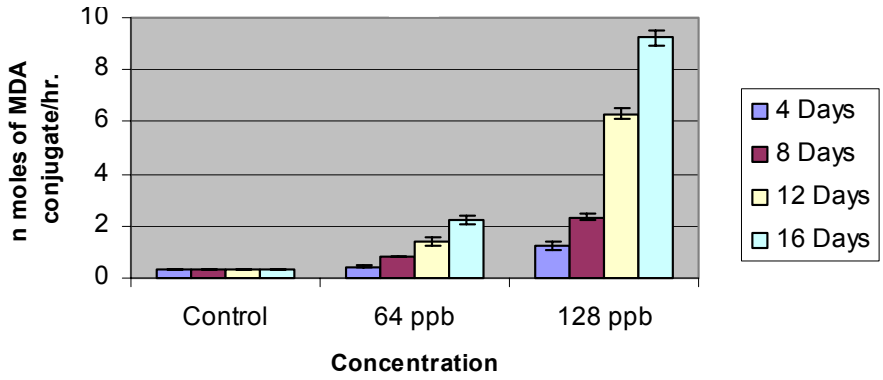


Fig. 4.3.1.17. Lipid Peroxidation in kidney after exposure to copper

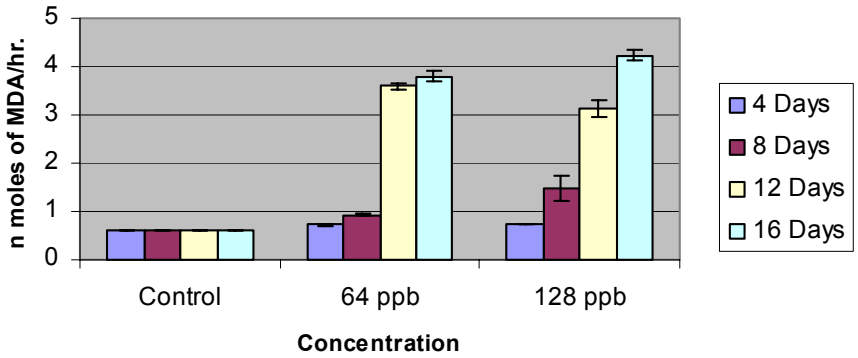


Fig. 4.3.1.18. Lipid Peroxidation in gills after exposure to copper

Table 4.3.1.7 a). Changes in reduced glutathione content in Tissues *Oreochromis mossambicus* of after exposure to copper.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	1751± 14.160	1751± 13.0900	1750± 12.0140	1748± 10.2800
	64 ppb	2435. 7± 32.3381	2438.5± 41.0488	2675.8± 102.3292	2715.8± 72.6394
	128 ppb	1324.5± 73.5166	1318.8± 36.691	1251. 7± 50.8165	1229.2± 63.8349
Kidney	Control	1018± 10.0700	1019± 9.1100	1018± 9.0510	1017± 12.0320
	64 ppb	2116.8± 57.224	2315.5± 35.3784	2250± 42.4495	2263.3± 41.6330
	128 ppb	927.2± 62.0412	913.2± 39.9408	914.2± 37.4833	913.5± 51.0488
Gills	Control	1313± 60.040	1312± 45.1700	1313± 53.300	1313± 58.1400
	64 ppb	1627.8± 56.7528	1712. 7± 71.8619	1728.5± 72.2248	1733. 7± 43.8297
	128 ppb	1133.8± 65.7528	1139± 83.2649	1074. 7± 90.5164	1084. 7± 71.0328

Table 4.3.1.7 b). ANOVA for changes in levels of reduced glutathione in relation to copper exposure.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	4.705	2	2.352	203.494	.002
Duration	.006	3	.002	.179	.910
Concentration	17.157	2	8.578	742.120	.024
Error	2.404	208	.012		
Total	24.272	215			

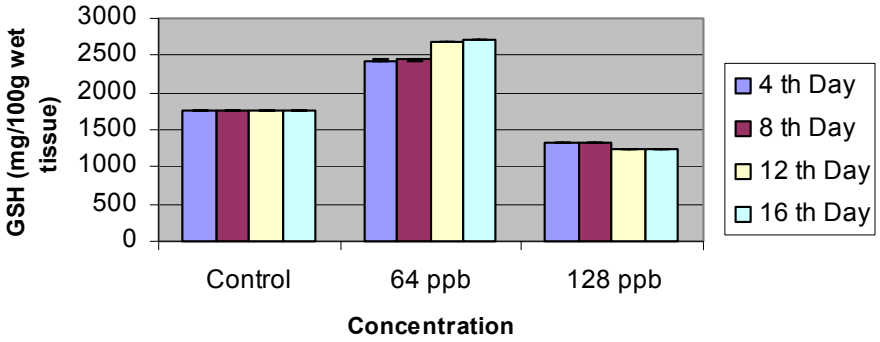


Fig. 4.3.1.19. Reduced Glutathione (GSH) in liver after exposure to Copper

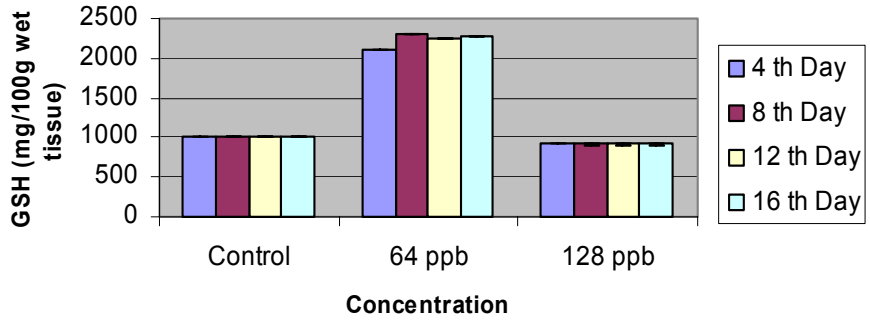


Fig. 4.3.1.20. Reduced Glutathione (GSH) in kidney after exposure to Copper

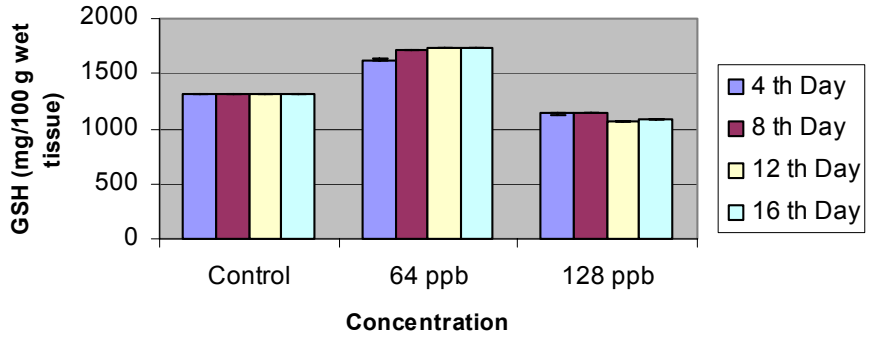


Fig. 4.3.1.21. Reduced Glutathione (GSH) in gill after exposure to Copper

Table 4.3.1.8. Multiple Comparison Test (Dunnett *t*) for Enzymatic Activities in *Oreochromis mossambicus* after exposure to copper.

	Groups	Catalase	Superoxide dismutase	Glutathione peroxidase	Glutathione S transferase	Glutathione reductase	Lipid peroxidation	Reduced glutathione
Tissue	Liver & Kidney	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.588 [d]	0.000 [a]
	Liver & Gills	0.000 [a]	0.125 [d]	0.173 [d]	0.000 [a]	0.000 [a]	0.755 [d]	0.000 [a]
	Kidney & Gills	0.000 [a]	0.000 [a]	0.000 [a]	0.003 [b]	0.000 [a]	0.208 [d]	0.005 [b]
Day	4 Days & 8 Days	0.067 [d]	0.548 [d]	0.465 [d]	0.000 [a]	0.647 [d]	0.610 [d]	0.904 [d]
	4 Days & 12 Days	0.009 [b]	0.979 [d]	0.020 [c]	0.000 [a]	0.986 [d]	0.000 [a]	0.962 [d]
	4 Days & 16 Days	0.000 [a]	0.104 [d]	0.112 [d]	0.000 [a]	1.000 [d]	0.000 [a]	0.943 [d]
	8 Days & 12 Days	0.891 [d]	0.790 [d]	0.457 [d]	0.000 [a]	0.833 [d]	0.000 [a]	0.997 [d]
	8 Days & 16 Days	0.236 [d]	0.770 [d]	0.854 [d]	0.000 [a]	0.631 [d]	0.000 [a]	0.999 [d]
	12 Days & 16 Days	0.644 [d]	0.235 [d]	0.908 [d]	0.000 [a]	0.985 [d]	0.000 [a]	1.000 [d]
Concentration	Control & 64 ppb	0.025 [c]	0.002 [b]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	Control & 128 ppb	0.000 [a]	0.453 [d]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	64 ppb & 128 ppb	0.000 [a]	0.057 [d]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]

The values are significant at a=P<.001, b=P<.01 and c=P<.05 and not significant at d.

Histopathological Effects of Copper.

Estuaries and coastal water bodies are veritable suite for various types of toxic chemicals employed for the treatment and for chemical control of the hydrology of culture systems. These chemicals are potentially hazardous and could also be emitted from industrial and chemical sources. In recent times, emphasis has been given to the evaluation of the causative relations between contaminant exposure and observable biological effects in aquatic organisms. Fish diseases, pathological effects, and related aetiology are being increasingly used as indicators of environmental stress since they provide a definite biological end point of historical exposure (Mathiessen., *et al* 1993). Further, biological biomarkers provide powerful tools to detect and characterise the biological end points of toxicant and exposure. As such, the utility of histological lesions as sensitive and reliable indicators of the health of wild fish populations has been demonstrated in aquatic systems (Myers *et al*., 1998). Several laboratory and mesocosm studies have also demonstrated causal links between exposures to xenobiotics and the development of toxicopathic lesions in different cardinal tissues of fishes (Stein, *et al.,* 1990). It is generally accepted that certain hepatic lesions in marine flatfish can be induced by environmental contaminants and that these represent an ecologically relevant biological end point of exposure to pollution. The increasing emphasis on the assessment and monitoring of aquatic ecosystems has highlighted the need to develop appropriate biological indices and measurements as monitoring strategies.

The normal teleost liver is a tubular gland and the functional unit is a tubule of hepatocytes surrounding a biliary structure, the lumen. Exocrine functions of the liver include production of bile by the hepatocytes and its transport within a hierarchy of biliary passageways. The hepatocytes are

the most conspicuous cells and occupy the greatest percentage of the organ volume. The hepatocyte is an example of a single cell serving both exocrine and endocrine functions. Biliary epithelial cells have been categorized by the portion of the intrahepatic biliary tree that they are associated with, i.e., bile preductular epithelial cells, ductular or epithelial cells, small and large bile ductular epithelial cells, or, by morphology, as oval, cuboidal, short and columnar epithelial cells (Fig. 4.3.1.22).

As a result of exposure to copper, substantial damage was observed in the liver of *Oreochromis mossambicus*. The stained sections of liver have shown pycnotic nuclei and necrosis at low and high concentrations of the toxicant. The severity of damage was assessed at high concentration (128 ppb) on 8^{th} and 16^{th} day after exposure. Almost 60 percent of the nuclei have become pycnotic by the 8^{th} day with necrotic effects spreading to 40 percent of the area. The severity of the damages became more intense on 16^{th} day as evidenced by the number of condensed nuclei and necrotic patches encompassing over 60 percent leading to a loss of normal architecture of the organ (Fig. 4.3.23, 24 and 25).

The kidney of *Oreochromis mossambicus* (Fig.4.3.1.25) is of mesonephric type and opisthonephric in position consisting of glomeruli encircled by cup shaped Bowman's capsules and proximal renal tubules identifiable by the basal position of nuclei. The distal tubules with the central location of nuclei lead to collecting duct (Hentschel and Fink'enstabt", 1980).

When exposed to high concentration of copper, the kidney exhibited morphological changes. Glomeruli were swollen with a concomitant increase in space of Bowman's capsule. Moreover, desquamation of tubular epithelia and pycnotic nuclei were observed on 8^{th} day of exposure, however, this was

very prominent on 16th day (Fig. 4.3.1. 26, 27, 28 and 29). Hyalinised nature of tubular epithelial cytoplasm with castings was another feature that set in on 16th day after exposure to copper.

The toxic insult with 128 ppb of copper has elicited morphological changes in the gills of the fish, the details of which are shown in figure 4.3.1.31 and 32 and the changes in the extent of damage with respect to advancement in exposure period is illustrated in figure 4.3.1.33. The apices of secondary lamellae have become clavate in shape and hyperplasia of respiratory lamellae accompanied by lamellar curling set in after 8th day of exposure. The shortening of secondary lamellae with concomitant epithelial lifting and squamous metaplasia were obvious on 16th day. Separation of epithelial cells from one another classically seen in the deeper cell layers of the epidermis following intra cytoplasmic oedema (balloon degeneration) was evident in certain areas on the gill.

Sublethal exposure of copper induced structural damages in renal tissue which included hyperchromatinised (pycnotic) nuclei, hyaline cytoplasm, glomerular swelling and desquamation of tubular epithelia. The lesions progressed with prolongation of exposure period. Deposition of tubular casts was the major change developed after 16th day of exposure to copper (Fig.4.3.1.26, 27 and 28).

The normal gill of *Oreochromis mossambicus* (Fig.4.3.1.29 a & b) bears two rows of gill filaments (primary lamellae), which on both sides bear a series of alternately arranged respiratory lamellae (secondary lamellae). Alternately arranged pillar cells and blood channels, through which usually one or two cells can pass, constitute the vascular component of the gills. A very thin barrier layer of respiratory epithelium covers the vascular component of the gill lamellae. Goblet mucus cells are always present in the

inter lamellar epithelial lining as well as on the distal tip of the gill filament. Chloride cells are regularly present at different loci on the gills.

The prevalence of histopathological alterations observed in the gill showed a higher incidence of aneurism, hyperplasia and fusion of secondary lamellae. Major changes in the gill were lamellar curling, lamellar fusion, hyperplasia, and exfoliation (Fig.4.3.1.30, 31 and 32).

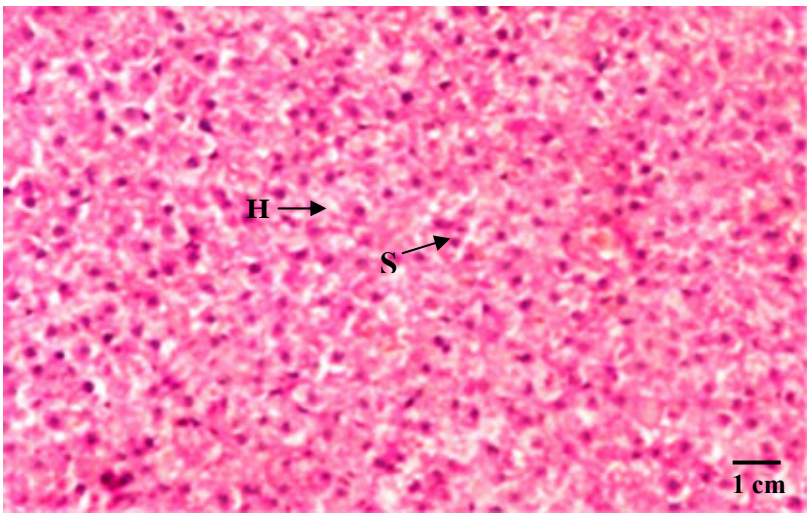


Fig.4.3.1.22. Liver (Normal) with hepatocytes enclosing almost even sized nuclei (H) and sinusoidal lamina (S). H&E. 40x.

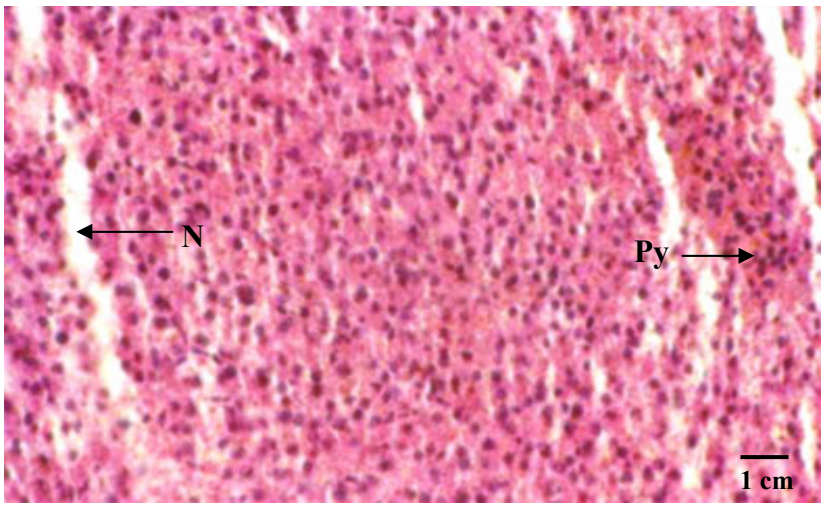


Fig. 4.3.1.23. Liver on 8th day of exposure to copper at 128 ppb. Necrotic areas (N) and pycnotic nuclei (Py) are evident. 39% of the total area is affected by necrosis and 61% of the area is affected by pycnosis. H & E. 40x

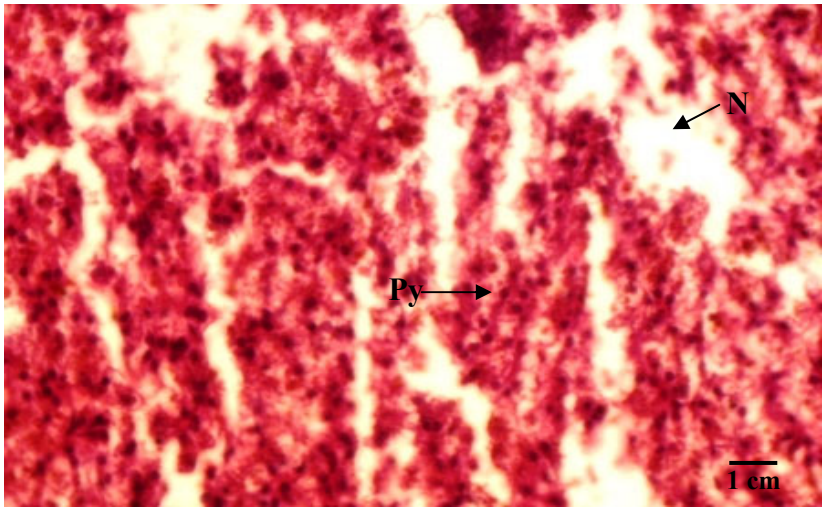


Fig.4.3.1.24. Liver on 16th day of exposure to copper at 128 ppb. Necrotic areas (N) and pycnotic nuclei (Py) are evident. 61% of the total area is affected by necrosis and 70.5% of the area is affected by pycnosis. Architectural loss sets in. H & E. 40x

Table 4.3.1.9. Histopathology of liver after exposure to 128 ppb of copper.

Treatment	Percentage damage		Additional feature
	Pycnosis	Necrosis	
Normal	0.00	0.00	Nil
8th day	60.00	39.00	Nil
16th day	70.50	61.00	Architectural loss

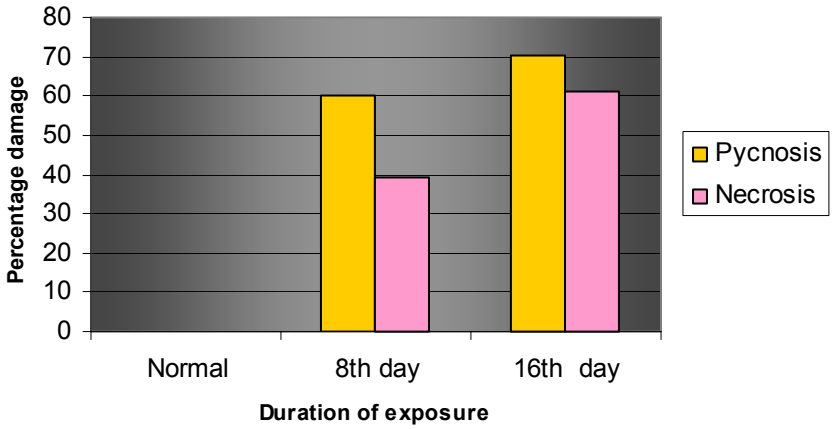


Fig. 4.3.1.25. Histopathology of liver after exposure to copper

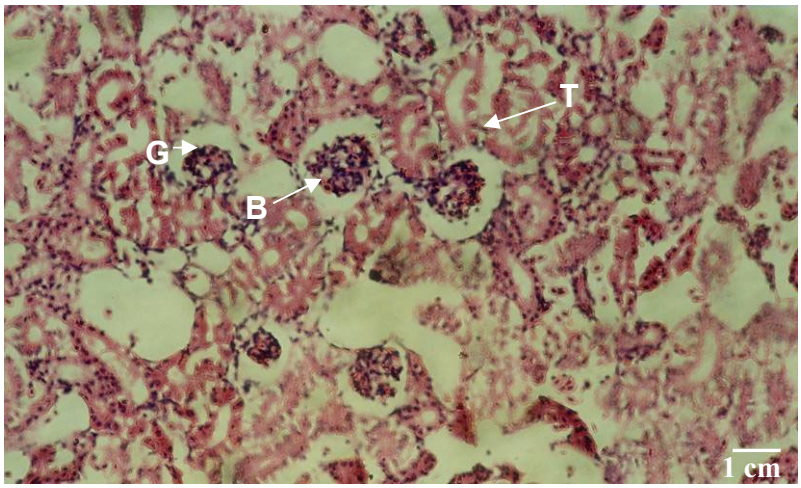


Fig. 4.3.1.26. Kidney (Normal) with well formed glomerulus (G), Bowman's capsule (B) and renal tubule (T). H&E.20x.

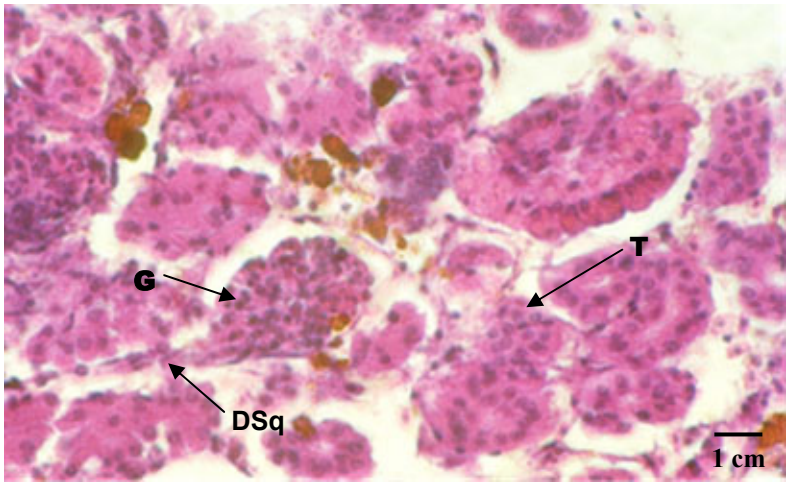


Fig. 4.3.1.27. Kidney on 8th day of exposure to copper at 128 ppb showing swollen glomerulus (G), thickened Bowman's capsule (B) and renal tubule with pycnotic nuclei (T). Desquamation of tubular epithelia begins. H & E. 40x

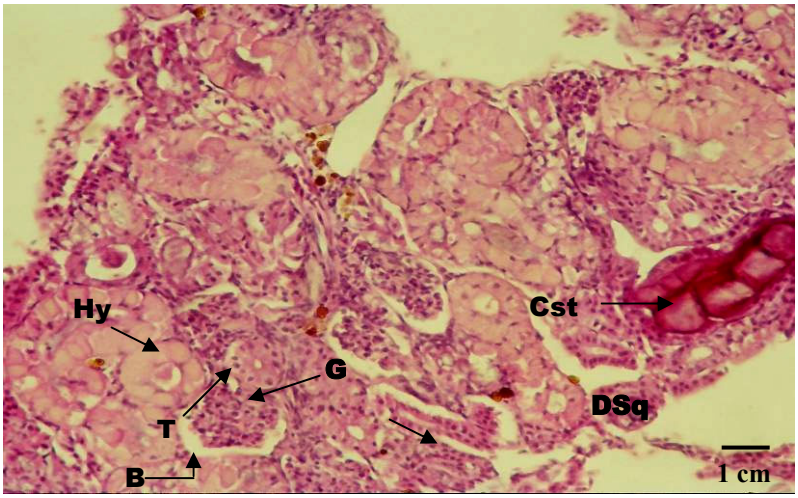


Fig. 4.3.1.28. Kidney on 16th day of exposure to copper at 128 ppb showing swollen glomerulus (G), thickened Bowman's capsule (B) and renal tubule with pycnotic nuclei (T). Tubular epithelia are desquamated (DSq) and hyalinated (Hy) and castings (Cst) appear. H & E. 20x.

Table 4.3.1.10. Histopathology of kidney after exposure to 128 ppb of copper.

Treatment	Percentage damage				
	Hyalinisation	Casts	Pycnosis	Desquamation	Number of swollen glomeruli and Bowman's capsules
Normal	0.00	0.00	24.00	0.00	0.00
8th day	0.00	0.00	65.00	10.00	2.00
16th day	66.70	9.30	83.60	10.00	3.00

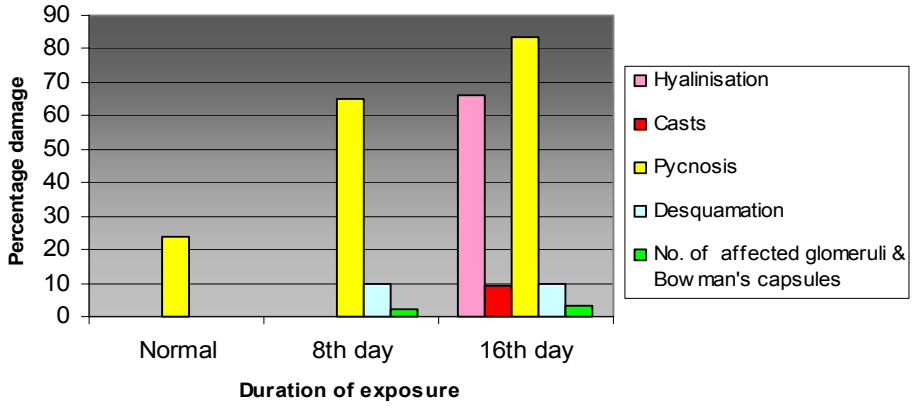
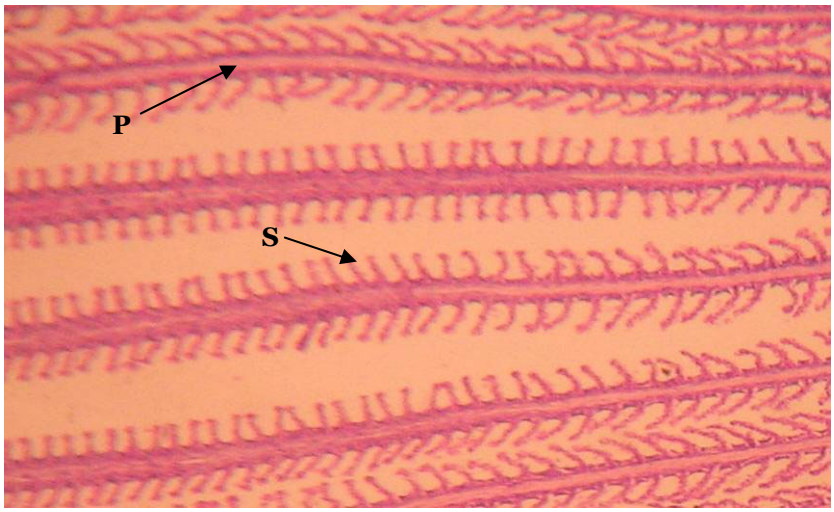
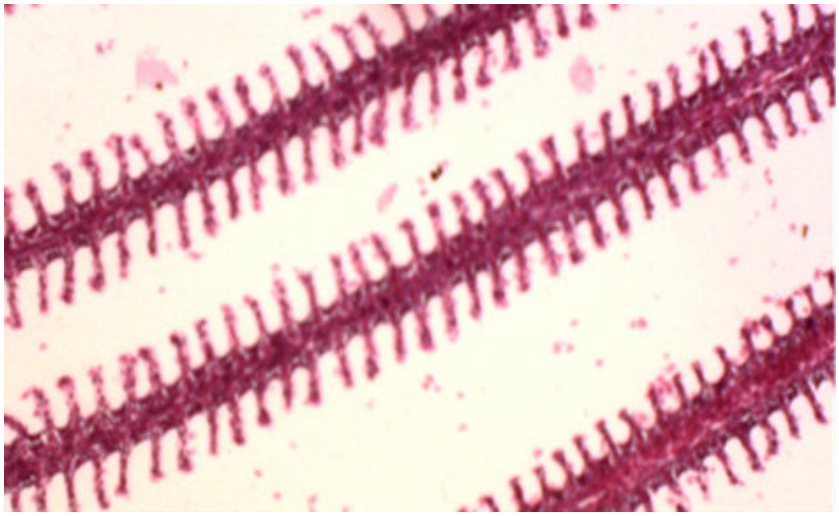


Fig. 4.3.1.29. Histopathology of Kidney after exposure to 128 ppb of copper



(a)



(b)

Fig. 4.3.1.30. a) Gill (Normal) of *Oreochromis mossambicus* with well formed primary lamellae (P) and intact secondary lamellae (S). H & E, 10x. b) the same 20x.

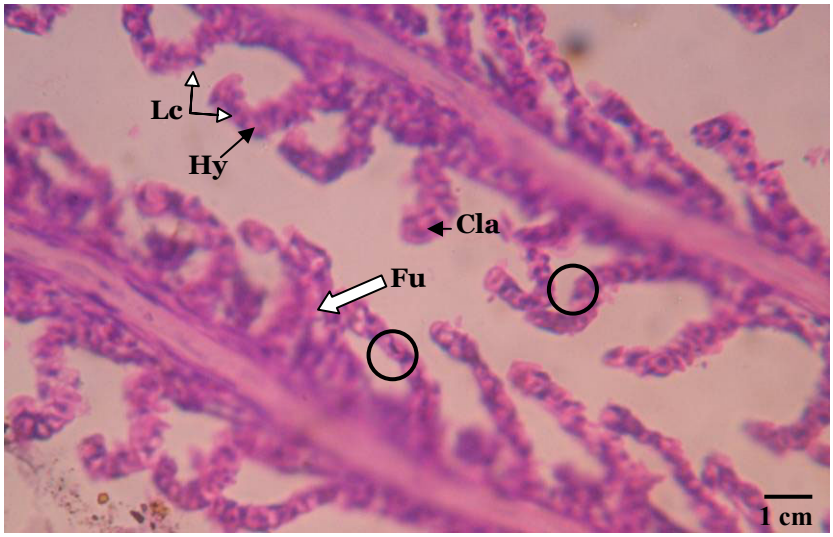
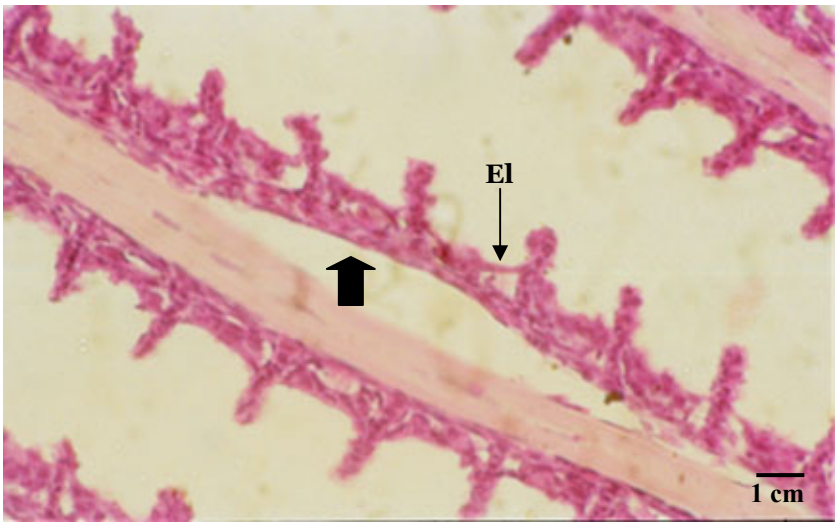
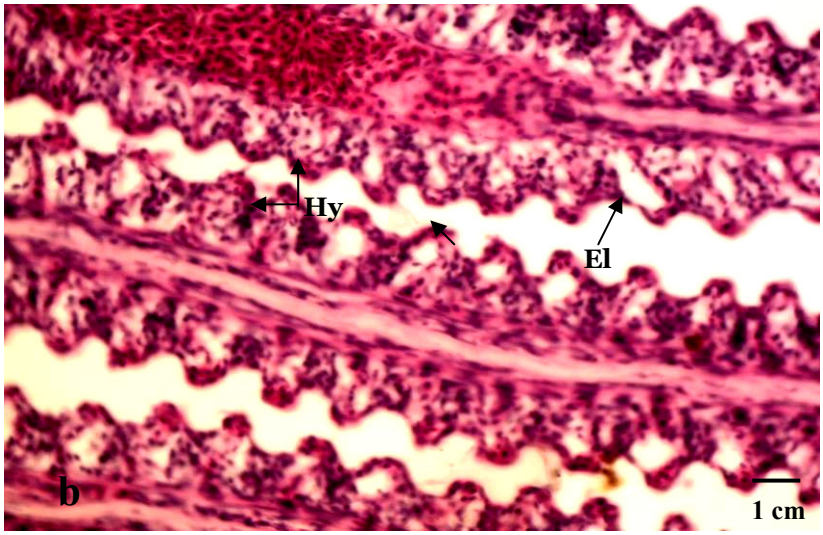


Fig. 4.3.1.31. Gill of *Oreochromis mossambicus* on 8th day of exposure to 128 ppb of copper, indicating clavate lamellae (Cla), secondary lamellar curling (Lc) leading to lamellar fusion (Fu) and hyperplasia (Hy) of lamellar epithelium and basal laminar cells containing chloride cells and mucus cells. Pillar cells alternating with lamellar blood sinuses are shown (circles). 40x.



(a)



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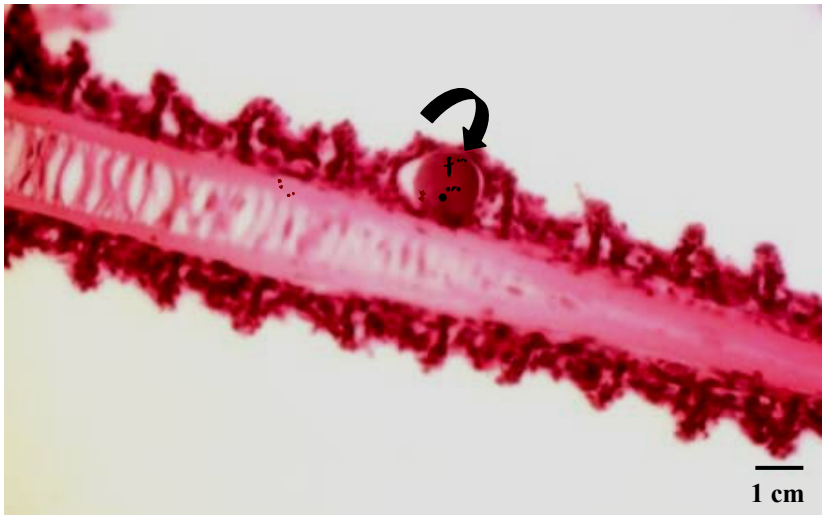

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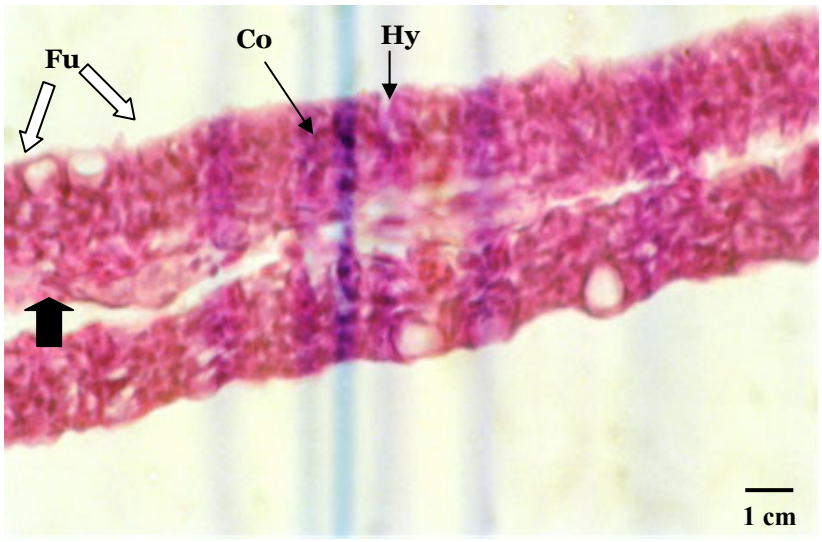


(d)

Fig. 4.3.1.32. Gill 16th day of exposure to 128 ppb of copper with more advanced lesions. a) Severe hyperplasia of filamental and secondary lamellar epithelia (Hy) shortening of secondary lamellae and slight epithelial lifting (El) squamous metaplasia (black block arrows).b) Spreading of epithelial lifting to other lamellae accompanied by squamous metaplasia and exfoliation (black block arrows).c) Balloon degeneration (curved block arrow). d) Vascular congestion or hyperemia (Co), total fusion of secondary lamellae (white block arrows) and advanced squamous metaplasia (black block arrows). 40x.

Table 4.3.1.11. Histopathology of gill after exposure to 128 ppb of copper.

Treatment	Percentage damage					
	Lamellar curling	**Lamellar fusion and shortening**	**Epithelial lifting**	**Hyperplasia**	**Squamous metaplasia**	**Balloon degeneration**
Normal	0.00	0.00	0.00	1.50	0.00	0.00
8th day	29.50	5.00	0.00	15.21	0.00	0.00
16th day	56.00	74.00	100.00	86.00	65.38	0.90

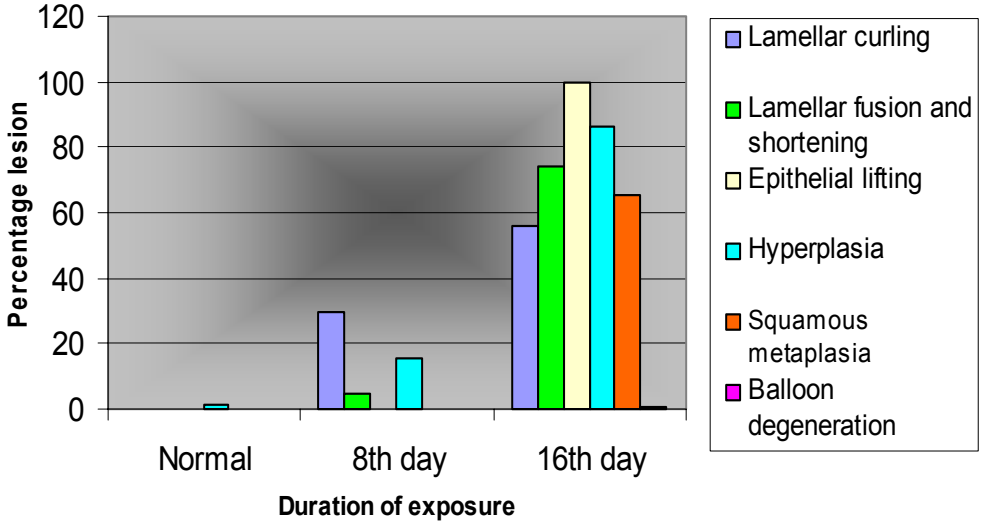


Fig. 4.3.1.33. Histopathology of gill after exposure to 128 ppb of copper

4.3.2 Effects of Exposure to Malachite Green

Malachite green is highly toxic to fish. The progress of intoxication is very rapid and the pathological anatomical picture of which is characterized by greenish tinge of their skin and increased production of skin slime. The gills are oedematous with excessive production of mucus and are discolored by the dye. Vessels in the body cavity were dilated, and muscle tissue and internal organs were often light green in colour (Machova *et al*., 2001). Similar observations were recorded in the present study also. Though the oxalate form of the dye is supposed to be less toxic, the recommended therapeutic concentrations are more often very close to and sometimes even higher than its lethal concentration (Sudova *et al*., 2007).

Except in the case of kidney, the activity of catalase was found to increase steadily and gradually under low concentration of malachite green. Nevertheless, kidney showed steady increase of catalase activity on 4^{th} and 8^{th} day and sharp decrease (below that of control animals)

thereafter. So also at higher concentration of the dye, catalase in liver and gills recorded higher activity when compared to that of control animals. In the three test tissues, significant reduction ($P<0.05$) was recorded on 16th day under 25 ppb level of the dye. Here also kidney was an exception where a gradual decrease appeared commencing from 4th day. Curiously enough, the enzyme activity registered catalase activity values below that of control animals on the 4th day (Table. 4.3.2.1 a & b, Fig.4.3.2.1, 2 and 3).

Under both low and high concentrations of malachite green, the level of superoxide dismutase in liver increased progressively up to 12th day with a significant declension on 16th day. However, in the gills, the activity enhancement was confined to 4th day only at low concentration. At high concentration, no enhancement was observed in the gill. On the contrary, the activity decreased from the 4th day onwards reaching the minimum on the 16th day. The values were below the control values. In kidney, at low concentration, the activity of this primary antioxidant enzyme decreased gradually at low concentration and showed a dwindling trend at high concentration (Table 4.3.2.2 a & b, Fig. 4.3.2.4, 5 and 6).

The activity of glutathione peroxidase resembles the response of catalase, recording gradual increase in liver and gills under low concentration of malachite green. However, at higher levels of the toxicant, the increase in activity up to 12th day has been observed only in liver. In gills, the enzyme behaved differently with alternate increase and decrease in activity. Kidney exhibited a sharp enhancement of glutathione peroxidase activity at low concentration on 4th day itself but the activity declined progressively reaching levels below that of control. However, by and large the enzyme activity in kidney though altered was below the

control levels at high concentration of the dye (Table 4.3.2.3. a & b, Fig. 4.3.2.7, 8 and 9).

The average activity level of glutathione S transferase was highest in liver when compared to that of the gills and kidney. The hierarchy of activity was in the order liver > gill > kidney. At low concentration of malachite green, the activity of this enzyme showed significant changes ($P< 0.05$). In the liver, the activity increased up to 12^{th} day only. However, at high concentration of the dye, the activity increased commendably on the 4^{th} day.

Liver has shown a progressive upturn in glutathione S transferase activity commencing from the 4^{th} day culminating in a decrease ($P<0.05$) on 16^{th} day. Interestingly, the trend was just the opposite at high concentration. After recording an enhancement on 4^{th} day, it decreased progressively reaching the minimum on 16^{th} day. At low concentration in the kidney, the activity of glutathione transferase decreased below that of the control level on the 4^{th} day itself, reaching minimal level on 16^{th} day of exposure. Though the activity of glutathione S transferase has shown similar trend at high concentration also, the activity approached below normal condition only on 8^{th} day and then continued the tendency to decrease up to the last day of sampling. The activity of this enzyme has recorded a decreasing trend (below control values) in the gills on 4^{th} day of sampling, and then showed a decreasing trend up to 12^{th} day. However, the activity enhanced on the 16^{th} day. At high concentration, the activity increased up to 8^{th} day decreasing progressively up to 16^{th} day (Table. 4.3.2.4. a & b, Fig. 4.3.2.10, 11 and 12).

Glutathione reductase activity data for liver has registered a rise up to 12th day at low concentration of malachite green. Similarly, at high concentration of the dye, the enzyme activity has increased up to 8th day and decreased thereafter. The increase and decrease were concentration dependent. The activity of this antioxidative enzyme exhibited an initial (4th day) increase and progressive decrease reaching the minimum on 16th day. In the gills, the exposure to malachite green resulted in a progressive rise in the activity of this enzyme up to 12th day at low concentration and up to 8th day at high concentration. A lowering of activity was the effect on 16th day at low concentration and this decrease began on 12th day itself at high concentration, approaching the minimal level on 16th day (Table. 4.3.2.5. a & b, Fig. 4.3.2.13, 14 and 15).

The rate of generation of malondialdehyde conjugates has shown a steady increase from 4th to 16th day in all the test tissues under the two experimental concentrations (Table. 4.3.2.6. a & b, Fig. 4.3.2.16, 17 and 18).

The level of reduced glutathione revealed significant ($P<0.05$) changes with respect to concentration, duration of exposure and tissues due to exposure to malachite green. In general, the reduced glutathione level was below control values in the tissues at the two experimental concentrations (Table. 4.3.2.7. a & b, Fig. 4.3.2.19, 20 and 21).

Table 4.3.2.1 a). Changes in catalase activity in tissues after exposure to different concentrations of malachite green.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	9.612± 0.0310	9.610± 0.0220	9.609± 0.0150	9.610± 0.4100
	12.5 ppb	10.13± 0.0787	10.92± 0.0907	15.10± 0.0893	18.13± 0.0677
	25 ppb	13.74± 0.2466	14.18± 0.1086	18.23± 0.1176	11.22± 0.1769
Kidney	Control	3.000± 0.0170	3.130± 0.0090	3.080± 0.0330	3.050± 0.0700
	12.5 ppb	2.953± 0.0288	3.447± 0.0327	1.365± 0.0207	1.130± 0.0110
	25 ppb	2.392± 0.0717	2.130± 0.0228	2.018± 0.0467	1.692± 0.0739
Gills	Control	14.91± 0.0230	14.90± 0.2000	14.91± 0.0410	14.87± 0.9100
	12.5 ppb	17.07± 0.2161	18.06± 0.5621	19.39± 0.1272	19.85± 0.0194
	25 ppb	17.46± 0.0194	20.94± 0.0718	17.12± 0.2845	13.34± 0.1275

Table 4.3.2.1 b) ANOVA for changes in the activity of catalase in relation to malachite green exposure.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	7935.007	2	3967.504	960.547	.000
Duration	36.826	3	12.275	2.972	.033
Concentration	226.507	2	113.253	27.419	.000
Error	859.136	208	4.130		
Total	9057.476	215			

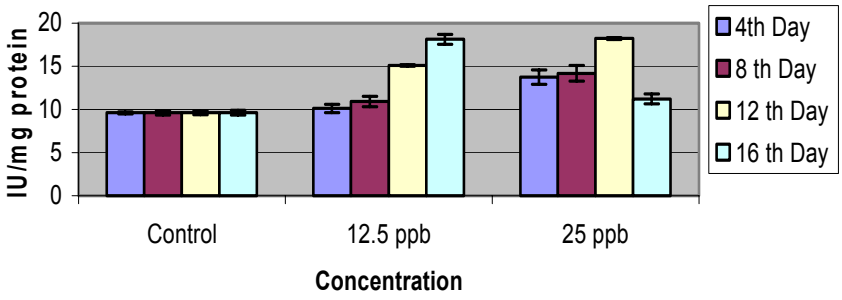


Fig. 4.3.2.1. Changes in catalase activity in liver after exposure to malachite green

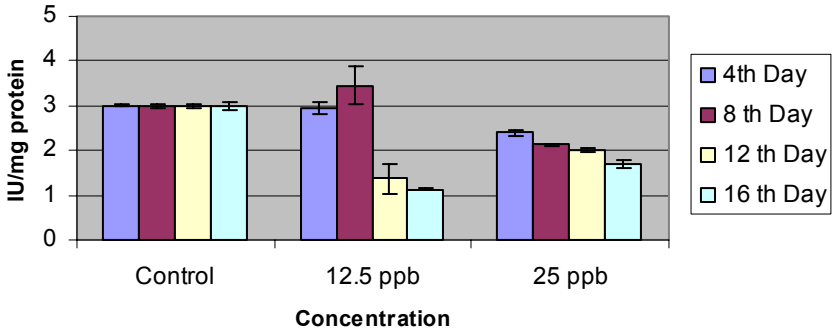


Fig. 4.3.2.2. Changes in catalase activity in kidney after exposure to malachite green

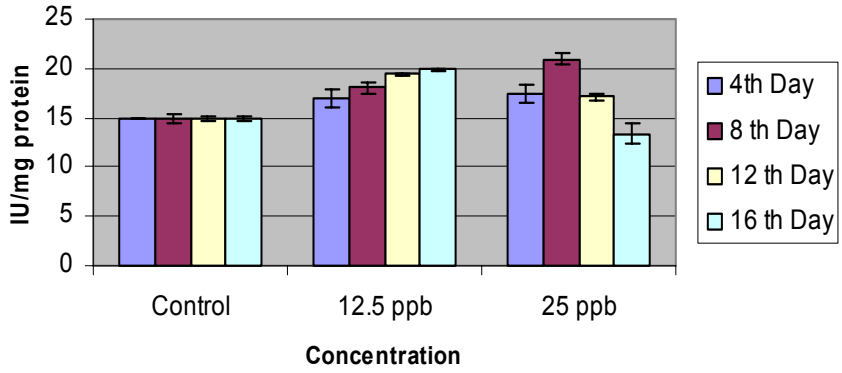


Fig. 4.3.2.3. Changes in catalase activity in gill after exposure to malachite green

Table 4.3.2.2 a). Changes in superoxide dismutase activity in tissues after exposure to different concentrations of malachite green.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	0.968± 0.0370	0.965± 0.1800	0.966± 0.0200	0.969± 0.1100
	12.5 ppb	1.218± 0.0117	3.089± 0.0455	9.332± 0.0496	4.558± 0.3056
	25 ppb	1.321± 0.0262	4.123± 0.0368	9.332± 0.0496	3.048± 0.0656
Kidney	Control	0.321± 0.0120	0.320± 0.5100	0.321± 0.0300	0.324± 0.0700
	12.5 ppb	0.283± 0.0135	0.225± 0.0259	0.175± 0.0057	0.140± 0.0268
	25 ppb	0.040± 0.0027	0.221± 0.3229	0.040± 0.0063	0.310± 0.0219
Gills	Control	0.984± 0.0300	0.982± 0.6100	0.980± 0.0080	0.981± 0.2000
	12.5 ppb	1.800± 0.0544	0.660± 0.0447	0.507± 0.0139	0.417± 0.0037
	25 ppb	0.691± 0.0556	0.317± 0.0234	0.224± 0.0102	0.121± 0.0228

Table 4.3.2.2 b). ANOVA for changes in the activity of superoxide dismutase in relation to malachite green exposure.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	398.778	2	199.389	79.873	.041
Duration	77.713	3	25.904	10.377	.011
Concentration	49.704	2	24.852	9.955	.001
Error	519.237	208	2.496		
Total	1045.432	215			

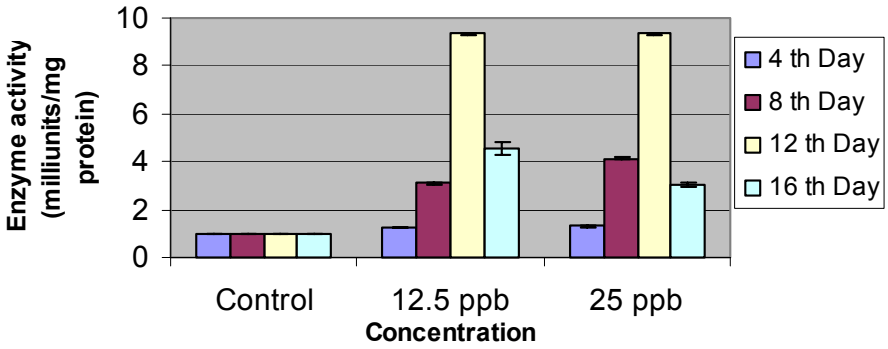


Fig. 4.3.2.4. Superoxide Dismutase activity in liver after exposure to malachite Green

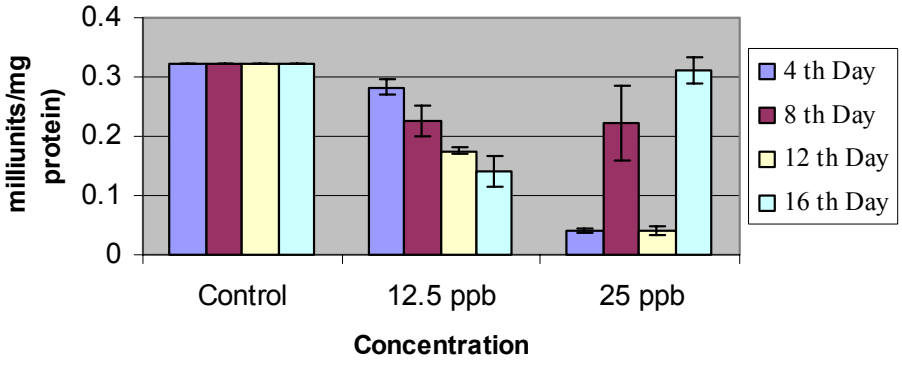


Fig. 4.3.2.5. Changes in superoxide dismutase activity in kidney after exposure to malachite green

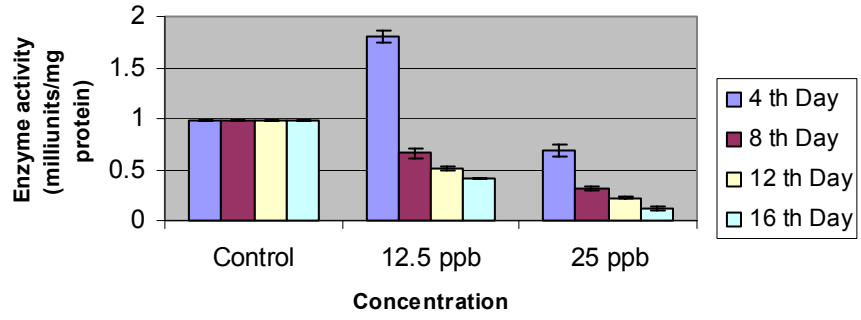


Fig. 4.3.2.6. Superoxide Dismutase activity in gill afterexposure to Malachite Green

Table 4.3.2.3 a). Changes in glutathione peroxidase activity in tissues after exposure to different concentrations of malachite green.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	7.563± 0.0470	7.561± 0.0220	7.558± 0.0100	7.560± 0.0500
	12.5 ppb	8.913± 0.1392	13.70± 0.2854	17.082± 0.4849	10.620± 0.0710
	25 ppb	15.65± 0.3792	18.19± 0.2282	23.26± 0.4298	16.05± 0.1660
Kidney	Control	4.080± 0.0470	4.083± 0.0300	4.079± 0.1200	4.080± 0.0114
	12.5 ppb	6.187± 0.2521	3.210± 0.2355	2.142± 0.0160	0.878± 0.0299
	25 ppb	2.112± 0.0436	1.185± 0.0599	1.878± 0.0387	0.908± 0.0417
Gills	Control	12.13± 0.0500	12.15± 0.0130	12.15± 0.0080	12.16± 0.0600
	12.5 ppb	10.53± 0.0454	13.16± 0.0234	18.07± 0.2851	13.74± 0.0907
	25 ppb	11.62± 0.1231	10.11± 0.1056	11.297± 0.1090	9.097± 0.0520

Table 4.3.2.3 b). ANOVA for changes in the activity of glutathione peroxidase in relation to malachite green exposure.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	4433.915	2	2216.957	235.708	.001
Duration	192.989	3	64.330	6.840	.015
Concentration	203.869	2	101.935	10.838	.010
Error	1956.345	208	9.406		
Total	6787.118	215			

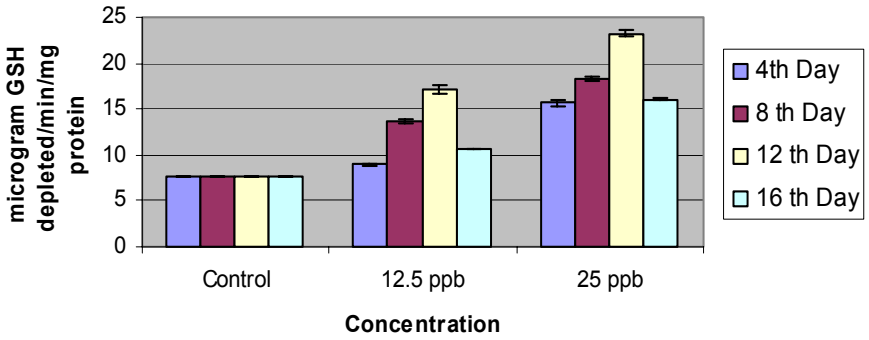


Fig. 4.3.2.7. Changes in glutathione peroxidase activity in liver after exposure to malachite green

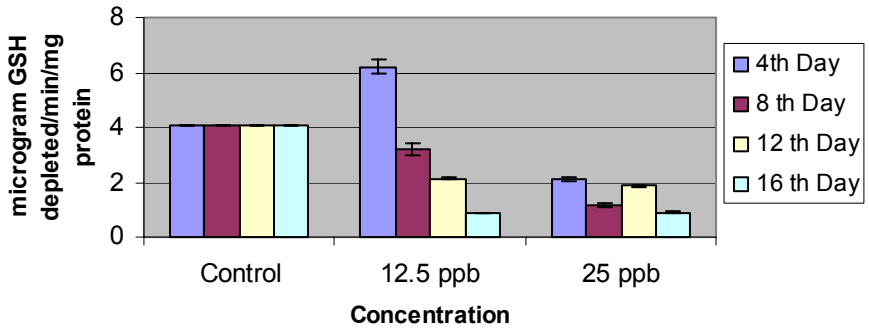


Fig. 4.3.2.8. Changes in glutathione peroxidase activity in kidney after exposure to malachite green

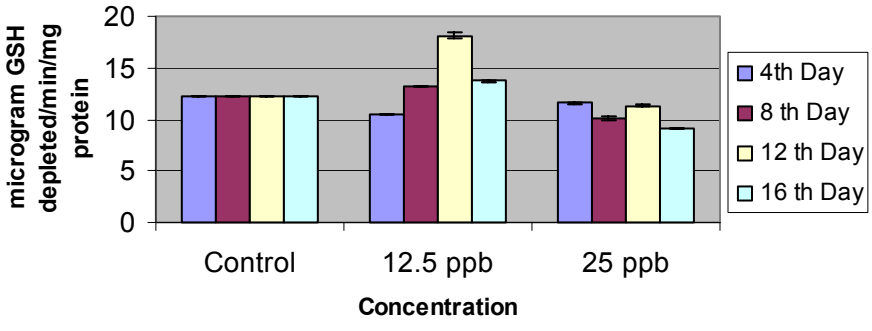


Fig. 4.3.2.9. Changes in glutathione peroxidase activity in gill after exposure to malachite green

Table 4.3.2.4 a). Changes in glutathione S transferase activity in tissues after exposure to different concentrations of malachite green.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	22.74± 0.0550	22.74± 0.0570	22.72± 0.0230	22.74± 0.0810
	12.5 ppb	30.01± 0.2633	33.17± 0.0561	39.89± 0.2778	23.33± 0.4715
	25 ppb	40.90± 0.5224	35.37± 0.4862	27.40± 0.3984	20.71± 0.2354
Kidney	Control	12.43± 0.0520	12.43± 0.0800	12.40± 0.0020	12.43± 0.0930
	12.5 ppb	10.02± 0.2134	6.048± 0.0975	4.137± 0.1621	3.403± 0.1632
	25 ppb	13.97± 0.3090	10.91± 0.2313	7.485± 0.1310	2.425± 0.0709
Gills	Control	14.96± 0.0057	14.92± 0.0200	14.94± 0.0090	14.96± 0.7000
	12.5 ppb	14.01± 0.5693	12.20± 0.2535	8.528± 0.0360	13.68± 0.1580
	25 ppb	15.48± 0.1323	17.98± 0.2349	11.44± 0.1143	9.903± 0.3084

Table 4.3.2.4 b). ANOVA for changes in the activity of glutathione S transferase in relation to malachite green exposure.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	14916.992	2	7458.496	391.606	.041
Duration	1005.428	3	335.143	17.597	.013
Concentration	71.233	2	35.616	1.870	.157
Error	3961.549	208	19.046		
Total	19955.202	215			

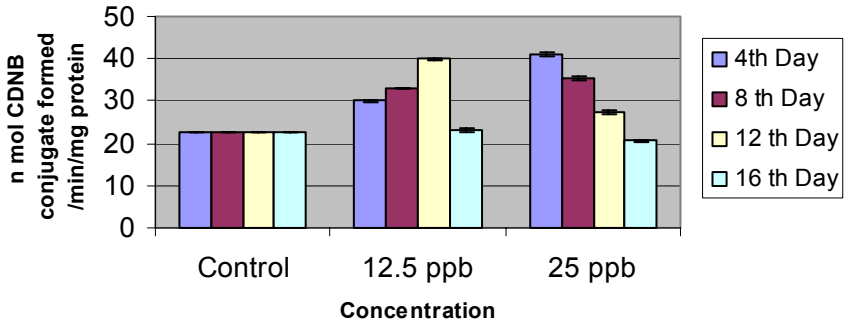


Fig. 4.3.2.10. Changes in glutathione S transferase in liver after exposure to malachite green

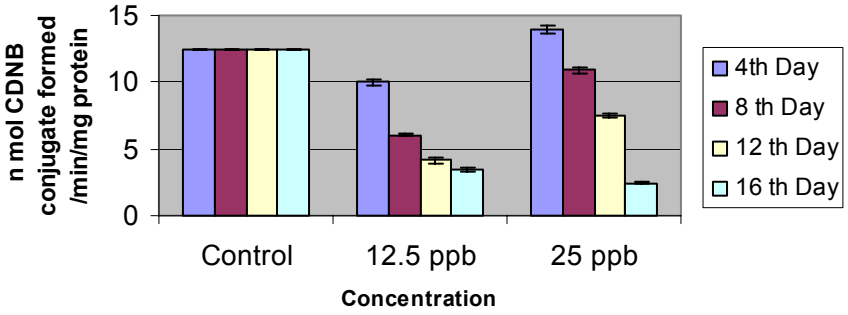


Fig. 4.3.2.11. Changes in glutathione S transferase activity in kidney after exposure to malachite green

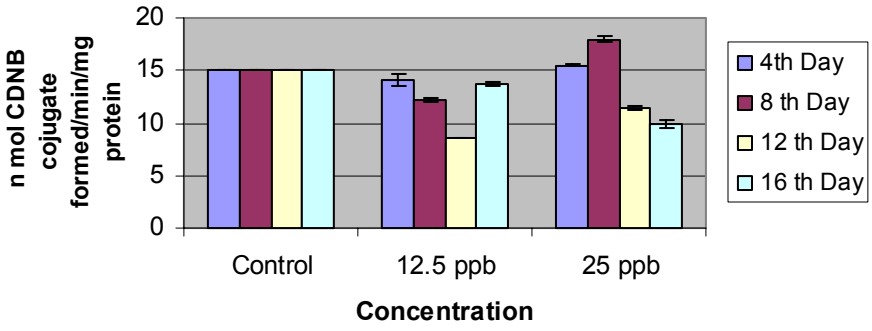


Fig. 4.3.2.12. Changes in glutathione S transferase activity in gill after exposure to malachite green

Table 4.3.2.5 a). Changes in glutathione reductase activity in tissues after exposure to different concentrations of malachite green.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	4.060± 0.0600	4.057± 0.0150	4.060± 0.0200	4.061± 0.0830
	12.5 ppb	9.163± 0.2893	16.11± 0.1862	19.37± 0.3859	14.44± 0.2014
	25 ppb	9.677± 0.2774	13.27± 0.2878	6.170± 0.1135	2.223± 0.1737
Kidney	Control	2.420± 0.4100	2.423± 0.0400	2.422± 0.0720	2.418± 0.0060
	12.5 ppb	2.052± 0.1795	1.027± 0.0891	0.532± 0.0232	0.138± 0.0194
	25 ppb	3.247± 0.2202	1.647± 0.0771	0.435± 0.0327	0.097± 0.0081
Gills	Control	3.970± 0.1200	3.100± 0.3200	3.108± 0.0800	3.170± 0.0430
	12.5 ppb	5.080± 0.1349	9.180± 0.1151	11.41± 0.0980	10.76± 0.1317
	25 ppb	7.390± 0.1035	9.180± 0.1151	4.177± 0.0948	1.610± 0.0696

Table 4.3.2.5 b). ANOVA for changes in the activity of glutathione reductase in relation to malachite green exposure.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	1853.170	2	926.585	83.467	.006
Duration	150.680	3	50.227	4.524	.004
Concentration	553.553	2	276.776	24.932	.026
Error	2275.749	205	11.101		
Total	4833.152	212			

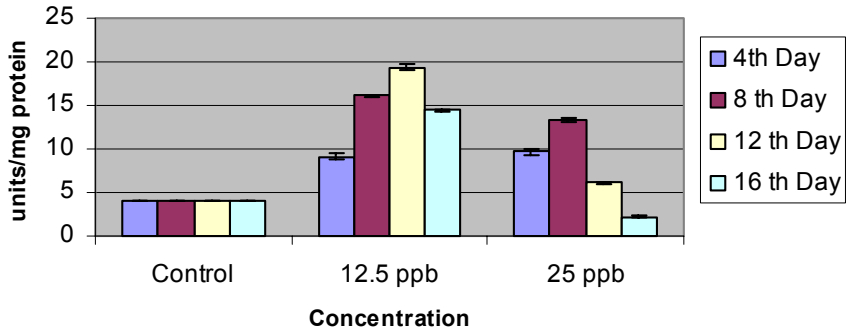


Fig. 4.3.2.13. Changes in glutathione reductase activity in liver after exposure to malachite green

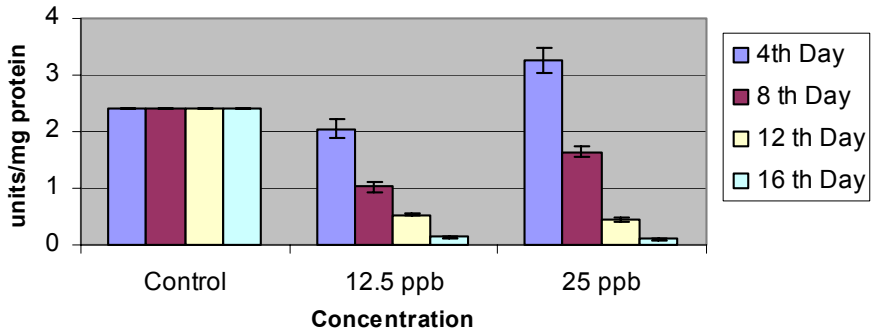


Fig. 4.3.2.14. Changes in glutathione reductase activity in kidney after exposure to malachite green

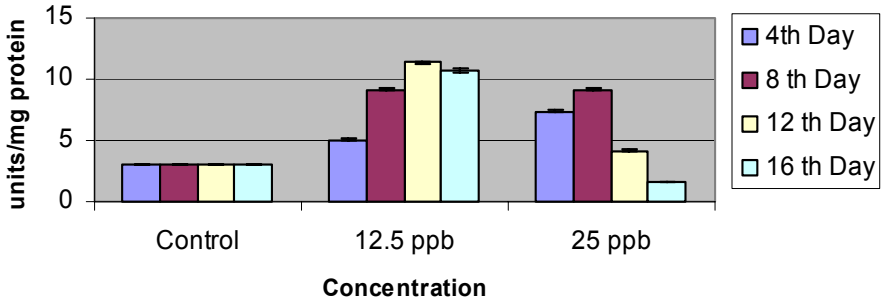


Fig. 4.3.2.15. Changes in glutathione reductase activity in gill after exposure to malachite green

Table 4.3.2.6 a). Changes in lipid peroxidation in tissues after exposure to different concentrations of malachite green.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	0.894± 0.0230	0.892± 0.4500	0.897± 0.0750	0.891± 0.0130
	12.5 ppb	0.853± 0.0090	0.870± 0.0048	1.287± 0.4271	4.168± 0.0359
	25 ppb	0.916± 0.0055	1.154± 0.0238	3.632± 0.0809	7.112± 0.3266
Kidney	Control	0.383± 0.0055	0.382± 0.0660	0.381± 0.0070	0.383± 0.0270
	12.5 ppb	0.724± 0.0237	0.817± 0.0350	1.041± 0.1079	2.398± 0.0987
	25 ppb	0.948± 0.0271	2.278± 0.0880	4.583± 0.2545	5.185± 0.0476
Gills	Control	1.080± 0.0074	1.083± 0.0700	1.082± 0.0500	1.081± 0.0840
	12.5 ppb	4.148± 0.0271	11.47± 0.0632	14.67± 1.6051	15.40± 0.0828
	25 ppb	4.430± 0.0645	12.52± 0.1430	19.26± 0.4062	19.36± 0.0634

Table 4.3.2.6 b). ANOVA for changes in lipid peroxidation in relation to malachite green exposure.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	2357.745	2	1178.872	79.929	.011
Duration	666.201	3	222.067	15.056	.007
Concentration	320.991	2	160.496	10.882	.027
Error	3067.803	208	14.749		
Total	6412.740	215			

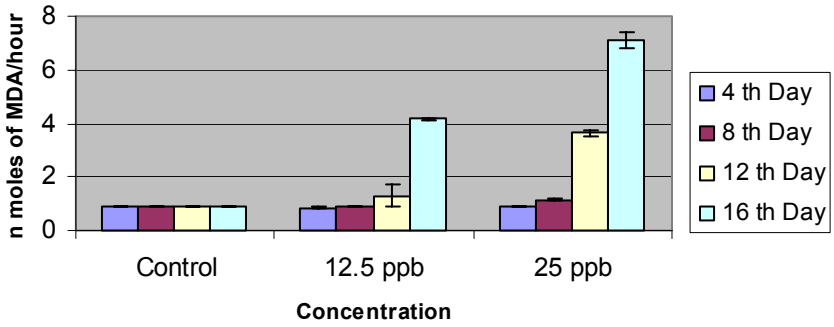


Fig. 4.3.2.16. Lipid peroxidation in liver after exposure to malachite green

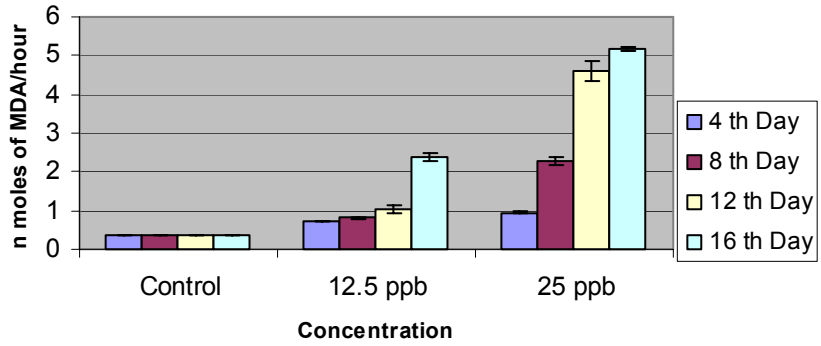


Fig. 4.3.2.17. Lipid peroxidation in kidney after exposure to malachite green

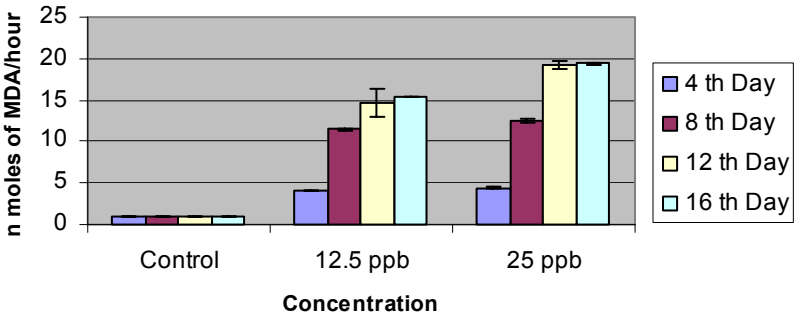


Fig. 4.3.2.18. Lipid peroxidation in gill after exposure to malachite green

Table 4.3.2.7 a). Changes in reduced glutathione level in tissues after exposure to different concentrations of malachite green.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	1745± 0.0087	1748± 0.0021	1745± 0.0090	1748± 0.0710
	12.5 ppb	1230. 7± 1.2111	1017.2± 1.8349	975.9± 0.9559	917.9± 0.2499
	25 ppb	1213. 7± 1.0328	964.6± 0.6802	937.5± 1.0078	670.9± 0.2535
Kidney	Control	1018± 0.0500	1019± 0.0300	1017± 0.0210	1017± 0.0430
	12.5 ppb	1026.2± 0.3755	1411.9± 0.4273	1033.1± 0.9438	976.6± 0.5162
	25 ppb	1018.9± 0.4454	1074.1± 0.2101	975.2± 0.2422	969.0± 0.2637
Gills	Control	1313± 0.0065	1313± 0.0700	1314± 0.3200	1313± 0.0140
	12.5 ppb	1204± 0.1506	1147.2± 0.9940	1020.2± 0.2070	997.0± 0.9957
	25 ppb	1212.9± 0.5288	1199.4± 1.4460	1027.9± 0.8271	1025.8± 1.1465

Table 4.3.2.7 b). ANOVA for changes in reduced glutathione level in relation to malachite green exposure.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	.724	2	.362	11.003	.001
Duration	.812	3	.271	8.221	.030
Concentration	.734	2	.367	11.148	.033
Error	6.845	208	.034		
Total	9.115	215			

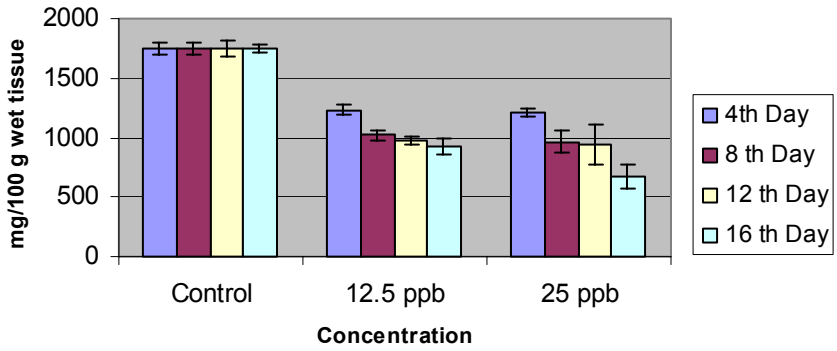


Fig. 4.3.2.19. Reduced glutathoione level in liver after exposure to malachite green

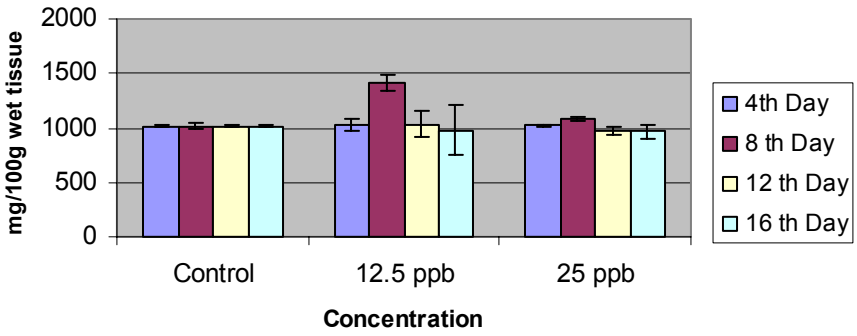


Fig. 4.3.2.20. Reduced glutathoione level in kidney after exposure to malachite green

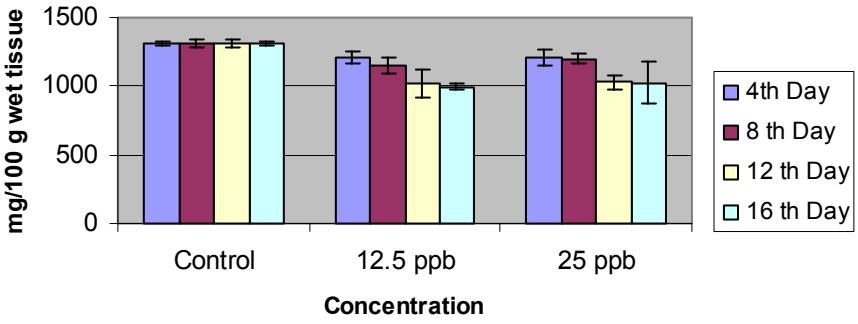


Fig. 4.3.2.21. Reduced glutathoione level in gill after exposure to malachite green

Table 4.3.2.8. Multiple Comparison Test (Dunnett *t*) for Enzymatic Activities in *Oreochromis mossambicus* after exposure to malachite green.

	Groups	Catalase	Superoxide dismutase	Glutathione peroxidase	Glutathione S transferase	Glutathione reductase	Lipid peroxidation	Reduced glutathione
Tissue	Liver & Kidney	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.857 [d]	0.000 [a]
	Liver & Gills	0.000 [a]	0.000 [a]	0.446 [d]	0.000 [a]	0.000 [a]	0.000 [a]	0.866 [d]
	Kidney & Gills	0.000 [a]	0.146 [d]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.001 [a]
Day	4 Days & 8 Days	0.333 [d]	0.628 [d]	0.828 [d]	0.657 [d]	0.098 [d]	0.053 [d]	0.986 [d]
	4 Days & 12 Days	0.038 [c]	0.000 [a]	0.003 [b]	0.005 [b]	0.778 [d]	0.000 [a]	0.034 [c]
	4 Days & 16 Days	0.968 [d]	0.638 [d]	0.897 [d]	0.000 [a]	0.581 [d]	0.000 [a]	0.000 [a]
	8 Days & 12 Days	0.745 [d]	0.000 [a]	0.040 [c]	0.121 [d]	0.516 [d]	0.099 [d]	0.082 [d]
	8 Days & 16 Days	0.609 [d]	1.000 [d]	0.407 [d]	0.000 [a]	0.003 [b]	0.002 [b]	0.001 [a]
	12 Days & 16 Days	0.117 [d]	0.000 [a]	0.000 [a]	0.005 [b]	0.120 [d]	0.516 [d]	0.416 [d]
Concentration	Control & 12.5 ppb	0.000 [a]	0.000 [a]	0.001 [a]	0.968 [d]	0.000 [a]	0.327 [d]	0.618 [d]
	Control & 25ppb	0.000 [a]	0.002 [b]	0.000 [a]	0.274 [d]	0.001 [a]	0.006 [b]	0.001 [a]
	12.5 ppb & 25ppb	0.731 [d]	0.686 [d]	0.867 [d]	0.178 [d]	0.000 [a]	0.000 [a]	0.000 [a]

The values are significant at a=P<.001, b=P<.01 and c=P<.05 and not significant at d.

Histopathological Effects of Malachite Green

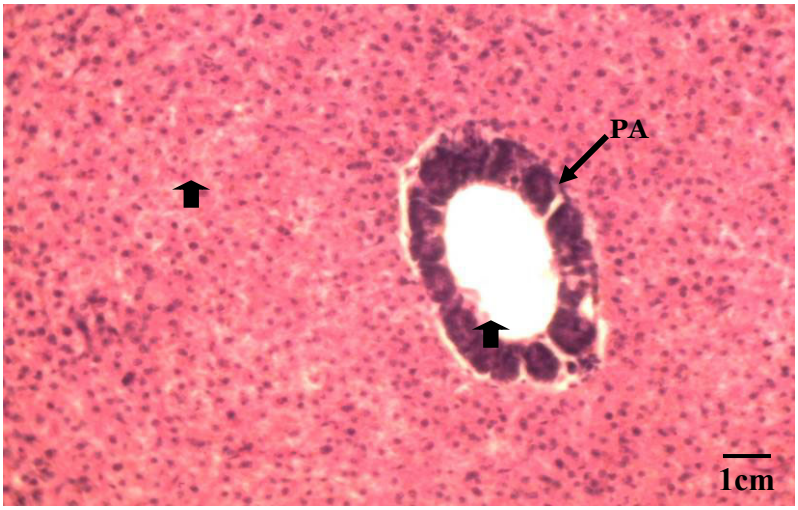
The exposure of *Oreochromis mossambicus* to 25 ppb of malachite green resulted in histological alterations in liver, kidney and gills. The deviations from normalcy in gill were, in general, not as severe as observed under exposure to copper.

The nuclei of hepatocytes became pycnotic by the 8th day after exposure to the dye which scaled up to more than 92 percent by 16th day. Necrosis and non fatty vacuolation were the additional features emerged by 16th day (Fig. 4.3.2.22. a, b, c and d, Table 4.3.2.8 and Fig. 4.3.2.23).

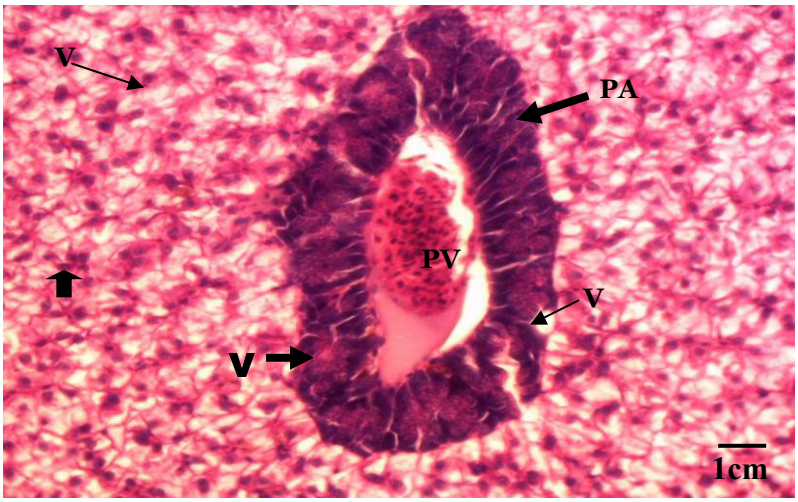
Kidney of the fish underwent extensive hyalinization, glomerular inflammation (swelling), widening of Bowman's spaces and pycnosis of nuclei due to malachite green exposure. There occurred a duration dependent increase in tubular constriction. The extent of hyalinization and nuclear condensation also progressed with duration of exposure (4.3.2.24 and 25). As s result of exposure to malachite green, kidney of the fish revealed a duration dependent increase in pycnotic nuclei. The average number of inflammated (swollen) glomeruli and associated Bowman's spaces remained constant after 8th and 16th day of exposure. There was no duration dependent change in glomerular leakage. However, the cytoplasm of tubules became progressively hyalinised recording 58.70 percent on 8th day after exposure and 73 percent on 16th day. The number of tubules with constricted lumen increased from 11 percent on 8th day to 69 percent on 16th day after exposure to the dye (Table. 4.3.2.9, Fig. 4.3.2.24 a & b, 25).

Except exfoliation, the gills were found to suffer no gross structural damage due to malachite green exposure. A negligible but duration

dependent declension was observed in secondary lamellar curling (Fig. 4.3.2.27) accompanied by a slight increase in lamellar fusion. The increase recorded in the hyperplasia of respiratory epithelia was also dependent on duration. However, exfoliation of basal laminar cells was a new change manifested after 16th day of exposure (Fig. 4.3.2.26. a & b).



(a)



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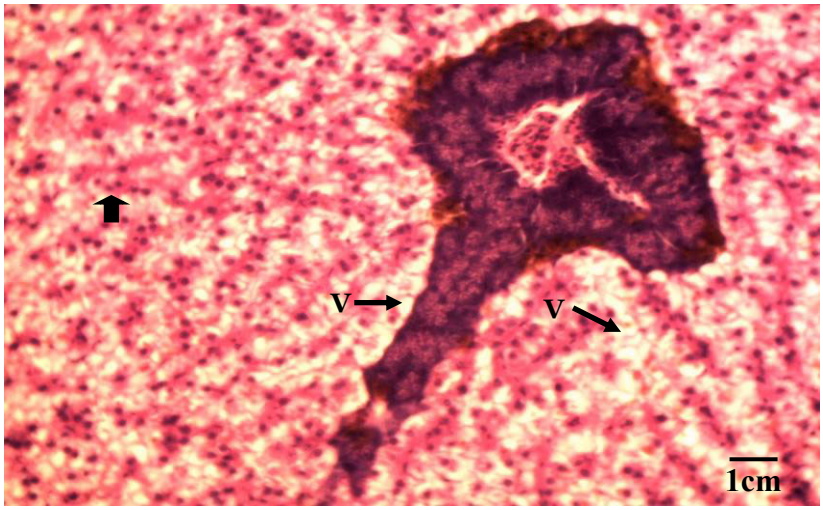

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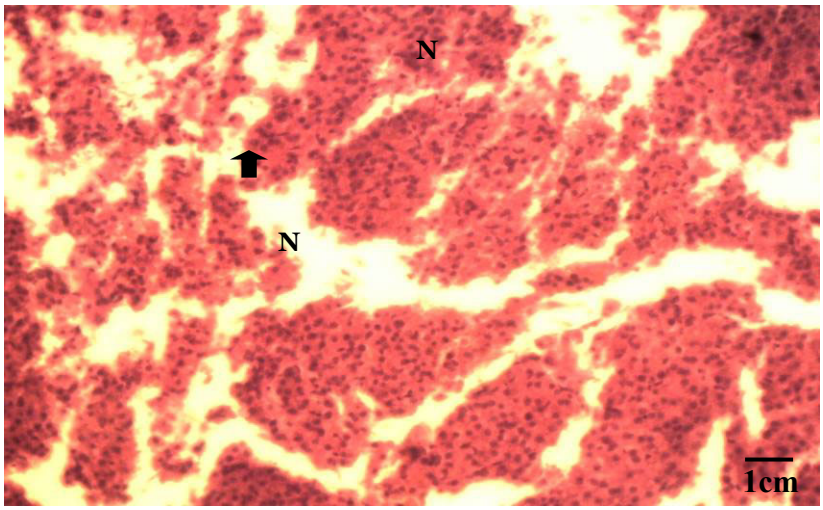


(d)

Fig. 4.3.2.22.a) Liver on 8th day of exposure to 25 ppb of malachite green denoting moderate and non fatty vacuolation of both the hepatocytes and pancreatic cells. 20x. b), c) and d) Liver on 16th day with the same concentration. 40x. Abbreviations and symbols: Vacuolation (V), pycnotic nuclei (block arrows), pancreatic acinus (PA), portal vein (PV), necrosis leading to architectural loss (N).

Table 4.3.2.9. Histopathology of liver after exposure to 25 ppb of malachite green.

Treatment	Percentage damage		
	Pycnosis	**Necrosis and architectural loss**	**vacuolation**
Normal	0.00	0.00	0.00
8th day	68.00	0.00	48.00
16th day	92.60	87.75	81.30

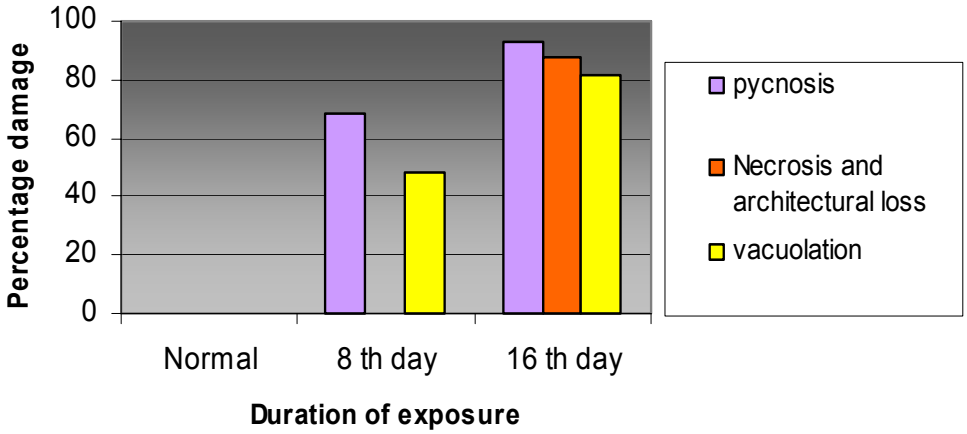
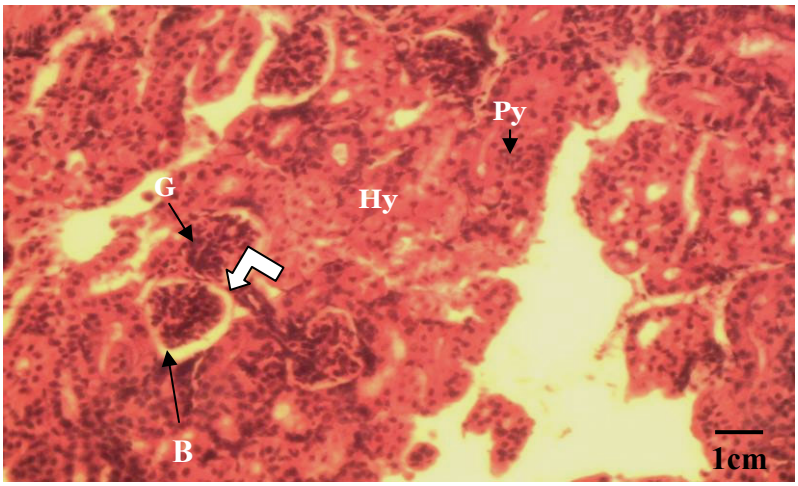
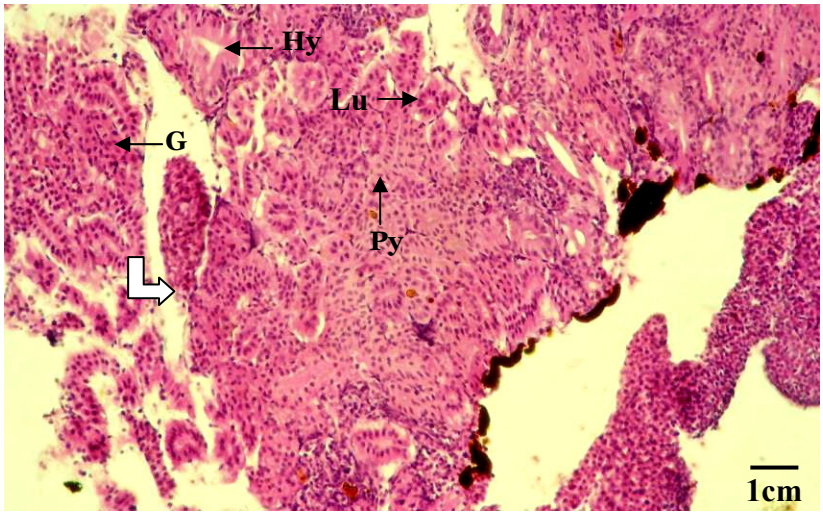


Fig.4.3.2.23. Histopathology of liver after exposure to 25 ppb of malachite green



(a)



(b)

Fig.4.3.2.24. a) and **b)** Kidney of *Oreochromis mossambicus* after exposure to 25 ppb of malachite green on 8th and 16th day respectively indicating hyalinised tubules (Hy), pycnotic nuclei (Py), swollen glomeruli (G), constriction of Bowman's capsules (B) glomerular leakage (white block arrow) and constriction of tubular lumen (Lu). H& E, 20x.

Table4.3.2.10. Histopathology of kidney in *Oreochromis mossambicus* after exposure to 25 ppb of malachite green.

Treatment	Percentage damage				
	Constriction of tubular lumen	Hyalinisation	Pycnosis	Glomerular leakage	Number of swollen glomeruli and Bowman's capsules
Normal	0.00	0.00	24.00	0.00	0.00
8th day	11.00	58.70	71.30	1.00	4.27
16th day	69.00	73.00	100.00	1.00	4.27

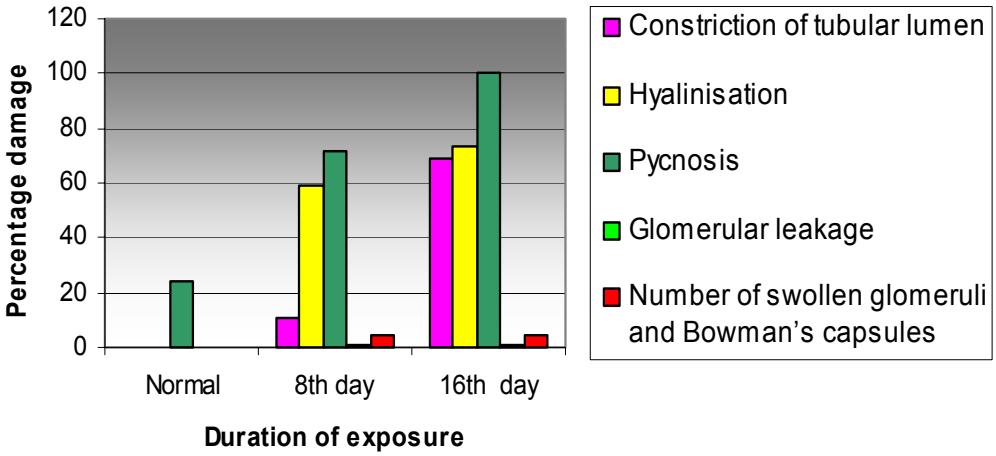


Fig. 4.3.2.25. Histopathology of Kidney after exposure to 25ppb of malachite green

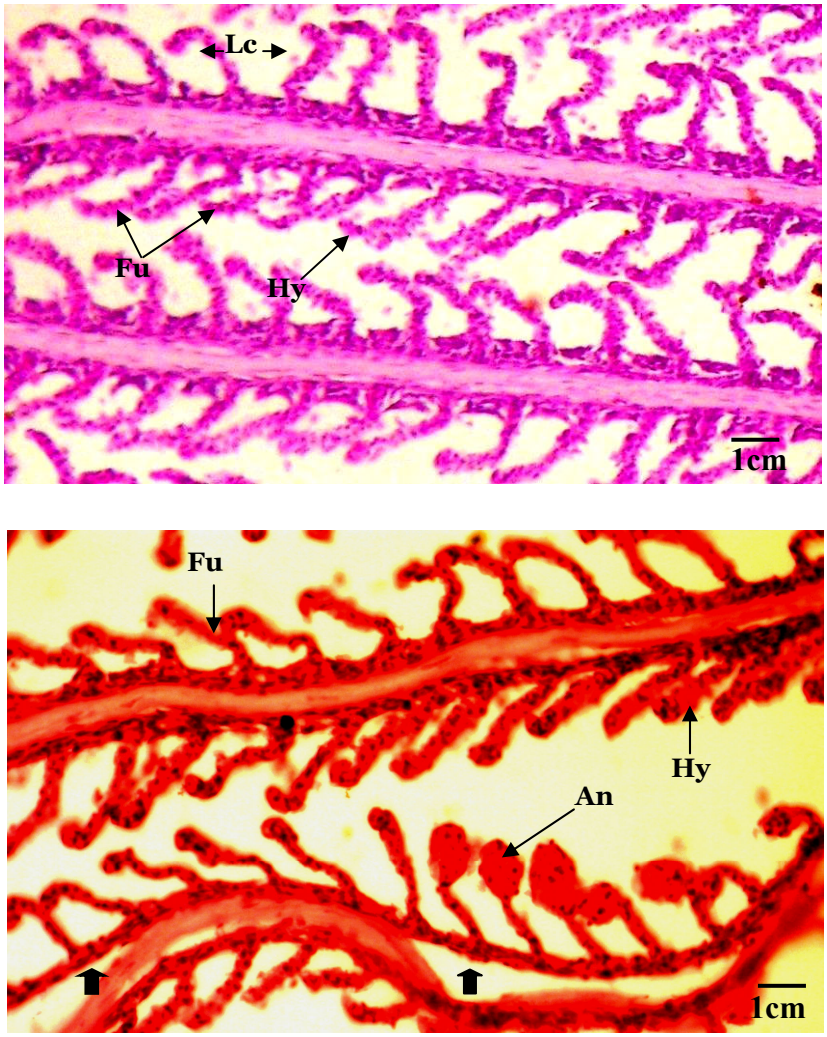


Fig. 4.3.2.26. Gill of *Oreochromis mossambicus* after exposure to 25 ppb of malachite green. a) 8th day b) 16th day. Abbreviations and symbols: Lc- Lamellar curling, Fu- Lamellar fusion, Hy-Hyperplasia, An- Aneurism, black block arrows- Exfoliation. 20x.

Table 4.3.2.11. Histopathology of gill in *Oreochromis mossambicus* after exposure to 25 ppb of malachite green.

Treatment	Percentage damage			
	Lamellar curling	Lamellar fusion	Hyperplasia	Exfoliation
Normal	0.00	0.00	1.50	0.00
8th day	46.80	12.00	14.40	0.00
16th day	43.60	16.43	17.85	14.06

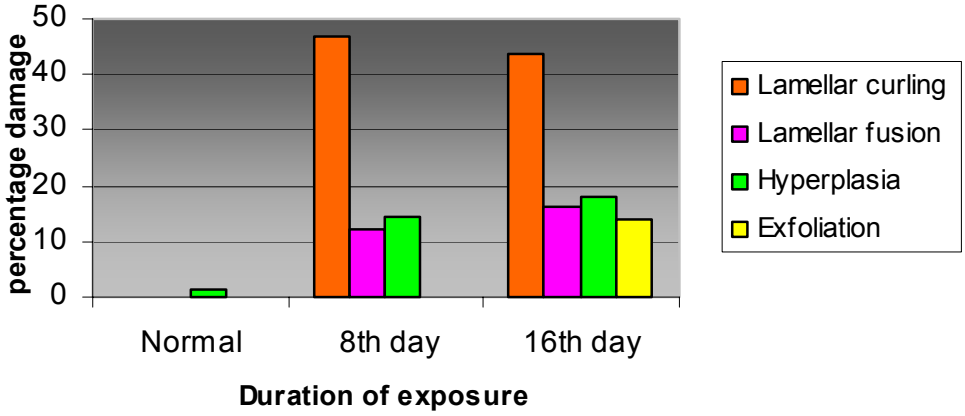


Fig. 4.3.2.27. Histopathology of gill after exposure to 25ppb of malachite green

4.3.3 Effects of Exposure to Metacid 50.

In liver, kidney and gill, the level of catalase was found to increase when exposed to Metacid 50. Low concentration of the toxicant elicited a progressive enhancement in catalase activity, reaching the maximum value on 16th day of exposure. However, at high concentration, the kidney and gills exhibited a reduction in activity on 16th day, whereas in liver, the enzyme was found to maintain high activity throughout the exposure period. However, none of the tissues showed activity below the control level under the influence of Metacid 50 (Table. 4.3.3.1 a & b, Fig. 4.3.3.1, 2 and 3).

The activity levels of superoxide dismutase in liver and kidney of test fishes were found to decrease considerably when compared to that of control fishes. The gills registered a more or less stable activity at low concentration. The activity was less on 16th day after exposure in high concentration. The kidney showed a continuous decrease in activity both at low and high concentrations of Metacid 50 (Table. 4.3.3.2 a & b, Fig. 4.3.3.4, 5 and 6).

In liver and gill, the activity of glutathione peroxidase enhanced significantly due to exposure to the pesticide, whereas in kidney, the activity recorded a reduction. Though the level of the enzyme exhibited an enhancement in gill also, it decreased after 8th day of exposure reaching the minimal level on 16th day (Table. 4.3.3.3 a & b, Fig. 4.3.3.7, 8 and 9).

Glutathione S transferase responded in a different manner in that it registered a continuous increase in activity at both the concentrations in liver and kidney, whereas a progressive reduction in activity occurred in gills (Table. 4.3.3.4 a & b, Fig. 4.3.3.10, 11 and 12).

The changes in the activity of glutathione reductase were more or less similar to the response of glutathione S transferase (Table. 4.3.3.5 a & b, Fig. 4.3.3.13, 14 and 15).

The lipid peroxidation values, as evidenced by malondialdehyde levels after exposure to the pesticide, were found to increase significantly ($P<0.05$) in the test tissues under both the experimental concentrations of the pesticide (Table. 4.3.3.6 a & b, Fig. 4.3.3.16, 17 and 18).

The level of reduced glutathione was lower in liver because of exposure to Metacid 50. Similar was the case with kidney. However, the gills showed a different response, a reduced level of the endogenous antioxidant on 4th and 8th day and then maintaining the levels almost equal to that of control group, on 12th and 16th day (Table. 4.3.3.7 a & b, Fig. 4.3.3.19, 20 and 21).

Table:4.3.3.1. a) Changes in catalase activity in tissues of a *Oreochromis mossambicus* after exposure to different concentrations of metacid 50.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	7.543± 0.0810	7.540± 0.0460	7.541± 0.0090	7.539± 0.1400
	0.62 ppb	11.63± 0.2148	12.37± 0.1489	13.21± 0.3024	14.72± 0.9922
	1.24 ppb	15.30± 0.1714	15.03± 0.2764	15.45± 1.0629	20.34± 0.6084
Kidney	Control	4.352± 0.0220	4.348± 0.0500	4.349± 0.0150	4.352± 0.0423
	0.62 ppb	5.083± 0.0455	8.143± 0.2978	9.303± 0.2373	14.37± 0.4254
	1.24 ppb	10.25± 0.1253	10.95± 0.3525	11.99± 0.6518	7.063± 0.2823
Gills	Control	13.22± 0.0670	13.22± 0.0440	13.22± 0.0130	13.22± 0.0500
	0.62 ppb	14.30± 0.3052	17.60± 0.1960	22.20± 0.1396	24.21± 0.1745
	1.24 ppb	15.99± 0.4727	17.67± 0.2707	25.60± 0.1029	14.99± 0.7215

Table: 4.3.3.1. b) ANOVA for changes in catalase activity after exposure to metacid 50.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	3073.843	2	1536.922	272.508	.001
Duration	284.857	3	94.952	16.836	.032
Concentration	1842.774	2	921.387	163.369	.004
Error	1173.102	208	5.640		
Total	6374.576	215			

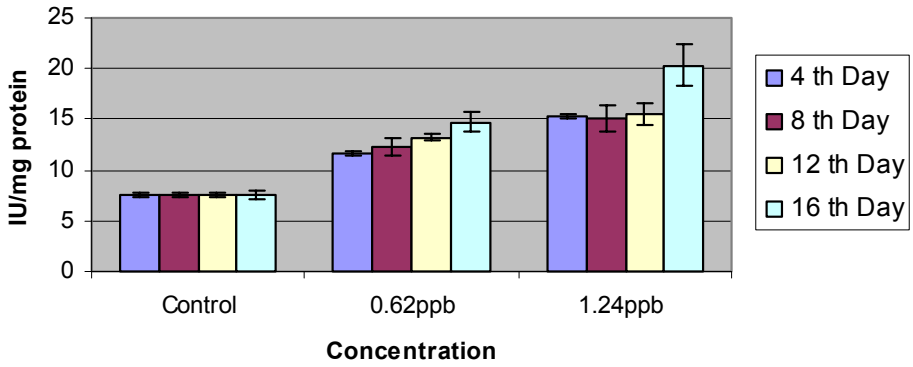


Fig.4.3.3.1. Changes in catalase activity in liver after exposure to different concentrations of Metacid 50

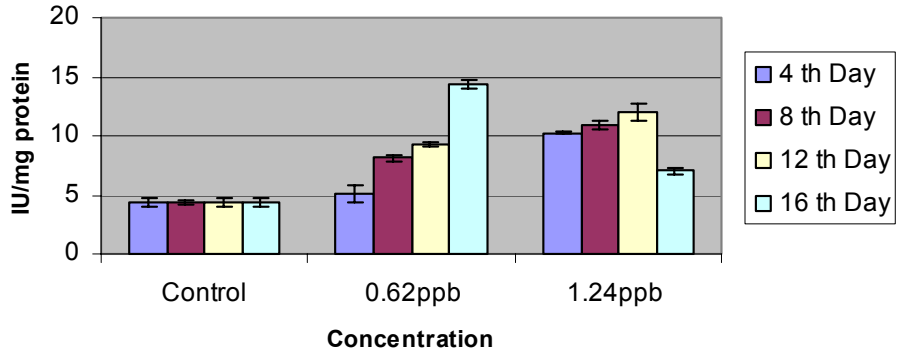


Fig.4.3.3.2. Changes in catalase activity in kidney after exposure to different concentrations of Metacid 50

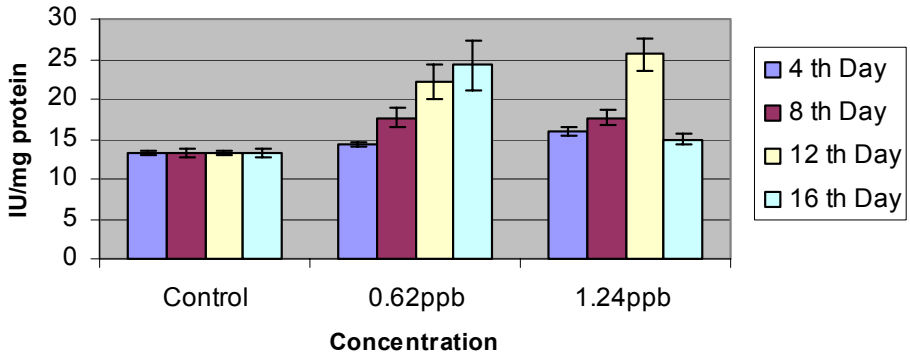


Fig.4.3.3.3. Changes in catalase activity in gills after exposure to different concentrations of Metacid 50

Table:4.3.3.2.a) Changes in superoxide dismutase activity in *Oreochromis mossambicus* after exposure to different concentrations of metacid 50.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	1.340± 0.0040	1.343± 0.0170	1.340± 0.0710	1.342± 0.0050
	0.62 ppb	0.818± 0.0313	0.495± 0.0418	0.335± 0.0105	0.733± 0.0301
	1.24 ppb	0.887± 0.0216	0.447± 0.0383	0.242± 0.0147	0.577± 0.0266
Kidney	Control	0.420± 0.0260	0.420± 0.0041	0.419± 0.0820	0.420± 0.0500
	0.62 ppb	0.292± 0.0147	0.247± 0.0151	0.230± 0.0127	0.103± 0.0015
	1.24 ppb	0.225± 0.0138	0.180± 0.0063	0.175± 0.0084	0.082± 0.0075
Gills	Control	2.491± 0.6200	2.490± 0.0470	2.493± 0.0200	2.487± 0.0530
	0.62 ppb	2.430± 0.0310	2.547± 0.333	2.597± 0.0766	2.183± 0.0459
	1.24 ppb	2.525± 0.0476	3.132± 0.0471	3.135± 0.0123	1.168± 0.0436

Table: 4.3.3.2. b) ANOVA for changes in superoxide dismutase activity after exposure to metacid 50.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	189.387	2	94.693	933.847	.003
Duration	2.350	3	.783	7.724	.045
Concentration	5.640	2	2.820	27.812	.025
Error	21.092	208	.101		
Total	218.469	215			

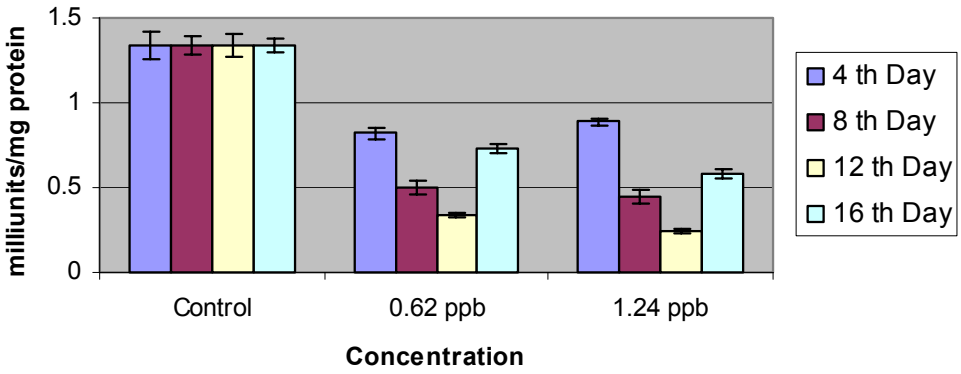


Fig.4.3.3.4. Changes in superoxide dismutase activity in liver after exposure to different concentrations of Metacid 50

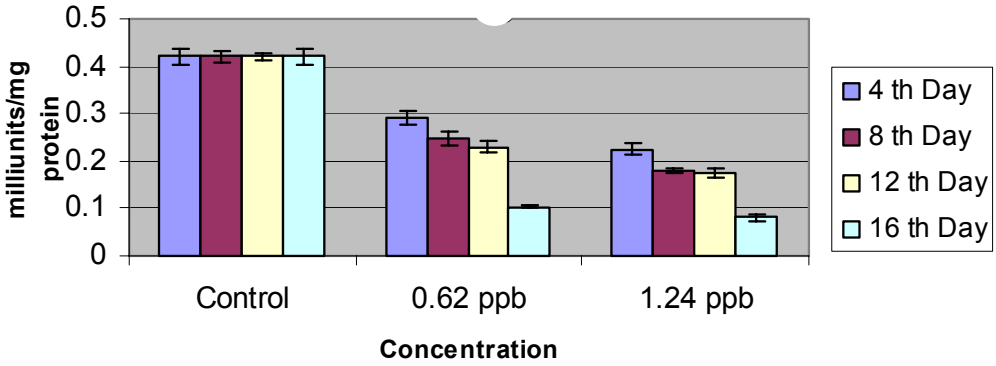


Fig.4.3.3.5. Changes in superoxide dismutase activity in kidney after exposure to different concentrations of Metacid 50

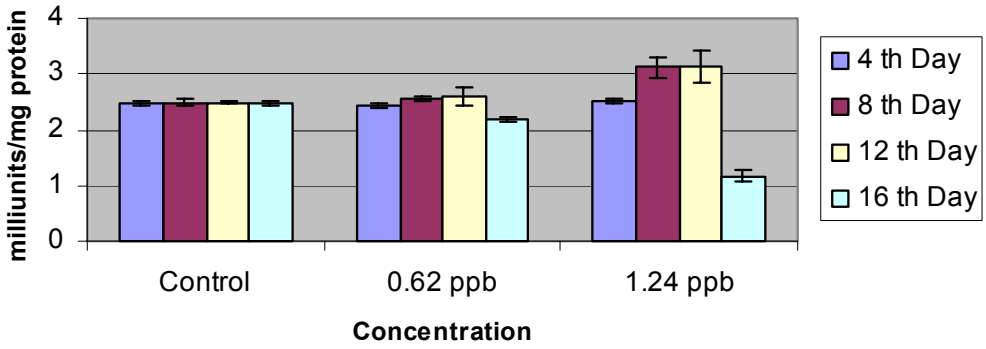


Fig.4.3.3.6. Changes in superoxide dismutase activity in gills after exposure to different concentrations of Metacid 50

Table: 4.3.3.3.a) Changes in glutathione peroxidase activity in tissues of *Oreochromis mossambicus* after exposure to different concentrations of metacid 50.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	7.630± 0.0330	7.627± 0.0800	7.632± 0.0090	7.635± 0.0700
	0.62 ppb	11.32± 0.1318	12.22± 0.6169	12.34± 0.0683	12.41± 0.0933
	1.24 ppb	11.43± 0.1336	13.21± 0.1487	15.26± 0.1247	15.82± 0.1009
Kidney	Control	5.043± 0.0540	5.036± 0.0077	5.044± 0.0820	5.037± 0.0900
	0.62 ppb	3.785± 0.4440	3.230± 0.1653	2.102± 0.0204	1.128± 0.0578
	1.24 ppb	1.763± 0.2015	1.217± 0.0186	0.977± 0.0572	0.327± 0.0103
Gills	Control	12.27± 0.0170	12.24± 0.0400	12.27± 0.0090	12.27± 0.0850
	0.62 ppb	17.16± 0.0327	18.11± 0.0210	10.07± 0.1093	5.153± 0.0509
	1.24 ppb	17.63± 0.1957	19.68± 0.4864	7.088± 0.0392	5.548± 0.2368

Table: 4.3.3.3.b) ANOVA for changes in glutathione peroxidase activity after exposure to metacid 50.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	3896.587	2	1948.293	196.343	.001
Duration	327.061	3	109.020	10.987	.041
Concentration	31.769	2	15.885	1.601	.204
Error	2063.963	208	9.923		
Total	6319.380	215			

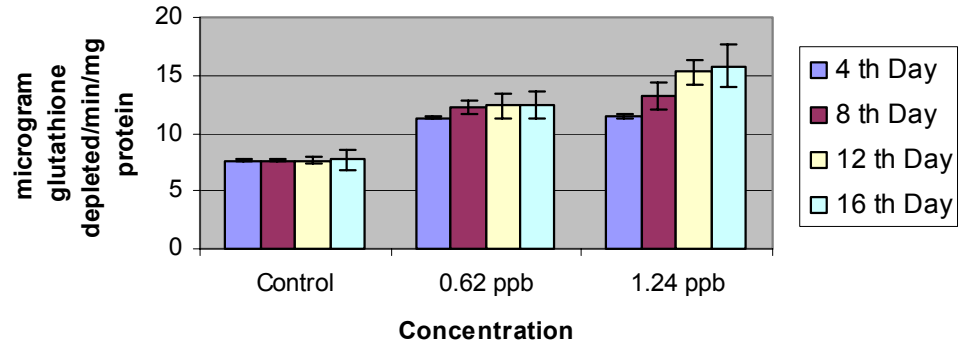


Fig.4.3.3.7. Changes in glutathione peroxidase activity in liver after exposure to different concentrations of Metacid 50

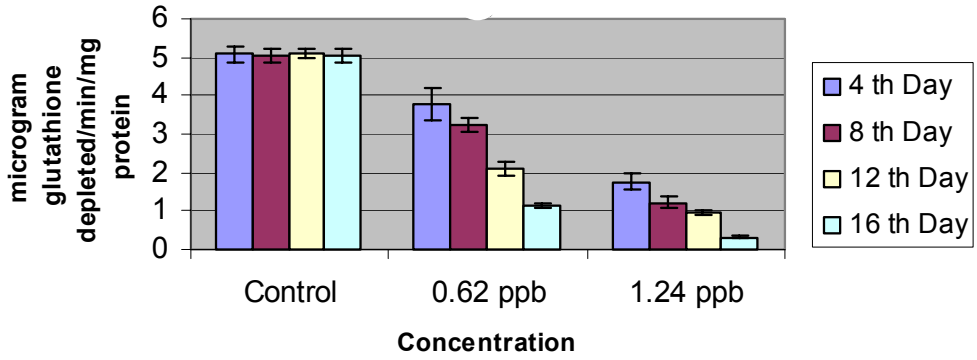


Fig.4.3.3.8. Changes in glutathione peroxidase activity in kidney after exposure to different concentrations of Metacid 50

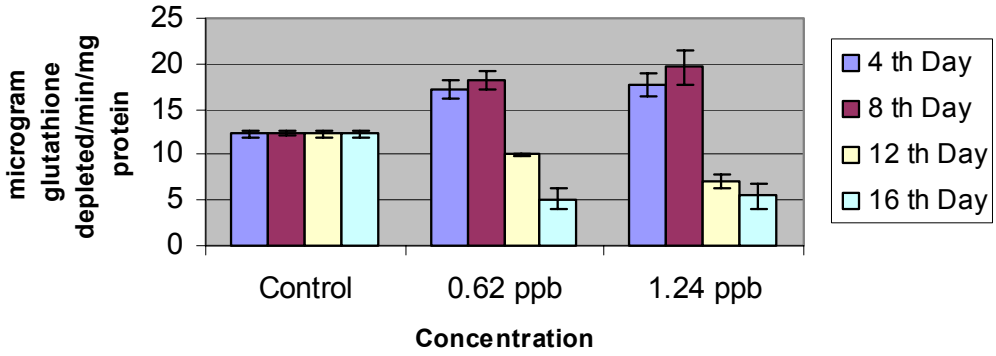


Fig.4.3.3.9. Changes in glutathione peroxidase activity in gills after exposure to different concentrations of Metacid 50

Table: 4.3.3.4. a) Changes in glutathione S transferase activity in tissues of *Oreochromis mossambicus* after exposure to different concentrations of metacid 50.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	19.73± 0.0000	19.73± 0.0000	19.73± 0.0000	19.73± 0.0000
	0.62 ppb	21.12± 0.1408	23.14± 0.0308	26.94± 0.1332	27.18± 0.1021
	1.24 ppb	25.36± 0.1317	33.14± 0.0388	33.50± 0.0896	34.07± 0.0398
Kidney	Control	11.25± 0.0000	11.25± 0.0000	11.25± 0.0000	11.25± 0.0000
	0.62 ppb	11.32± 0.0387	11.44± 0.0250	13.38± 0.0774	14.47± 0.2518
	1.24 ppb	12.07±± 0.0388	12.92± 0.4814	16.38± 0.0226	18.28± 0.0493
Gills	Control	13.19± 0.0000	13.19± 0.0000	13.19± 0.0000	13.19± 0.0000
	0.62 ppb	12.17± 0.0315	9.163± 0.0266	9.007± 0.0804	8.658± 0.0256
	1.24 ppb	11.21± 0.0449	9.308± 0.1556	6.440± 0.0127	5.225± 0.0666

Table: 4.3.3.4. b) ANOVA for glutathione S transferase activity after exposure to metacid 50.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	9184.244	2	4592.122	385.394	.026
Duration	87.867	3	29.289	2.458	.064
Concentration	453.209	2	226.604	19.018	.030
Error	2478.404	208	11.915		
Total	12203.724	215			

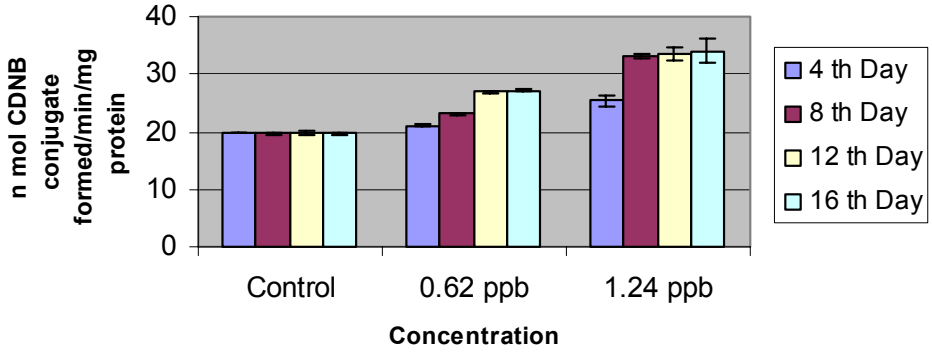


Fig.4.3.3.10. Changes in glutathione S transferase activity in liver after exposure to different concentrations of Metacid 50

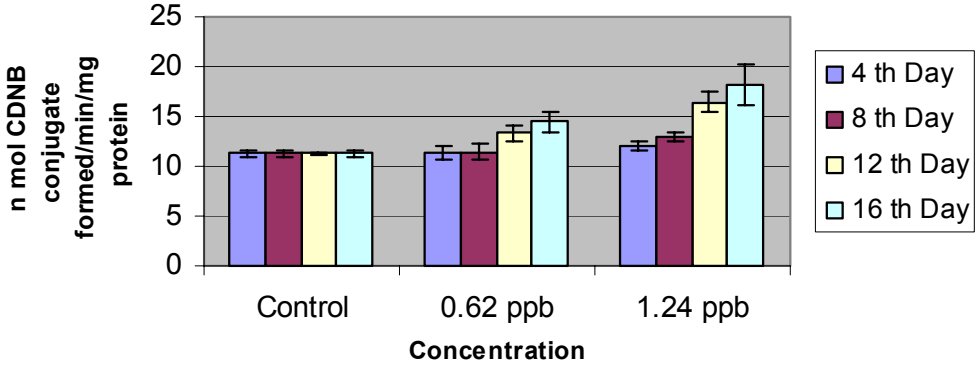


Fig.4.3.3.11. Changes in glutathione S transferase activity in kidney after exposure to different concentrations of Metacid 50

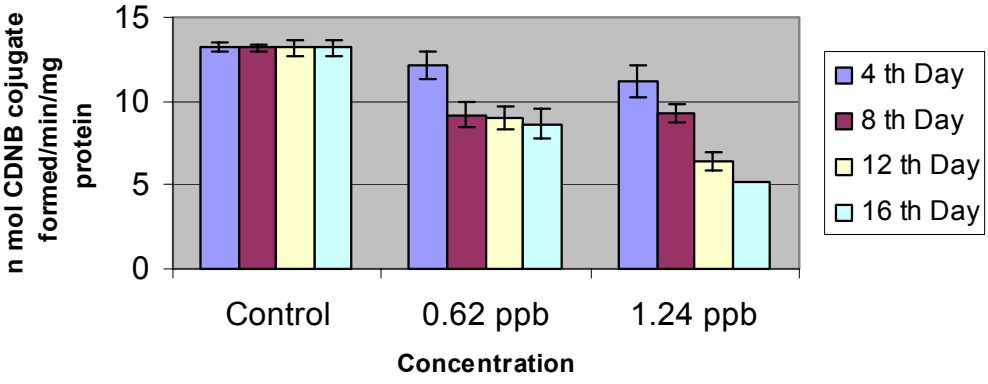


Fig.4.3.3.12. Changes in glutathione S transferase activity in gill after exposure to different concentrations of Metacid 50

Table: 4.3.3.5. a) Changes in glutathione reductase activity in tissues of *Oreochromis mossambicus* after exposure to different concentrations of metacid 50.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	2.935± 0.1300	2.937± 0.2800	2.944± 0.0770	2.943± 0.0350
	0.62 ppb	5.142± 0.0172	7.133± 0.0163	10.16± 0.0463	10.18± 0.0281
	1.24 ppb	10.22± 0.0207	10.79± 0.1050	13.47± 0.0590	13.75± 0.0417
Kidney	Control	1.570± 0.0603	1.567± 0.2200	1.573± 0.1700	1.567± 0.0640
	0.62 ppb	3.032± 0.0343	4.948± 0.0725	5.133± 0.0659	7.230± 0.0947
	1.24 ppb	5.447± 0.1075	7.543± 0.0520	9.467± 0.0250	11.15± 0.0302
Gills	Control	2.353± 0.0560	2.350± 0.0710	2.349± 0.1500	2.354± 0.0090
	0.62 ppb	3.658± 0.0360	5.335± 0.0442	7.153± 0.0294	4.132± 0.0760
	1.24 ppb	5.200± 0.0283	8.345± 0.0339	10.275± 0.0635	3.030± 0.1731

Table: 4.3.3.5. b) ANOVA for changes in glutathione reductase activity in tissues after exposure to metacid 50.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	393.905	2	196.952	80.271	.001
Duration	189.973	3	63.324	25.809	.017
Concentration	1658.917	2	829.459	338.058	.021
Error	510.349	208	2.454		
Total	2753.144	215			

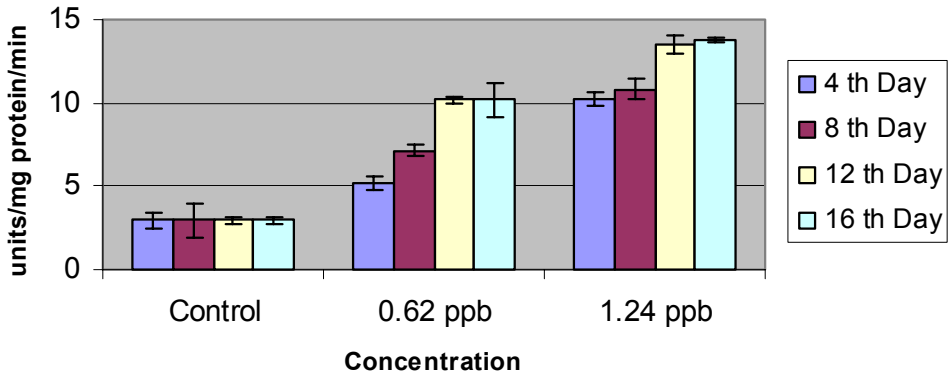


Fig.4.3.3.13. Changes in glutathione reductase activity in liver after exposure to different concentrations of Metacid 50

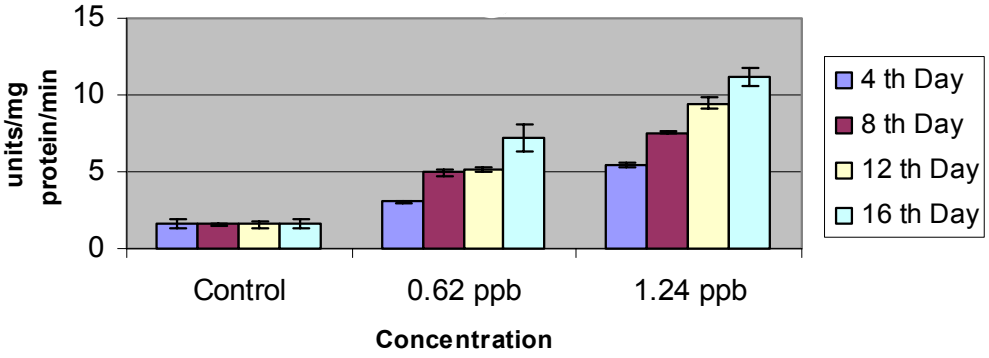


Fig.4.3.3.14. Changes in glutathione reductase activity in kidney after exposure to different concentrations of Metacid 50

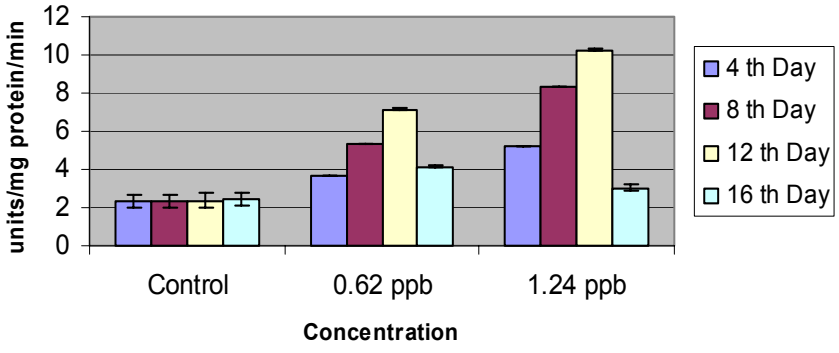


Fig.4.3.3.15. Changes in glutathione reductase activity in gills after exposure to different concentrations of Metacid 50

Table: 4.3.3.6. a) Changes in lipid peroxidation in tissues of *Oreochromis mossambicus* after exposure to different concentrations of metacid 50.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	0.693± 0.0050	0.691± 0.0340	0.687± 0.0110	0.690± 0.1000
	0.62 ppb	1.033± 0.1137	2.342± 0.0371	2.422± 0.0376	3.492± 0.0371
	1.24 ppb	2.458± 0.0319	5.108± 0.0194	5.498± 0.0884	6.170± 0.3276
Kidney	Control	0.343± 0.0070	0.338± 0.0400	0.340± 0.0170	0.340± 0.0510
	0.62 ppb	1.145± 0.0938	1.420± 0.4401	2.052± 0.0778	2.473± 0.1567
	1.24 ppb	1.398± 0.0720	2.683± 0.0557	3.152± 0.0264	4.055± 0.1043
Gills	Control	0.591± 0.0044	0.586± 0.0028	0.593± 0.0054	0.588± 0.0170
	0.62 ppb	1.013± 0.0216	2.653± 0.1398	4.102± 0.0954	4.067± 0.1490
	1.24 ppb	1.793± 0.1311	2.717± 0.0509	4.238± 0.2424	6.367± 0.1875

Table: 4.3.3.6. b) ANOVA for changes in lipid peroxidation in tissues of *Oreochromis mossambicus* after exposure to different concentrations of metacid 50.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	38.143	2	19.072	32.810	.001
Duration	113.689	3	37.896	65.195	.001
Concentration	384.809	2	192.405	331.003	.001
Error	120.906	208	.581		
Total	657.547	215			

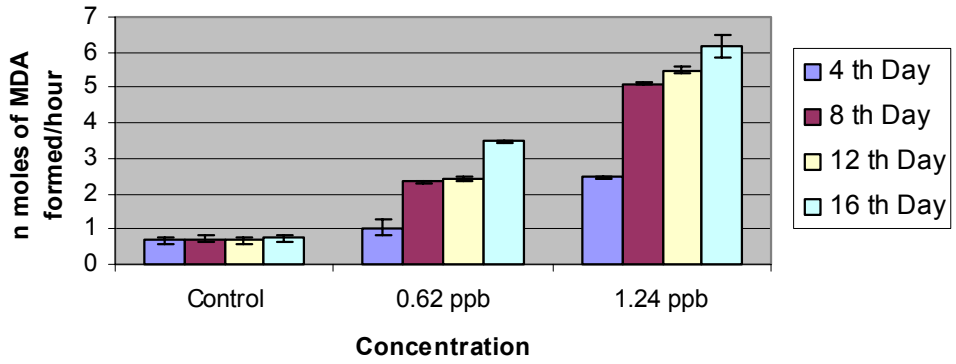


Fig.4.3.3.16. Changes in lipid peroxidation in liver after exposure to different concentrations of Metacid 50

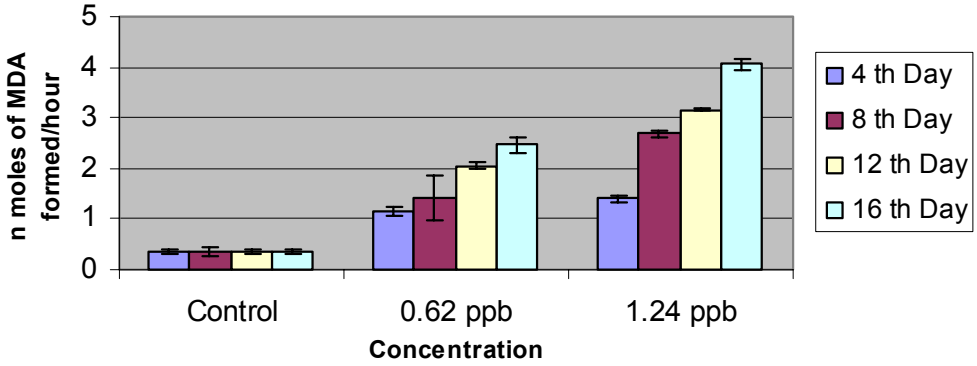


Fig.4.3.3.17. Changes in lipid peroxidation in kidney after exposure to different concentrations of Metacid 50

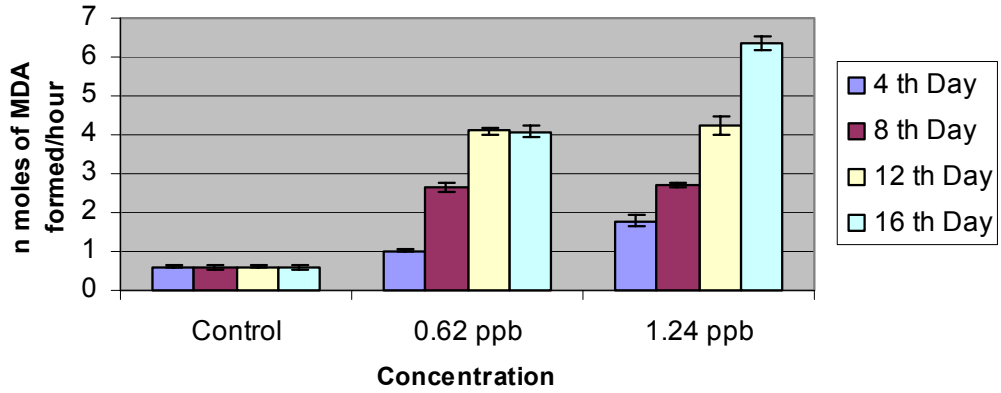


Fig.4.3.3.18. Changes in lipid peroxidation in gills after exposure to different concentrations of Metacid 50

Table: 4.3.3.7. a) Changes in reduced glutathione level in tissues of *Oreochromis mossambicus* after exposure to different concentrations of metacid 50.

Tissue	Concentration	Duration of exposure			
		4th Day	8th Day	12th Day	16th Day
Liver	Control	1756± 11.0800	1754± 14.0000	1762± 13.2500	1758± 20.3600
	0.62 ppb	1571.7± 38.401	1421.2± 45.349	1321.5± 21.8708	1264± 32.0000
	1.24 ppb	1453.5± 32.6742	1379.2± 45.4924	1227.5± 51.3784	1196.3± 61.2111
Kidney	Control	1011± 14.2500	1013± 10.7500	1015± 12.0800	1017± 13.2900
	0.62 ppb	2071.8± 43.8687	2065.8± 36.3715	2067.5± 54.3244	2043.3± 61.8854
	1.24 ppb	2106± 43.2863	2099± 52.3664	2070± 48.5323	2017.2± 40.7528
Gills	Control	1273± 14.0800	1276± 12.5800	1279± 11.3200	1275± 13.5500
	0.62 ppb	987.5± 31.3784	885.2± 42.1370	1276± 31.6733	1271.3± 32.4221
	1.24 ppb	962.2± 43.7224	863.0± 50.5492	1281.8± 38.7224	1272.7± 71.6330

Table: 4.3.3.7.b) ANOVA for reduced glutathione level in *Oreochromis mossambicus* after exposure to metacid 50.

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	4.742	2	2.371	45.609	.001
Duration	.117	3	.039	.749	.524
Concentration	.474	2	.237	4.559	.012
Error	10.813	208	.052		
Total	16.146	215			

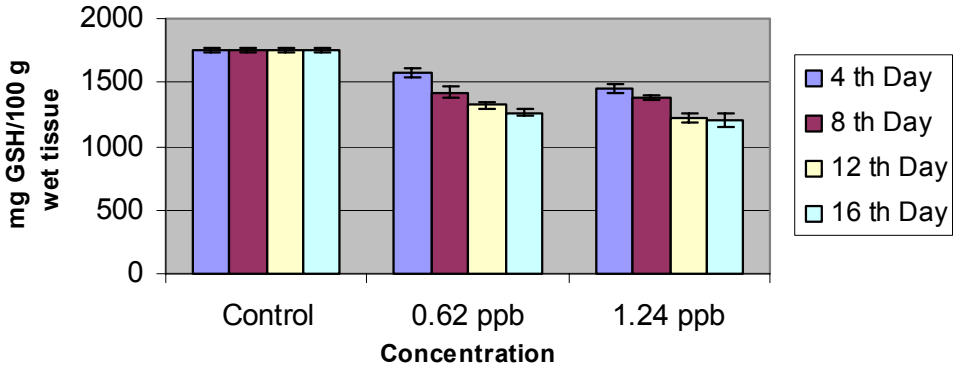


Fig. 4.3.3.19 Changes in reduced glutathione (GSH) level in liver after exposure to different concentrations of Metacid 50

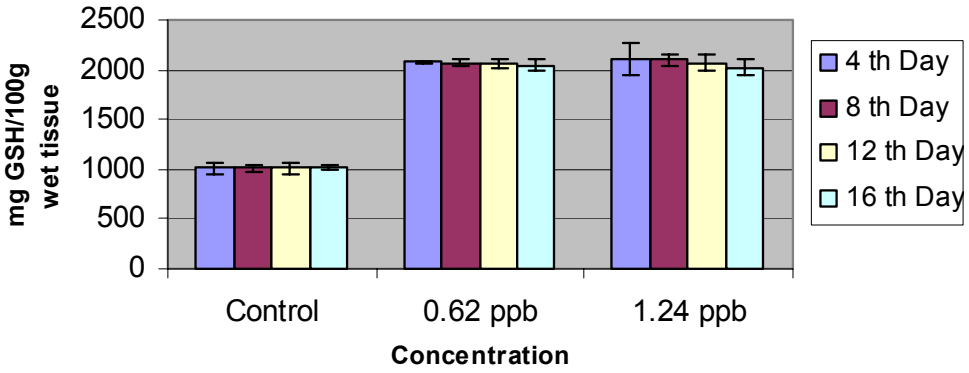


Fig. 4.3.3.20 Changes in reduced glutathione level in kidney after exposure to different concentrations of Metacid 50

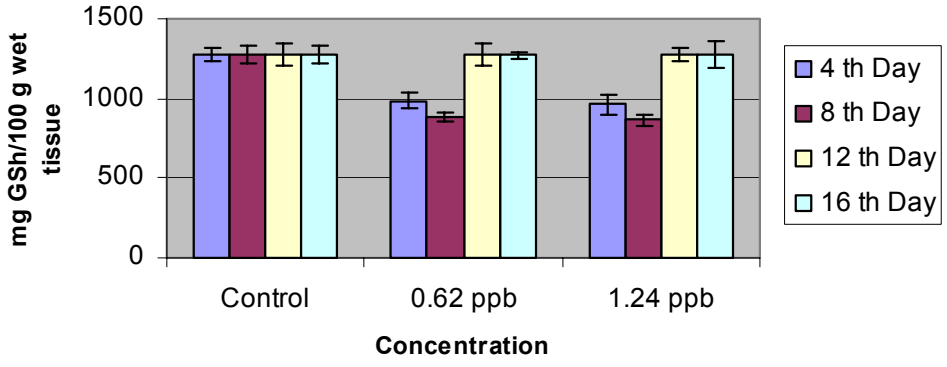


Fig. 4.3.3.21 Changes in reduced glutathione level in gills after exposure to different concentrations of Metacid 50

Table 4.3.3.8 Multiple Comparison Test (Dunnett *t*) for Enzymatic Activities in *Oreochromis mossambicus* after exposure to Metacid 50.

	Groups	Catalase	Superoxide dismutase	Glutathione peroxidase	Glutathione S transferase	Glutathione reductase	Lipid peroxidation	Reduced glutathione
Tissue	Liver & Kidney	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.022 [c]
	Liver & Gills	0.000 [a]	0.000 [a]	0.048 [c]	0.000 [a]	0.000 [a]	0.402 [d]	0.000 [a]
	Kidney & Gills	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.466 [d]	0.000 [a]	0.000 [a]
Day	4 Days & 8 Days	0.116 [d]	0.995 [d]	0.836 [d]	0.760 [d]	0.000 [a]	0.000 [a]	0.774 [d]
	4 Days & 12 Days	0.000 [a]	0.835 [d]	0.029 [c]	0.165 [d]	0.000 [a]	0.000 [a]	0.968 [d]
	4 Days & 16 Days	0.000 [a]	0.000 [a]	0.000 [a]	0.071 [d]	0.000 [a]	0.004 [b]	0.999 [d]
	8 Days & 12 Days	0.001 [a]	0.931 [d]	0.002 [b]	0.694 [d]	0.000 [a]	0.000 [a]	0.490 [d]
	8 Days & 16 Days	0.005 [b]	0.001 [a]	0.000 [a]	0.460 [d]	0.199 [d]	0.000 [a]	0.680 [d]
	12 Days & 16 Days	0.958 [d]	0.005 [b]	0.521 [d]	0.982 [d]	0.105 [d]	0.001 [a]	0.991 [d]
Concentration	Control & .62ppb	0.000 [a]	0.000 [a]	0.307 [d]	0.232 [d]	0.000 [a]	0.000 [a]	0.013 [c]
	Control & 1.24 ppb	0.000 [a]	0.000 [a]	0.240 [d]	0.000 [a]	0.000 [a]	0.000 [a]	0.061 [d]
	.62 ppb & 1.24 ppb	0.014 [c]	0.927 [d]	0.988 [d]	0.000 [a]	0.000 [a]	0.000 [a]	0.834 [d]

The values are significant at a=P<.001, b=P<.01 and c=P<.05 and not significant at d.

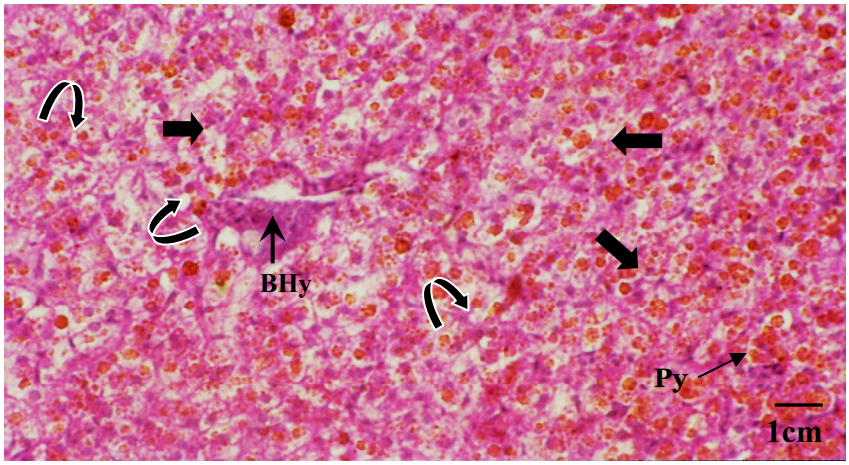
Histopathological Effects of Metacid 50.

Liver of tilapia revealed macrophage aggregates, hyperplasia of biliary epithelium and nuclear condensation of hepatocytes after 8th day after exposure to 1.24 ppb of

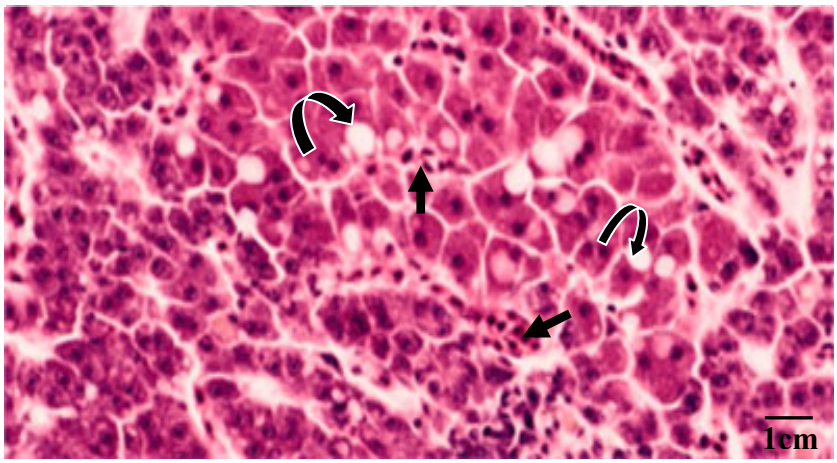
Metacid 50. Except melanomacrophages, other features became pronounced by 16th day after exposure to the pesticide (Figure 4.3.3.22 and 23).

Glomerular shrinkage, swelling of Bowman's capsules, intercapillary thickening ('wire loops') of glomeruli and presence of tubular casts were the morphological aberrations exhibited by the kidney of the fish due to sublethal exposure to 1.24 ppb of Metacid 50 (Fig. 4.3.3.24 and 25).

Metacid 50 induced structural changes in the gill of *Oreochromis mossambicus* which included lifting and hyperplasia of respiratory epithelia, hyperemia, lamellar aneurisms, squamous metaplasia, swelling of primary lamellar tips (not shown in photomicrographs), lamellar fusion and vascular congestion. In addition to necrotic changes, some of the primary lamellae were seen torn apart through the longitudinal axis (Fig. 4.3.3.26). Details of duration dependent increase/decrease in the intensity of changes are depicted in Fig. 4.3.3.27).



(a)



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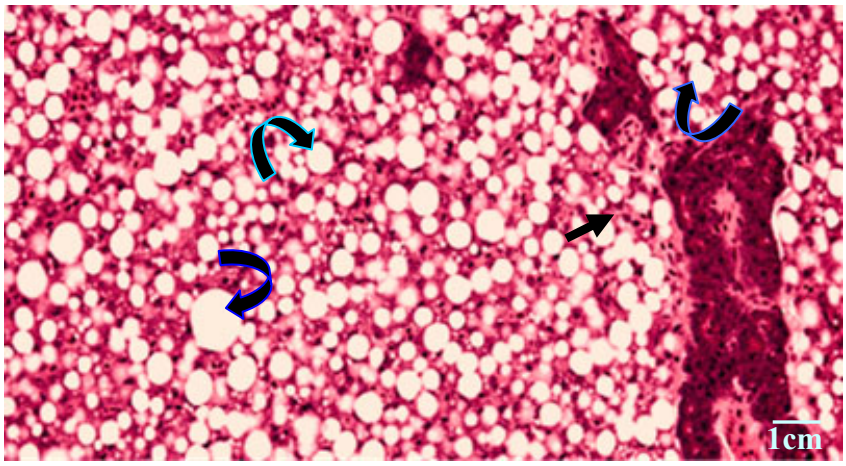


(c)

Fig. 4.3.3.22. a) Liver of *Oreochromis mossambicus* after exposure to 1.24 ppb of Metacid 50. **a)** 8th day showing biliary hyperplasia (BHy) and ceroid type macrophage aggregates (block arrows) and pycnotic nuclei (Py) and fatty vacuoles (curved arrows). H &E, 20x. **b)** & **c)** 16th day. Symbols: Fatty vacuolation (curved arrow), pycnotic nuclei (straight arrow). H & E, 40x.

Table 4.3.3.9. Histopathological effects of metacid 50 (1.24 ppb) on the liver of *Oreochromis mossambicus*.

Treatment	Percentage damage			
	Biliary hyperplasia	Macrophage aggregates	Pycnosis	Fatty vacuolation
Normal	0.00	0.00	0.00	0.00
8th day	3.41	83.70	31.59	18.65
16th day	18.00	0.00	63.27	94.8

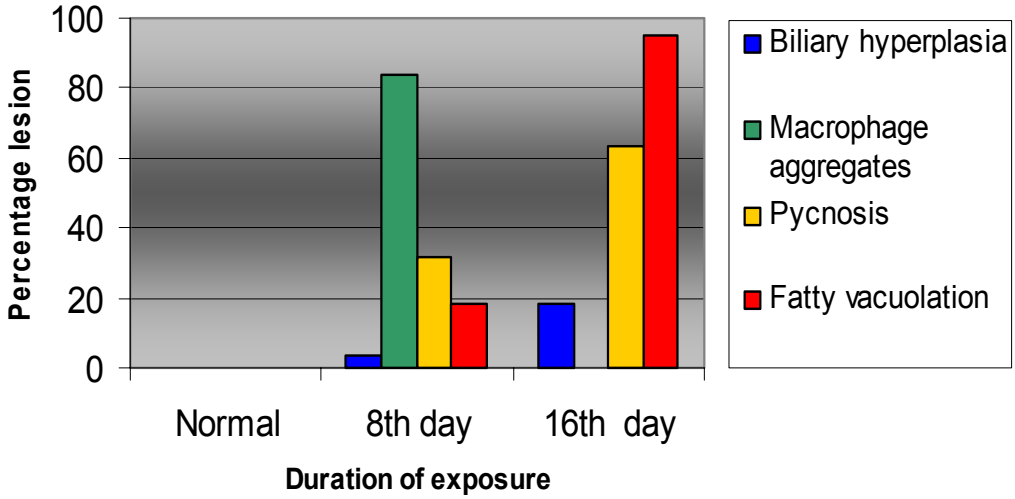
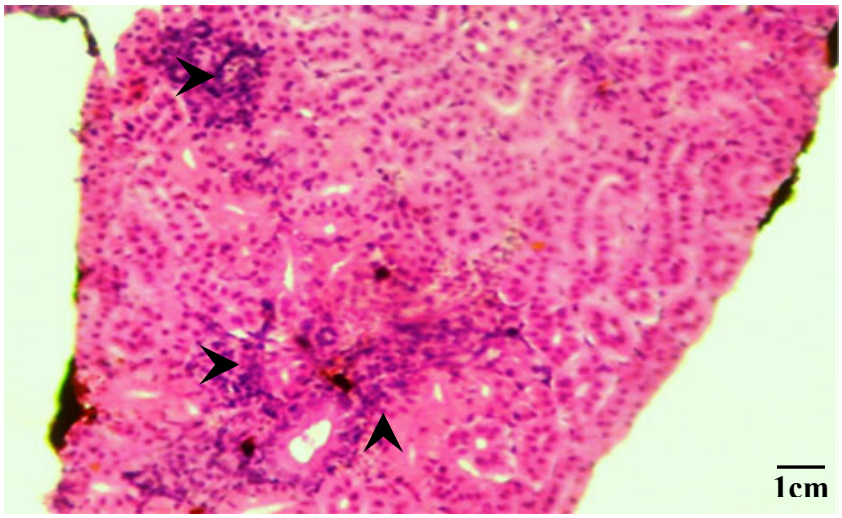
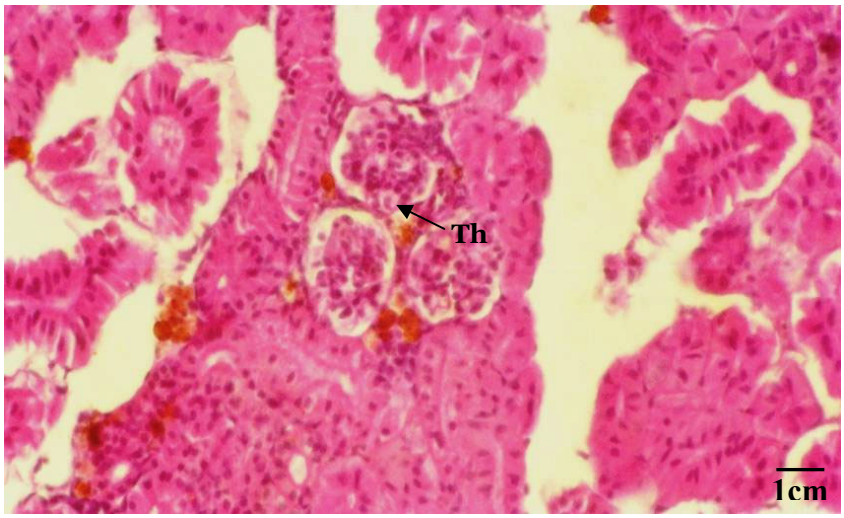


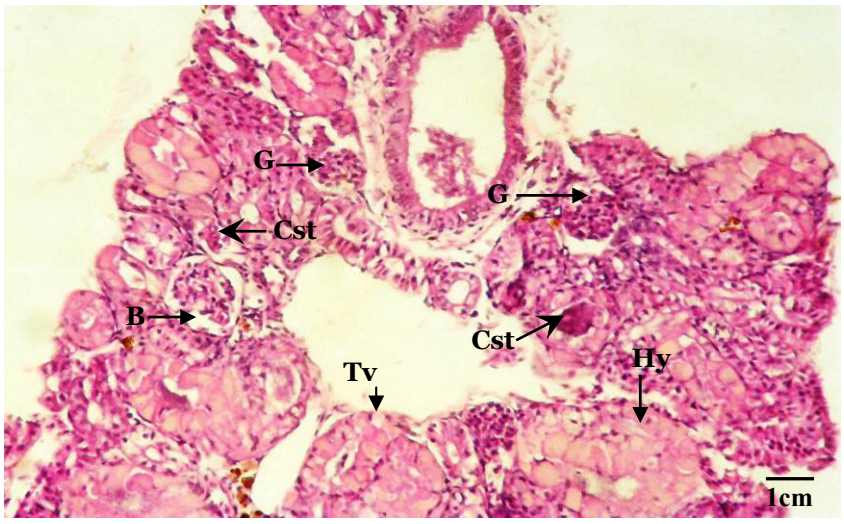
Fig 4.3.3.23. Histopathological changes in liver of Oreochromis mossambicus after exposure to 1.24 ppb of Metacid 50.



(a)



(b)



(c)

Fig.4.3.3.24. Histopathological effects of Metacid 50 (1.24 ppb) on the kidney of *Oreochromis mossambicus*. **a)** & **b)** 8th day after exposure. Arrowheads indicate basophilic areas demarcating caseous necrosis and presence of too many nuclei denotes hyperemia. Inter- capillary thickening (Th) of glomeruli is also noticed H& E, 20x. **c)** 16th day after exposure, showing glomerular shrinkage (G), swelling of Bowman's capsules (B), hyaline cytoplasm (Hy), tubular casts (Cst) and tubular vacuolation and degeneration(Tv). H & E, 20x.

Table 4.3.3.10. Histopathological effects of Metacid 50 (1.24 ppb) on the kidney of *Oreochromis mossambicus*.

Treatment	Percentage damage						
	Basophilic areas.	Glomerular shrinkage.	Glomerular inter-capillary thickening.	Capsular thickening.	Tubular vacuolation and degeneration.	Hyalinization.	Tubular casts.
Normal	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8th day	24.57	0.00	14.52	0.00	0.00	0.00	0.00
16th day	0.00	2.56	0.00	6.83	27.35	76.15	2.53

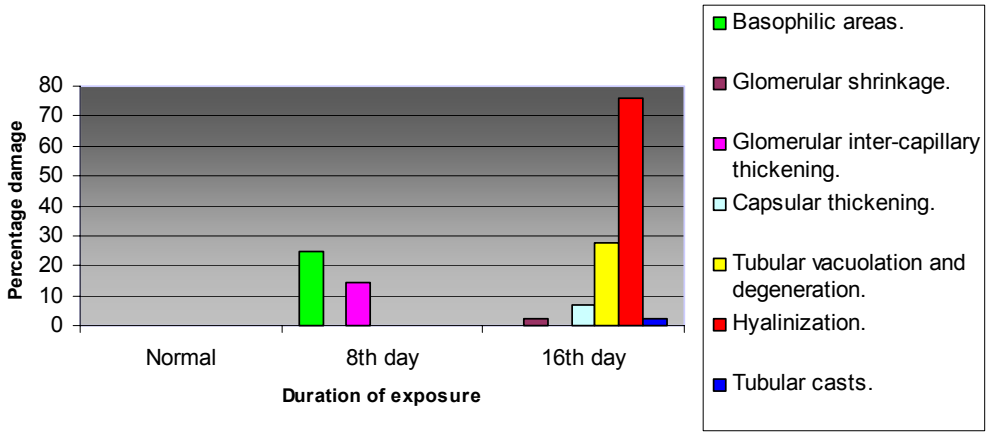
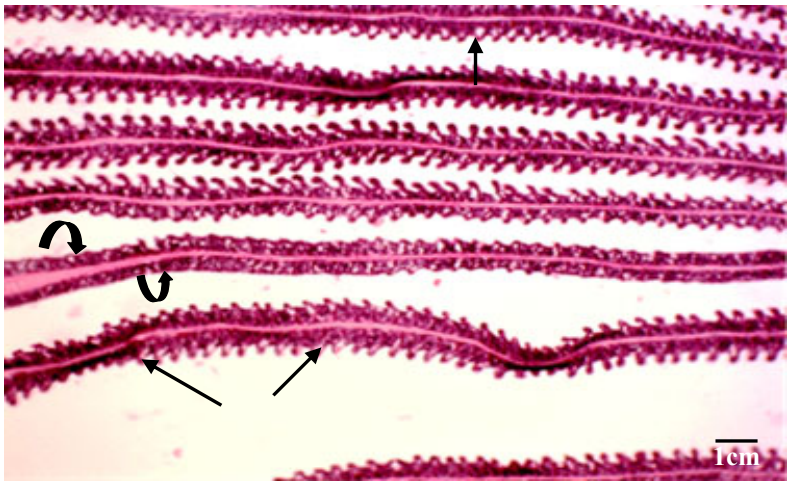
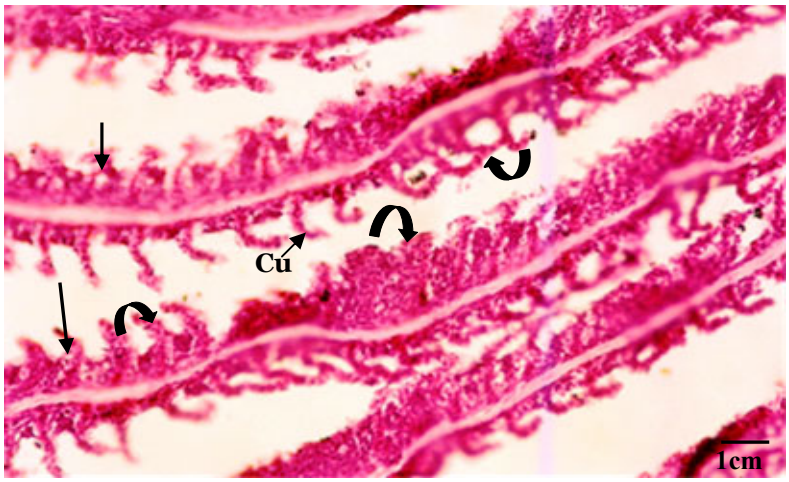


Fig. 4.3.3.25. Histopathological effects of Metacid 50 on the Kidney of *Oreochromis mossambicus*



(a)



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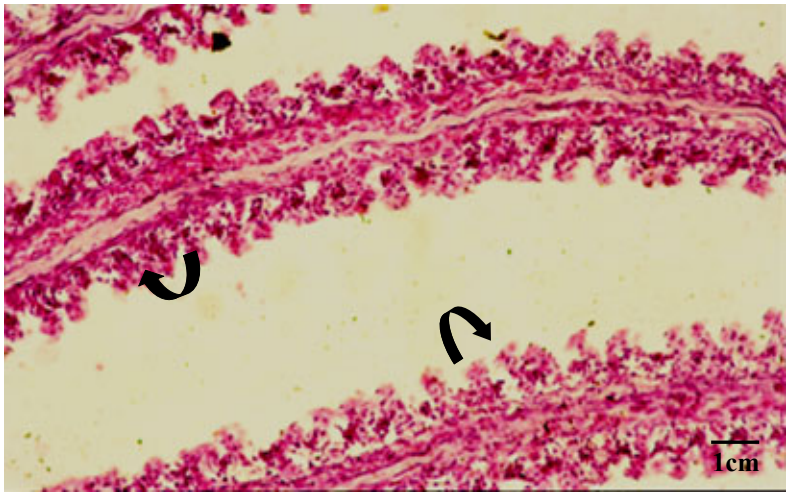

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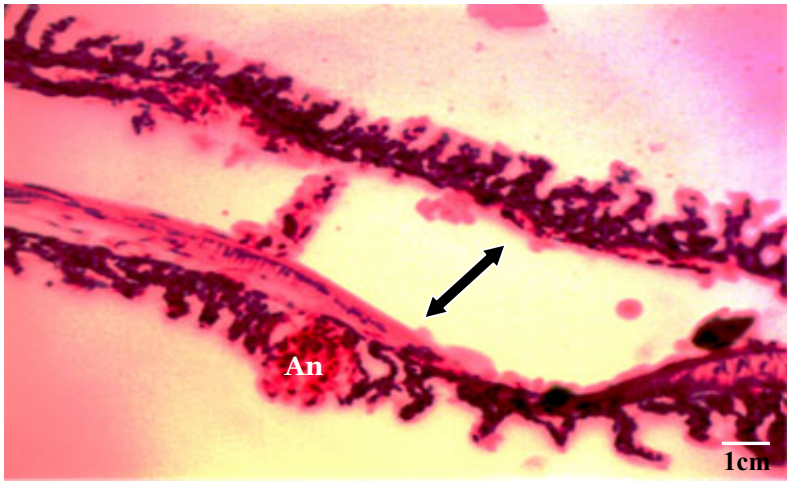

(d)

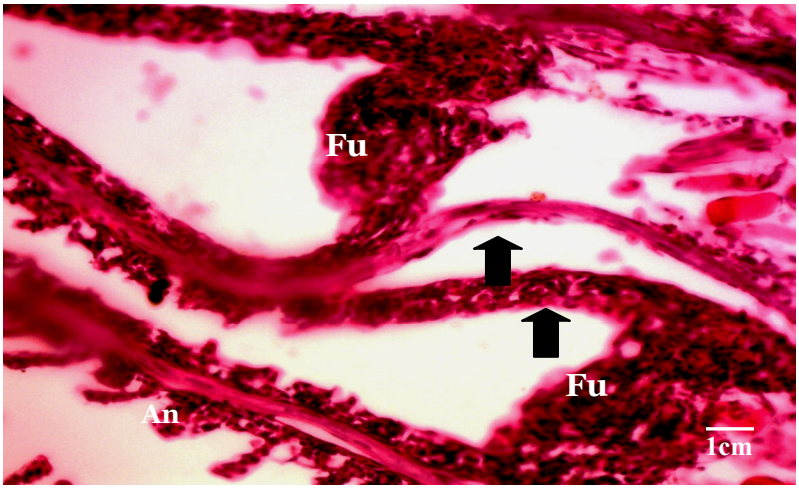

Fig. 4.3.3.26. Histopathological alterations in the gill of *Oreochromis mossambicus* after exposure to1.24 ppb of Metacid 50.

a) & **b)** 8th day after exposure showing extensive lifting of epithelia (straight arrows),lamellar curling (Cu) and secondary lamellar fusion accompanied by hyperplasia (curved arrows) in some areas. H & E. **a)** 20x and **b)** 40x.

c)16th day after exposure illustrating epithelial lifting (straight arrows) and severe secondary lamellar fusion with hyperplasia (curved arrows). H&E, 40x. **d)** & **e)** 16th day after exposure showing aneurism (An), longitudinal splitting of primary lamellae (double headed arrow), squamous metaplasia and exfoliation (straight block arrows) and fusion and extreme hyperplasia of secondary lamellae of adjacent primary lamellae (Fu). H&E, 40x.

Vascular congestion (edema or hyperemia) is evident in all sections. Abundance of apparent pycnotic nuclei is an indicator of coagulative necrosis.

Table 4.3.3.11. Histopathological effects of Metacid 50 (1.24 ppb) on the gill of *Oreochromis mossambicus*.

Treatment	Percentage damage							
	Lamellar curling	Lamellar fusion	Hyperplasia	Aneurism	Epithelial lifting	Necrosis	Squamous metaplasia	Exfoliation
Normal	0.00	0.00	1.50	0.00	0.00	0.00	0.00	0.00
8th day	31.60	20.51	43.34	0.00	82.47	33.00	7.65	0.00
16th day	5.12	68.00	62.17	6.83	7.10	72.35	14.50	7.00

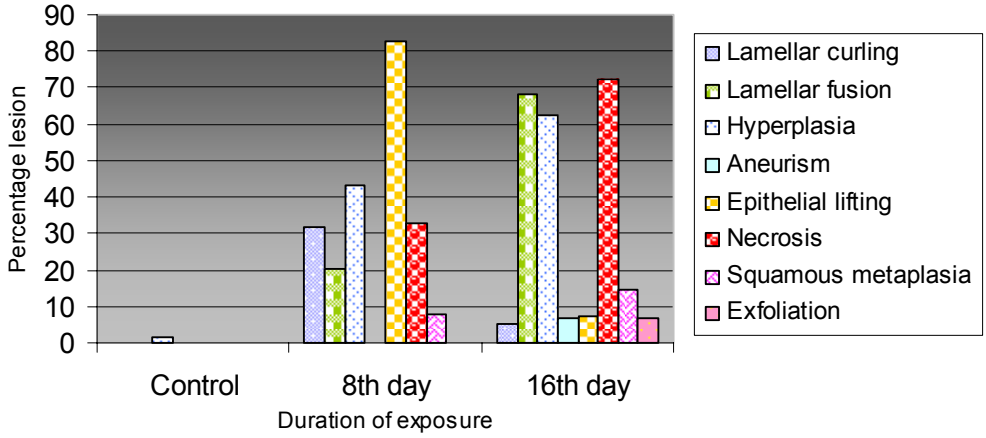

Fig. 4.3.3.27 Histopathological changes in the gill of *Oreochromis Mossambicus* after exposure to 1.24 ppb of Metacid 50

4.4. Discussion.

The continuous increase in the activity of catalase in liver as a result of exposure to copper is indicative of oxidative stress which induces greater activity of the enzyme. The highest activity recorded on 16th day at high concentration suggests that the activity is proportional to both concentration as well as duration. At the cellular level, it has been indicated that metal ions may compete with the required metabolic cofactors at the binding site affecting enzymatic activity. After entering the liver, the heavy metal might have undergone metabolism in cells to release reactive oxygen species which contributed to catalase activation (Chen *et al*., 2000).

The increased activity of catalase in liver in conjunction with increasing duration of exposure and concentration of copper was found to be accompanied by high level of lipid peroxidation. Lipid peroxidation, though considered as a criterion for tissue damage, the increased activity of the enzyme could be interpreted as the tissue making the maximum effort to counteract the toxicity of copper. Moreover, the activities of

glutathione peroxidase, glutathione S transferase and glutathione reductase were found to be high in liver accompanied by the high levels of glutathione (Fig. 4.3.1.7,10,13 and19). Literature shows that glutathione peroxidase is exceptionally active in teleost liver cells (Hassipieler *et al*, 1994). Glutathione is a cofactor for glutathione S transferase (Gallagher *et al*, 1992) and gets regenerated due to the activity of glutathione reductase. Therefore, it can be assumed that the activity of glutathione related enzymes mentioned above and glutathione itself are operating to counter the damage. The high activity of catalase in liver observed in the present study is in contrast to the finding by Singh and Sivalingam (1982) who reported copper as the strongest inhibitor of liver catalase in *Sarotherodon mossambicus*. Moreover, as in a previous work (Atli *et al*., 2006), it was found that liver is the organ of maximal catalase activity and stronger into the face of oxidative stress than other tissues and a uniform organ with the highest antioxidant enzyme activities (catalase and superoxide dismutase) in fishes. This could be connected to the fact that the liver is the site of multifaceted oxidative reactions and maximal free radical generation (Avci *et al*., 2005).

The gradual decrease in activity of catalase in kidney at 64ppb of copper is clearly an indication of inhibition resulting from the toxic action of copper. But as explained in the case of liver, the high level of glutathione which itself function as an oxyradical scavenger and indirectly augment the activity of glutathione S transferase and glutathione peroxidase, guards the organ from total oxidative damage. However, at high concentration of copper, by the 12^{th} day, peroxidative damage increased and the glutathione reserves declined (Fig.4.3.1.16 and 20) culminating in a declension of activity towards the 16^{th} day. The sporadic increase in activity at high concentration can be understood as the kidney

attempting to revive the activity of catalase due to the reduced levels of glutathione and associated declension in glutathione peroxidase activity (Fig. 4.3.1.8 and 20).

The gills of *Oreochromis mossambicus*, being an organ exposed directly to the toxicants in the medium was found to maintain more or less high activity of catalase comparable to that of control animals at both experimental concentrations (Table. 4.3.1.1 and Fig. 4.3.1.3). Gill is also the first affected organ when fish are exposed to metals. However, the activity decreased by 12^{th} and 16^{th} day at high concentration. It was determined that there was no significant change in catalase activity in the liver and gill and this was associated with the high activity of glutathione peroxidase, which acts as defense against the formation of hydrogen peroxide (Atli *et al*., 2006). It can also be deduced that at low toxic levels of copper (64 ppb), the gill is capable of maintaining higher activity of catalase, but the tissue cannot sustain this condition for longer duration at high concentration (128 ppb) leading to a collapse by 16^{th} day.

The high activity of catalase in liver and gills at low copper concentration was accompanied by high activity of glutathione peroxidase in these tissues. Since hydrogen peroxide, a non – radical reactive oxygen species capable of destroying biomembranes and disrupting the enzymatic machinery is the common substrate of both of these enzymes. The increased activity of these enzymes in liver and gill could be due to severe oxidative stress (Yilmaz *et al*., 2006

Induction of superoxide dismutase occurred in the three experimental tissues as given in table 4.3.1.2 a & b, Fig. 4.3.1.4, 5 and 6 and it is the first enzyme to handle the oxyradicals in biological systems. It has been given elsewhere that the high activity of glutathione peroxidase and catalase in

liver at low level of copper is indicative of oxidative stress. In this context, the high activity of superoxide dismutase should be the source of hydrogen peroxide that forms the substrate for these enzymes. But at high concentration of the heavy metal, the liver exhibiting meager superoxide dismutase activity might be due to the failure of the enzyme to catalyse the dismutation on superoxide anion to hydrogen peroxide and oxygen.

The very low activity of superoxide dismutase in kidney under low and high copper stress is presumably due to the increased activity of the enzyme in liver as it forms the main organ of storage and detoxification of copper the increased lipid peroxidation might also be the cause of decreased activity of decreased superoxide dismutase activity in kidney during 12^{th} and 16^{th} day of exposure at low copper concentration and throughout the experimental period at high concentration. But the exceptionally high activity of the enzyme on 8^{th} day following the exposure to low concentration of copper is indicative of the induction of superoxide dismutase activity. The transport of copper to kidney prior to the full fledged activity of superoxide dismutase in liver might be the cause of this situation (Rome'o *et al*., 2000). Furthermore, fish kidney contains a cystine rich copper binding protein which is thought to have either a detoxifying or storage function as reported by Luckey and Venugopal (1977).

As an organ of direct contact with water, the gills of *Oreochromis mossambicus* were found to possess high activity of superoxide dismutase at high concentration of copper (Fig. 4.3.1.6). Contrary to earlier report (Pandey *et al*., 2003, Farombi *et al*., 2007) the gill was found to possess high activity of glutathione related antioxidant enzymes at low copper concentration. But at high concentration of copper, the activity of glutathione peroxidase and glutathione reductase decreased though

glutathione S transferase maintained its high activity (Fig. 4.3.1.9, 12 and 15). However, the activity of superoxide dismutase regained its high activity at high copper concentration. The above situations are suggestive of superoxide dismutase and glutathione S transferase involvement in oxyradical scavenging at higher copper concentrations and the glutathione related antioxidant machinery is operating at low concentration of copper.

Significant differences ($P< 0.001$) were found in the activity of glutathione peroxidase activity in liver, kidney and gills of control fishes, the maximum being recorded in gills (Fig. 4.3.1.7,8 and 9). The redox defense system against oxidative damage, involving superoxide dismutase and glutathione peroxidase was inferred to be functioning in liver under low concentration of copper (Fig.4.3.1.4 and 7). Similar assessments were made by Almeida *et al*., (2002) in *Oreochromis niloticus*. The high level of the substrate (reduced glutathione) for glutathione peroxidase available in liver is supporting this judgment (Fig. 4.3.1.19).

The variations in the activity of glutathione peroxidase in kidney should be perceived in conjunction with corresponding levels of lipid peroxidation and glutathione content (Fig.4.3.1.17 and 20). The increased activity under low concentration of copper up to 12^{th} day is proportional to the enhancement in level of lipid peroxidation and glutathione. But the declension of glutathione peroxidase activity on 16^{th} day even under high glutathione content is attributed to much higher level of lipid peroxidation leading to tissue damage. Similarly at high concentration of copper, the activity changes of this enzyme followed the very same pattern as if under low concentration, the only difference being in the overall decrease in activity when compared to that noted under low copper concentration ($P < 0.05$). This is explainable with the progressive increase in lipid peroxidation and drastic reduction in glutathione reserve (Fig.4.3.1.17 and 20).

The explanation given above is equally applicable to the trend of glutathione peroxidase activity in gills also, under low and high concentrations of copper. But the gills succumbed more easily to damage than kidney at high concentration of the metal as indicated in Fig. 4.3.1.9 evidently due to its proximity to the exposure medium.

As observed by George (1994), the high activity of glutathione S transferase in liver, kidney and gill under low and high concentrations of copper in the present study clearly points towards the enzyme's involvement in defense against oxidative damage and peroxidative products of lipids. Basha and Rani (2003) reported a continuous elevation (7 – 30 day) of glutathione S transferase activity in liver and kidney of *Oreochromis mossambicus* due to cadmium intoxication.

In gills, as observed in the case of superoxide dismutase, duration dependent elevation was recorded at 64 ppb concentration of copper for glutathione S transferase also, underlining the alertness of branchial antioxidant system. This is in contrast to the findings by Atif *et al*., (2005) and Perry (1997) who reported a weak antioxidant activity including that of glutathione S transferase in gills in the presence of heavy metals.

Statistically significant ($P< 0.05$) temporal elevation in the activity of glutathione reductase in liver is supportive of the active role of the organ in defending oxidative stress. The production of glutathione during the reduction of oxidised glutathione contributes to the activity of glutathione S transferase and glutathione peroxidase. Since a rather high activity of the three resident enzymes of glutathione family (glutathione peroxidase, glutathione S transferase and glutathione reductase) accompanied by high levels of glutathione is observed in liver, especially under exposure to low concentration of copper (64 ppb), efficient

protection against oxidative damage is indicated. However, upon exposure to high concentration, glutathione peroxidase activity was found to decrease in the hepatic tissue probably due to reduction in glutathione levels and/or high activity of catalase both of which are concerned with the elimination of hydrogen peroxide. Curiously enough, drastic reduction in glutathione content in liver at 128 ppb of copper seems to impart no declension in the activity of glutathione S transferase in liver, kidney and gills. This can be explained by the utilization of glutathione (reduced) by glutathione S transferase at the expense of reduced activity of glutathione peroxidase (Fig. 4.3.1.7, 10, 13, 16 and19). Exposure to xenobiotics seems to result in high activity of glutathione reductase in hepatic tissue of *Oreochromis niloticus* (Uner and Oruc, 2000) and that of *Channa punctata* (Atif *et al*., 2005). A situation akin to this is inferred in the current study. The peculiar behavior of glutathione reductase in kidney involving increased activity up to 12^{th} and 8^{th} day after exposure at 64 ppb and 128 ppb of copper respectively culminating in sharp declension afterwards indicates dose and time dependent variations. This could be due to the failure of the enzyme to counter the oxidative stress both at increasing concentration of the toxicant and prolongation of exposure. At this juncture, alternate pathways for supply of glutathione (exported from the cell, like some GSH-conjugates or the activity of γ-glutamylcysteine synthetase (γ -GCS; EC 6.3.2.2.), the rate-limiting enzyme in the de novo synthesis of glutathione) may be operating to make up the glutathione pool (Stephensen *et al*., 2002). The significant declension and leveling off of glutathione reductase activity after an initial shoot up at 64 ppb of copper can be assumed as due to enhancement in lipid peroxidation. But at high concentration (128 ppb), the increase in activity of the enzyme was significantly lower ($P<0.05$) from 4^{th} to 12^{th} day after exposure leading to

a collapse on 16th day, indicating the influence of continued increase in lipid peroxidation.

The rapidity with which lipid peroxidation of the cells of cardinal tissues like liver, kidney and gills takes place clearly indicated extensive damage of the cells. Lipid peroxidation which is an index of deterioration of cells and the functioning of the subcellular structures which control the normal defined roles of the tissues involved in detoxification, excretion and respiration is a direct index of toxicity resulting from the uptake, transport and detoxification of copper.

Glutathione (reduced) is often the first line of defense against oxidative stress. Its levels can be increased due to an adaptive mechanism to slight oxidative stress through an increase in its synthesis; however, a severe oxidative stress may suppress it levels due to the loss of adaptive mechanisms and the oxidation of glutathione to its oxidized form. Glutathione is converted to its oxidized form in the biotransformation of hydroperoxides to free alcohol in the presence of glutathione peroxidase. During this biotransformation process, oxidized glutathione is formed, and this provides a substrate for glutathione reductase. Oxidised glutathione is converted back to its reduced form by glutathione reductase, therefore high glutathione reductase activity means that the production of oxidized glutathione is high and also glutathione peroxidase activity is high (Zhang *et al*., 2005). Significant ($P<0.05$) and duration-independent decrease in the level of reduced glutathione in liver, kidney and gills under exposure to 128 ppb of copper points to the reverse trend of lipid peroxidation(Fig. 4.3.1.16, 17 and 18). The tissues undergoing peroxidative damage may not be able to maintain high level of glutathione under the stress of 128 ppb of copper. Moreover, the glutathione dependent enzymes are exploiting the glutathione resource at the

maximum possible extent to maintain their activity and thereby defending the oxidative stress. Even under this situation, liver, the prime tissue concerned with detoxification, was maintaining the high activity of glutathione reductase (Fig. 4.3.1.13) under severe (128 ppb) stress of copper thereby regenerating glutathione. Duration dependent high activity of glutathione S transferase in the three experimental tissues at higher experimental concentration of copper may be another reason for reduced levels of glutathione as the enzyme demands the same for detoxification processes.

Malachite green, being a chemical, strange to the physiology of *Oreochromis mossambicus*, was found to trigger the inducement of the primary antioxidant enzyme catalase in liver, kidney and gills. The increase in catalase activity in liver, significant ($P<0.05$), on 12^{th} and 16^{th} day after exposure at 12.5 ppb level of malachite green indicated oxidative stress. It seems inferable that under high concentration of the dye, the liver defended the stress via increased activity of the enzyme but the stress was so enormous that the activity lowered drastically by 16th day. Trunk kidney, the excretory apparatus of the fish, faced severe oxidative damage due to the dye exposure as evident from the significant declension of catalase activity at high concentration. The initial enhancement in the activity of the enzyme (4^{th} and 8^{th} day at 12.5 ppb concentration) is an indication of the positive reaction of the organ to counter the stress. The gills also suffered from the exposure at both the experimental concentration of the dye. Akin to liver, at low concentration the oxidative insult was defended by an enhanced activity of catalase throughout the experimental period. But the organ failed to keep the defense much earlier than liver due to declension of catalase activity from

12th day onwards at high concentration leading to minimal activity by 16th day.

The activity of superoxide dismutase in liver was found to maintain high level of activity above that of control fishes at both the experimental concentrations suggestive of efficient defense against oxidative stress. However, decreased activity after 12th day was highly significant ($P<0.05$) indicating the exhaustion of the enzymatic machinery. This may be explained in relation to histological alterations occurred in liver. At 25 ppb of the dye, the liver showed signs of degeneration with extensive pycnosis denoting coagulative necrosis and non fatty vacuolation (Fig. 4.3.2.22. a). When considering the advanced lesions suffered by liver on 16th day at high concentration, it can be assumed that the magnitude of the lesions mentioned above have increased drastically and the hepatic tissue was severely damaged with necrosis leading to the loss of compactness and morphological integrity. Superoxide dismutase being a secretion from cytoplasm and/or mitochondria, the tissue may not be able to retain the activity of the enzyme. The activity of the enzyme in kidney on the other hand, was found to defend the oxidative stress at low concentration of the dye, but failed to keep up the activity throughout the experimental period evidently due to the overwhelming toxicity of malachite green. The intermittent increase and decrease of the enzyme activity at high concentration (25 ppb) might be inferred as an adaptive tendency of the organ. Curiously enough, the progressive decrease of the enzyme activity reaching below control level even from 4th day after exposure seems to be paradoxical requiring further studies for confirmation. Since gills are exposed directly to the dissolved compounds in water, it is more likely that defense mechanisms are brought out easily to ward off undesirable effects. Sharp increase in superoxide activity induced in gills by 12.5 ppb

of malachite green by fourth day after exposure might be surmised as due to such response. The significant ($P<0.05$) declension of the enzyme activity from 8th day until it reaches the minimum on 16th day signifies the weakening of the enzymatic defense. Curiously enough, total collapse of superoxide dismutase in providing protection from oxidative stress under high concentration of the dye is manifested from the significant decrease in activity of the enzyme as it never touched even the near control level at any day of sampling. In liver, kidney and gills, the decreased activity of superoxide dismutase at any time was accompanied by increase in thiobarbituric acid reactive substances (TBARS) due to extensive peroxidation of lipids.

The escalation in the activity of glutathione peroxidase in liver of *Oreochromis mossambicus* under 12.5 as well as 25 ppb of malachite green resembled that of hepatic catalase activity in the current study verifying the fact that this enzyme shares the ability to detoxify hydrogen peroxide with catalase (Lackner, 1998). Conversely, the decreased activity of the enzyme on 16th day at both the test concentrations of the dye can be elucidated on the basis of maximal level of lipid peroxidation and minimal availability of glutathione (reduced) as the enzyme needs the latter for efficient scavenging of hydroperoxides and hydrogen peroxide (Fig.4.3.2.1, 7,16 and 19). The variation in the activity of the enzyme in kidney is reflective of oxidative stress. The increase in activity recorded on 4th day under low stress (12.5 ppb) of the dye represents effective defense and the subsequent declension below that of control animals denoting weakening of the detoxification mechanism. On the other hand the peculiar behavior of glutathione peroxidase activity in kidney with intermittent increase and decrease at high concentration (25 ppb) signifies the failure of the enzyme in protecting the cell from oxidative damage

since the activity of the enzyme at 25 ppb of the dye was significantly low ($P<0.05$) throughout the assay intervals when compared to that of control fishes. Significantly ($P<0.05$) high activity of the enzyme in gills on 8^{th} and 12^{th} day under low concentration which approached near control values by 16^{th} day may be inferred as an adaptive change. This is because the morphology of the gill showed no serious injury even under high concentration of the dye (Fig.4.3.2.26. a & b, 4.3.2.27) and there was no significant declension in the level of branchial glutathione. Though slight decrease in activity of the enzyme occurred under high stress of malachite green, points to the insufficiency of the enzymatic activity to counter the damage.

Concentration dependent variation in the activity of glutathione S tansferase in liver of *Oreochromis mossambicus* is a clear reflection of oxidative stress as significant elevation was noticed in lipid peroxidation. However, the tissue seemed to shield the situation. The gradual increase in activity under low concentration may be associated with the declined levels of glutathione (Fig. 4.3.2.19) as the enzyme utilizes the same for conjugation of the toxicant thereby facilitating the detoxification process. According to Habig *et al*., (1974), glutathione S transferase makes use of glutathione in conjugation reactions with both exogenous and endogenous substrates and this enzyme is secreted in substantial amounts by fish liver. It catalyses the conjugation of electrophilic xenobiotics such as certain carcinogens to glutathione. This conjugate is more water soluble and is subsequently excreted after undergoing a sequence of changes. Malachite green is reported to possess carcinogenic properties in higher vertebrates and is capable of imparting serious damage to fish liver (Srivastava *et al*., 1998a). Nevertheless, statistically significant ($P<0.05$) declension at low concentration of the dye by 16^{th} day after exposure can be inferred as the

inhibition of glutathione S transferase due to the overwhelming toxicity of the compound. The sharp increase in tissue lipid peroxidation supports this assumption. Sudova *et al*., (2007) reviewed the inhibitory effect of malachite green in liver tissue of animals. The stepwise decrease of the enzyme activity at high concentration of the dye significant after every 96 hours can also be explained as initial defense against the damage the efficacy of which declines due to extensive lipid peroxidation. The histopathological features can justify this judgment (Fig. 4.3.2.22, a, b, c, and d). The kidney also suffered concentration and duration dependent damage due to malachite green insult. The maintenance and even an increase in the level of glutathione in renal tissues (Fig. 4.3.2.20) failed to keep the activity of glutathione S transferase in kidney though the activity showed an uptrend on 4^{th} day under exposure to 25 ppb of the dye (Fig. 4.3.2.11). The gills exhibited a leveling off of glutathione content after a slight decrease on 4^{th} and 8^{th} day after exposure at both experimental concentration of malachite green. Consequently, the activity of glutathione S transferase showed a declension in activity on 12^{th} and 16^{th} day with a regaining of moderately high activity (but low when compared to that of control animals) on 16^{th} day at low concentration of the dye notably indicating a tendency of tolerance. However, the branchial tissue was unable to regain the activity of the enzyme after 8^{th} day due to very high lipid peroxidation at high (25 ppb) concentration of malachite green.

Glutathione reductase has a crucial role in the maintaining of glutathione homeostasis (ratio between oxidized and reduced forms of glutathione) under stress conditions; however it is not involved in direct antioxidant defense in the same way as the primary antioxidant enzymes like catalase and superoxide dismutase. The pattern of activity of glutathione reductase in liver of the fish in response to malachite green

was the direct evidence of oxidative stress in the tissue which is dependent both on duration and concentration. The increase in activity of the enzyme at 12.5 ppb of the dye up to 12th day being accompanied by declension of glutathione levels and enhancement of lipid peroxidation is a direct signal of the enzyme giving way to reactive oxygen metabolites after an episode of defense. Peixoto *et al*., (2006) reported induction of glutathione reductase in the liver of *Oreochromis niloticus* after exposure to oxyfluorfen. Similarly the increase in activity of the enzyme was significantly low ($P< 0.05$) at high concentration of the dye when compared to the enhancement at low concentration. This is obviously due to the suppressive effect of oxidative stress. The activity of the enzyme reaching below that of control animals is indicative of extreme stress. The rise in the activity of glutathione reductase in kidney also is dependent on concentration of the dye in the medium; the lower the exposure concentration, the lower the activity enhancement. In any case, the activity decreased with the progress of exposure period. Evidently, lipid peroxidation also showed significant elevation which was dependent on concentration of the dye as well as duration of exposure. It is also noticed that the satisfactory level of glutathione failed to sustain the activity of all tested glutathione-dependent antioxidant enzymes (glutathione peroxidase, glutathione S transferase and glutathione reductase) suggests the vulnerability of renal tissue to oxidative damage. So also the early increase in the activity of glutathione reductase in gills which subsided later on denotes the concentration and duration dependent weakening of oxidative defense. But the structural integrity of gills was found not seriously affected especially on 8th day after exposure at high concentration, probably due to rather high level of glutathione during the corresponding period (Fig. 4.3.2.26. a & b).

The liver of *Oreochromis mossambicus* was found to defend the oxidative stress resulting from the exposure to Metacid 50 as the tissue experienced high activity of catalase and the three glutathione associated enzymes (glutathione peroxidase, glutathione S transferase and glutathione reductase). This was so at low (0.62 ppb) and high (1.24ppb) concentrations of the pesticide. Significant elevation of lipid peroxidation at both the experimental pesticide concentration seemed to impart no effect on the activity of these enzymes. The declension in glutathione content in hepatic tissues should be understood as this non – enzymatic antioxidant is diverted for the activity of glutathione S transferase and glutathione peroxidase. The upsurge in glutathione reductase activity might have maintained the level of glutathione (reduced) pool. Nevertheless, the decreased activity of superoxide dismutase; another primary antioxidative enzyme signifies the inability of the enzyme in countering the toxic effects of the pesticide. However, the enzyme activity was found to revive on 16^{th} day after exposure to Metacid 50 both at low and high concentrations. Notwithstanding, the extensive damage to liver noticed in histological examination suggests considerable damage to the tissue.

The primary enzymatic antioxidant machinery of the kidney of the fish comprising catalase and superoxide dismutase showed contrasting activities, the former showing duration and concentration related enhancement and the latter exhibiting a declension also dependent on duration and concentration (Fig. 4.3.3.2 and 5). However, the activity of catalase at high concentration (1.24 ppb) of the pesticide showed a significant ($P<0.05$) decrease when compared to that at low (0.62 ppb) concentration. The existence of high activity of catalase was in contrast and the decreased activity of superoxide dismutase was in agreement with

the observations made by Huang *et al*., (2007) in *Cyprinus carpio*. As the activity of glutathione peroxidase showed significant declension (Fig. 4.3.3.8) at both the concentrations, it is deduced that the removal of hydrogen peroxidase emerging from oxidative stress is done solely by the catalase. At the same time superoxide dismutase failed to cop up with the increasing oxidative stress (as evidenced by enhanced level of lipid peroxidation) and the accumulating superoxide radicals might have resulted in tissue injury. The inducement of glutathione S transferase in kidney with corresponding increase in the activity of glutathione reductase at high as well as low concentration of the pesticide vividly indicates the glutathione conjugation of the pesticide or its metabolites due to the activity of glutathione S transferase facilitating the conjugate excretion. The significantly high levels of glutathione in the renal tissue when compared to that of control animals at both the experimental pesticide concentrations underlines the increased activity of glutathione reductase which aids in the regeneration of tissue glutathione (Fig. 4.3.3.20). The gills of the fish was found to get protection from oxidative injury due to the action of the two primary antioxidative enzymes; catalase and superoxide dismutase, though the last day of sampling revealed slight declension in activity due to continued stress. The situation was slightly different at high concentration of the pesticide gaining comparatively high activity on 8^{th} and 12^{th} day after exposure which can be due to the machinery making maximum effort to minimize the damage and ultimately succumbing to it. However, the exposure to low concentration seemed to impart no significant declension in the activity of catalase ensuring the scavenging of hydrogen peroxide. At this instant, the increased activity of superoxide dismutase came down on 16^{th} day probably due to the weakening of the enzymatic machinery. The glutathione dependent enzymes showed erratic behavior in the gills. The

declension in activity of glutathione peroxidase during 12th and 16th day after exposure is accompanied by corresponding increase in the activity of catalase indicating the scavenging of hydrogen peroxide is entirely by catalase. Similarly, the induction of glutathione S transferase in branchial tissue was not potent enough to defend the oxidative challenge as indicated by the continued declension in its activity from 4th day of exposure. The high level of glutathione recorded in gills especially on 12th and 16th day after exposure might therefore be derived from pathways other than glutathione reductase, like γ-glutamylcysteine synthetase (Stephensen *et al*., 2002).

High degree of lipid peroxidation observed in liver, kidney and gills of the fish might also be due to the direct interaction of the pesticide with cellular plasma membrane, as organophosphorous compounds were found to possess such a potential (Durmaz *et al*., 2006).

The present study demonstrates that the liver of control fish exhibits a normal architecture and there were no pathological abnormalities. The hepatocytes present a homogenous cytoplasm and a large central or sub central spherical nucleus (Fig.4.3.1.22).

The histology showed that copper caused some alterations of the liver parenchyma, like vacuolization and necrosis. The liver histological changes observed were more evident in fish exposed to high copper concentrations and the severity of damage was dependent on duration of exposure (Fig.4.3.1.23, 24 and 25). The nuclei of hepatocytes became condensed (pycnosis). These alterations are often associated with a degenerative-necrotic condition. Several studies demonstrated that alterations in number, size and shape of the hepatocyte nucleus can be due to contaminants. Alterations in the size of nucleus have been previously

regarded by Paris-Palacios *et al*. (2000) in *Brachydanio rerio* exposed to sublethal concentrations of copper sulphate. Braunbeck *et al*. (1990) referred that alterations in size and shape of nucleus have often been regarding as signs of increased metabolic activity but may be of pathological origin. The progression of damage to normal architecture of the liver is assumed as the cause of significant increase in lipid peroxidation observed on 16th after exposure to 128 ppb of copper.

The normal trunk kidney of *Oreochromis mossambicus* is of mesonephric type and opisthonephric in position consisting of glomeruli encircled by cup shaped Bowman's capsules and proximal renal tubules identifiable by the basal position of nuclei. The distal tubules with the central location of nuclei lead to collecting duct (Fig. 4.3.1.25). The renal tissue exhibited duration dependent changes due to exposure to high concentration of copper in the medium. There occurred inflammation of glomeruli with thickening of Bowman's capsules. Cells of renal tubular epithelium became pycnotic and the tubular epithelia showed desquamation. Such necrotic changes set in after 8th day of exposure. In addition to the increased severity of these changes, the kidney showed widespread oesinophilic and inclusion of tubular casts after 16th day of exposure (Fig. 4.3.1.26, 27 and 28). As indicated by Baker (1969), it is inferred that copper can induce inflammatory changes in kidney. The observation of deposition of casts in tubular lamina, though rare in literature, was documented by Reichenbach-Klinke (1975) in teleosts after treatment with copper sulphate. Contrary to the degenerative changes occurred in liver, the pathological alterations developed in kidney emerged as defensive measures as indicated by oesinophilic infiltration. However, the toxic insult of copper afflicted the kidney so badly that it became degenerative as indicated by enhanced lipid peroxidation.

Pathological changes shown by gills due to copper exposure indicated defensive as well as degenerative features (Fig.4.3.1.30, 31 and 32). Enhanced mucus production in gills observed in the present study (evidenced by hyperplasia of mucus cells) seemed as a defensive mechanism (Gardner, 1975). A hypersecretion of mucus is considered a defense response to contaminant exposure rather than a direct effect of toxicants (Mallatt, 1985). Mucous cells contain mucins, polyanions composed of glycoproteins that can be effective in trapping toxicants and aid in the prevention of toxicant entry into the gill epithelium. Although mucous cell proliferation (hyperplasia) may be beneficial in reducing toxicant entry, the consequence is an increase in the distance for gas exchange along the secondary lamellae, potentially reduces the efficiency of gas exchange and causing hypoxic conditions (Nero *et al*., 2006). The edema, epithelial lifting as well as lamellar fusion observed in the current study also are defense mechanisms that reduce the branchial superficial area in contact with the external milieu. These mechanisms also increase the diffusion barrier to the pollutant. Similar features were recorded by Fernandes, *et al* (2007) after the exposure of *Oreochromis niloticus* to water-borne copper. Ballooning degeneration and lamellar shortening observed in the gills in the present investigation are clear indications of disintegration of the tissue. Studies by earlier workers (Gardner, 1975 and Wobeser, 1975b) demonstrated such features in gills of fishes after exposure to chemical contaminants. Commonly reported gill lesions are of two categories detailed to hypothesise two different kinds of reactions to irritants. One type deals with necrosis and rupture of the branchial epithelium. These alterations are believed to reflect the direct deleterious effects of irritants. It is evident that epithelial necrosis and rupture are among the most dose - dependent branchial lesions. However, this is more reported in connection with lethal toxicity than with chronic sub lethal

toxicity. Branchial cell death and rupture could develop via two different mechanisms viz. autolysis induced by the cells' own enzymes following the toxicant induced disruption of cell processes or rapid lyses caused by the direct lytic action of toxicants on cell constituents. Often, direct action of the toxicant on the gill may manifest and could be interpreted as defense reactions. Epithelial necrosis and rupture are among the most dose dependent branchial lesion types and they are clearly more often reported under lethal than under sublethal conditions. Commonly under the most highly toxic conditions, necrosis and rupture are the only gill lesions found reported. Mucus hyper secretion, which results in preventing toxicant entry and chloride cell proliferation, also indicate the role of structures on toxicant extrusion or neutralisation. Lifting, swelling and hyperplasia of the lamellar epithelium could serve a defense function as these alterations increase the distance across which water borne irritants must diffuse to reach the blood stream. Lamellar fusion could be protective in that it diminishes the area of vulnerable gill surface.

The histopathological signs indicated by liver (necrosis and loss of architecture), kidney (tubular degeneration) and gills (hyperplasia) are in accordance with the observations made by Krishnani *et al* (2003) in *Lates calcarifer*. The structural abnormalities developed in gills due to copper exposure are in agreement with the findings of Karan *et al* (1998) who studied copper-induced histopathology in carps.

Morphological changes triggered by malachite green in the branchial, hepatic and renal tissues of *Oreochromis mossambicus* showed some qualitative resemblances to those that occurred due to exposure to copper. Exposure to the experimental high concentration of the dye (25ppb) resulted in led to pycnosis of hepatocytes and necrotic changes developed by 16th day after exposure (4.3.2.22 and 23). Nevertheless, the

stemming of non-fatty vacuolation remained a new feature when compared to the changes elicited due to copper intoxication. It causes sinusoidal congestion and focal necrosis in liver, damages mitochondria and also causes nuclear alterations (Gerundo *et al*., 1991). Hypertrophy and vacuolisation followed by necrosis and cirrhosis have been observed in hepatocytes of *Heteropneustes fossilis* following treatment with malachite green (Srivastava *et al*., 1998a). The duration dependent advancements in hepatic lesions registered in the present study indicate the risk associated with long term exposure to malachite green as the organ failed to provide sufficient protection from the dye-induced oxidative damage, underlining the activity profile of antioxidant enzymes after the exposure.

Duration dependent histological alterations occurred in kidney under high concentration of malachite green were somewhat analogous to the observations made by Srivastava *et al* (1998b) in the kidney of *Heteropneustes fossilis*. The dye caused desquamation of epithelial lining cells and glomerular leakage and widespread pycnosis of cells (4.3.2.24 and 25). However, glomerular swelling, constriction of tubular lumina and hyalinization of almost all renal cells indicated inflammatory condition of the organ. As in the case of liver, the pathological features exhibited by kidney also point out the undesirable consequences of long term exposure of the fish to the dye.

The gills showed no gross morphological abnormality due to malachite green exposure, except for exfoliation of basal laminar cells after 16^{th} day of exposure (Fig. 4.3.2.26 and 27). The occurrence and duration based increase of hyperplasia of epithelial cells, secondary lamellar curling and lamellar fusion represents defensive strategies to minimize the area exposed to the chemical compound. Occasional

occurrence of lamellar aneurisms illustrates release of blood due to the break down of vascular integrity. The collapse of pillar system supplements this damage leading to apical bulging effected by the pushing of blood from inside. Even under this context, the pathological symptoms observed in liver and kidney dictates the gills as an almost easy gateway for malachite green.

Metacid 50 elicited changes in the morphology of the experimental tissues of *Oreochromis mossambicus* which were typical of an organophosphorous pesticide. The liver showed proliferative (biliary hyperplasia) as well as degenerative (pycnotic nuclei of hepatocytes and copious fatty vacuolation) due to exposure to 1.24 ppb of the pesticide (Fig. 4.3.3.22 and 23). Similar changes were recorded by Sarkar *et al*., (2005) in *Labeo rohita* after exposure to cypermethrin and carbofuran. The yellowish brown bodies (ceroid pigments) observed on 8th day after exposure indicates bile stagnation. Similar results were recorded in *Corydoras paleatus* by Fanta *et al*., (2003) as a consequence of exposure to organophosphorous pesticide Folidol 100. The progression of fatty vacuolation (both in size and number) and pycnosis of hepatocytes recorded after 16th day of exposure under the same concentration of Metacid 50 indicated duration dependent severity of degenerative changes and that of pancreatic tissue showed progression of proliferative changes. Like mammals, fish have an enterohepatic cycling mechanism (Gingerich, 1982) for the processing of substances that were not metabolized during their first pass through the liver. As in mammals, enterohepatic cycling may prolong the removal and/or biotransformation in fishes (deBethizy and Hayes, 2001). Thus, the delay in detoxification processes might be the cause of persistence and progression of the lesions.

Kidney of fishes receives abundant volume of post branchial blood, and therefore, renal lesions might be expected to be good indicators of toxicant exposure (Cengiz, 2006). Kidney of *Oreochromis mossambicus* exhibited nephrotoxic lesions, including degenerative changes in tubular epithelium (cytoplasmic vacuolation and pycnotic nuclei), dilation of tubular lumina, and casts within tubular lumina (probably resulting from protein leakage), tubular necrosis and/or epithelial desquamation and necrosis of interstitial hematopoietic tissues. The presence of glomerular inter-capillary thickenings noted on 8th day after exposure (Fig. 4.3.3.24 b) to the pesticide, forms the precursor of protein leakage culminating in the deposition of casts in tubular lumen(Fig. 4.3.3.24 c) due to prolongation of toxic insult. Changes of this kind seemed possible as given by Anees (1976). Loss of differential staining with disintegration of tissue structures and cell membranes led to the sighting of basophilic areas (homogeneous mixture of granular debris that is basophilic to purple with hematoxylin and eosin) which is an indication of necrotic changes (Fig. 4.3.3.24 a). Basophilic areas giving way to tubular vacuolation, degeneration and casts, glomerular shrinkage and capsular thickening as observed on 16th day after exposure dictates the temporal increase in renal lesions due to Metacid 50 (Fig.4.3.3.25). Velmurugan *et al*., (2007) observed wide spread changes, more similar to that registered in the present study, in the kidney of *Cirrhinus mrigala* due to exposure to the pesticide; fenvalerate.

Extensive temporal changes cropped up in the gills of *Oreochromis mossambicus* indicated different degrees of defensive responses and degenerative changes during prolonged exposure to Metacid 50 (Fig. 4.3.3.26 and 27). The lifting of respiratory epithelia and necrotic changes observed on 8th day after exposure; indicate direct responses induced by

the action of the pesticide. The hypersecretion of branchial mucus observed during the experimental period indicated localized immune response to defend the pesticide-stimulated perturbations. Prolongation of the sublethal toxic exposure led to the coalescence of epithelia due to extensive hyperplasia and consequent reduction in the degree of epithelial lifting (Fig. 4.3.3.27). Severe vascular congestion (hyperemia) denotes circulatory adjustments facilitating the maximal utilization of available dissolved oxygen under the pesticide-induced stress situation. Lifting of respiratory epithelia, their hyperplasia and lamellar fusion could be protective in that it diminishes the amount of vulnerable gill surface area. Necrotic changes and desquamation of epithelia are direct reactions to exposure to the pesticide. The lifting up of respiratory epithelium increases the diffusion distances through which the toxicant has to travel to reach the blood stream. Hyperplasic condition of respiratory cells leads to a decrease in the respiratory surface and consequent increase in the toxicant-blood diffusion distance. This defensive mechanism since operates at the expense of respiratory efficiency of the gills, eventually get surpassed by respiratory impairment, culminating in the collapse of protective effect.

Secondary lamellar aneurism results due to the break down of the pillar cell system and loss of vascular integrity with a release of copious amount of blood that push the lamellar epithelium outward (Cengiz, 2006). Exfoliation of basal laminar cells, severe necrosis and longitudinal tearing of primary lamellae indicated serious mutilation of respiratory machinery of *Oreochromis mossambicus* triggered by Metacid 50.

The results of the study on the sublethal toxicity assessed by biochemical assay of the prominent enzymes did exhibit the possibility of utilizing these as biomarkers of contamination under controlled

conditions. A judicious assessment of activity of such enzymes has to be carried out by including all possible enzymes so that the chances of error in deciding effects could be reduced. This is necessary to understand the interplay enzymes springing into action under oxidative stress. The utilization of histopathology of affected tissues to support biochemical effects is essential although it is not possible to conduct the biopsy of such tissues which exhibit biochemical damages concomitantly. Therefore, such investigations have to be done with utmost care. There are many practical difficulties associated with the use of same animal or population for different types of studies. Furthermore, the morphological aberrations from normalcy in the test tissues employed in the present study showed no toxicant-specific responses except for fatty degeneration developed in liver as a result of exposure to Metacid 50. Indiscriminate and long term use of malachite green, even for therapeutic purposes in fishes, may invite damage to vital organs. Similarly, exposure to copper and Metacid 50 can elicit oxidative damage in tissues and therefore their input into water bodies should be controlled to ensure a healthy and congenial culture conditions in commercial aquaculture.

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Chapter -5

EFFECT OF VITAMIN C ON TOXICITY

Contents

5.1 Introduction

The existence and need for a dietary supply of ascorbic acid (Vitamin C) for growth and development were substantiated in a number of freshwater and marine species of fish. Ascorbic acid is a common cofactor of many hydroxylating enzymes. It is essential for the synthesis of the important skin component collagen by the hydroxylation of pro – collagen and is also involved in the formation of the cartilage and of the endothelium of blood vessels. However, most fishes cannot synthesize this vitamin because of the loss of a liver enzyme, L glucono-γ-lactone oxidase, during the course of evolution (Deshpande *et al*. 1996., Chatterjee, 1973). Natural resources of ascorbic acid are also often insufficient for natural growth of fish. Thus, an exogenous supply of this nutrient is desirable for optimal growth and production of fish. Blanco and Meade (1980) indicated that dietary supply of ascorbic acid not only helps in promoting growth but also protects the fish from the toxicity of many chemicals. Agrawal *et al.,* (1978) and Guha *et al.,* (1993) observed that ascorbic acid could be used as an antitoxic agent against

pesticide toxicity to fish. Vitamin C is a potent scavenger of reactive oxygen species capable of repairing tissue damage.

This chapter deals with the assessment of ameliorative effect of ascorbic acid on the antioxidative machinery of *Oreochromis mossambicus* and the restorative changes in test tissues.

5.2 Material and Methods

The general protocol for adopted for sublethal toxicity studies for this episode is the same as those described under section 4.2. The preparation of test solutions was done according to the methods detailed in Table 4.2.1 The assay procedures for antioxidant enzymes, lipid peroxidation and estimation of protein remain the same as given under section 4.2.2. The methods adopted for histopathological assessment are the same as given under section 4.2.3. The modifications made to suite the experimental situation are given below.

The copper intoxicated specimens (receiving 128 ppb of copper) were supplied with the experimental basal diet the composition of which is given in table 5.1. The test group was fed with vitamin C enriched feed containing L-ascorbic acid. The components of the feed mix for test group contained in addition to those of the basal diet, vitamin C in two separate concentrations, one set containing 250mg/kg and another set containing 500mg/kg diet. Rovimix® Stay C® was used as the vitamin C supplement containing L-ascorbyl-2-polyphosphate as the active ingredient. The feed constituents other than vitamins were made into a paste, extracted through a suitable sieve, and dried in a hot air oven at 70°C . Vitamin mix was added after drying the feed half way. Excess heating was avoided to prevent the degradation of vitamins. The feed mix was prepared afresh for the days' feeding to check the loss of vitamins on storage.

The data of antioxidant enzyme assays, lipid peroxidation study and reduced glutathione content were statistically evaluated using one-way analysis of variance utilizing SPSS for Windows version 15.0 and the results subjected to post hoc analysis by multiple comparison test using Dunnett's test (Dunnett *t*) to find out which individual groups were significantly different from control and intoxicated animals. All the data were stated as mean ± standard deviation of the mean of six observations.

Table 5.2.1. Composition of the experimental diet (control). (After Jaya Kumari and Sahoo , 2005).

Ingredients.	Amount (gm/kg diet).
Fish meal.	300
Groundnut oil cake.	250
Soybean meal.	150
Rice bran.	100
Wheat flour.	30
Carboxymethyl cellulose.	8
Vitamin mix (except Ascorbic acid).	15

5.3 Results

The results of assays performed for antioxidant enzymes, reduced glutathione levels and lipid peroxidation carried out individually to assess the effect of vitamin supplementation (two concentrations) on the intoxication of (i) Copper (ii) Malachite green and (iii) Metacid 50 are presented here. The alterations revealed by the test organs of intoxicated fishes after complementing with vitamin C are depicted after the summarizing the results of biochemical considerations.

5.3.1 Effect of Vitamin C on Copper Toxicity.

From the previous chapter it became evident that copper is capable of eliciting biochemical as well as structural variations in liver, kidney

and gills of *Oreochromis mossambicus* making the fish to confront deleterious consequences. The fishes were subjected to vitamin C supplementation to check whether there is any restorative change from toxicity. The results are detailed below.

Table 5.3.1.1 a) Changes in the activity of catalase in the tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation.

Tissue	Concentration	Duration of Exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	11.38± 0.0901	11.38± 0.0518	11.40± 0.0737	11.37± 0.0496
	128 ppb (copper intoxicated)	11.46± 0.0082	13.65± 0.2969	15.60± 0.3684	18.51± 0.3827
	250 mg vit C/kg diet	11.64± 0.0103	13.54± 0.0549	12.89± 0.0609	12.23± 0.0331
	500 mg vit C/kg diet	11.68± 0.0593	13.43± 0.0349	12.62± 0.0167	12.12± 0.0225
Kidney	Control	2.962± 0.1807	2.928± 0.2392	2.898± 0.3368	3.003± 0.1735
	128 ppb (copper intoxicated)	2.858± 0.1098	3.870± 0.4265	3.678± 0.2998	2.117± 0.0750
	250 mg vit C/kg diet	2.858± 0.0098	2.903± 0.0273	2.930± 0.0374	3.077± 0.0339
	500 mg vit C/kg diet	2.928± 0.0319	2.967± 0.0103	3.260± 0.0179	3.183± 0.0234
Gills	Control	10.38± 0.0517	10.40± 0.0565	10.40± 0.0831	10.39± 0.2741
	128 ppb (copper intoxicated)	13.32± 0.6127	13.44± 0.5983	12.30± 0.3729	12.40± 0.0763
	250 mg vit C/kg diet	13.33± 0.0356	13.34± 0.0242	11.76± 0.0356	11.38± 0.0512
	500 mg vit C/kg diet	12.71± 0.0527	12.77± 0.0418	11.70± 0.0286	11.24± 0.0098

Table 5.3.1.1 b). ANOVA for catalase activity in the tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	3238.066	2	1619.033	807.033	.000
Duration	26.812	3	8.937	4.455	.005
Concentration	28.891	1	28.891	14.401	.000
Error	274.843	137	2.006		
Total	3568.612	143			

Table 5.3.1.1 c). ANOVA for catalase activity in the tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation (comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	2406.684	2	1203.342	1853.564	.000
Day	2.018	3	.673	1.036	.379
Concentration	46.195	1	46.195	71.156	.000
Error	88.941	137	.649		
Total	2543.838	143			

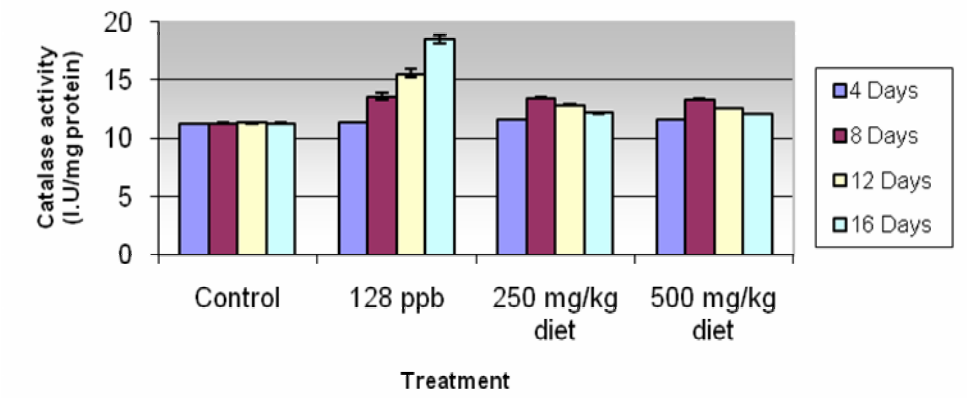


Fig. 5.3.1.1. Variation in catalse activity in the liver of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

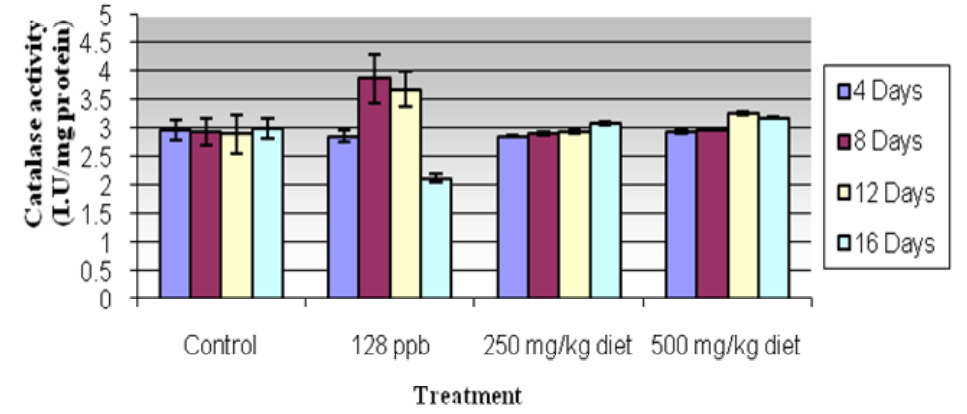


Fig. 5.3.1.2. Variation in catalse activity in the kidney of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

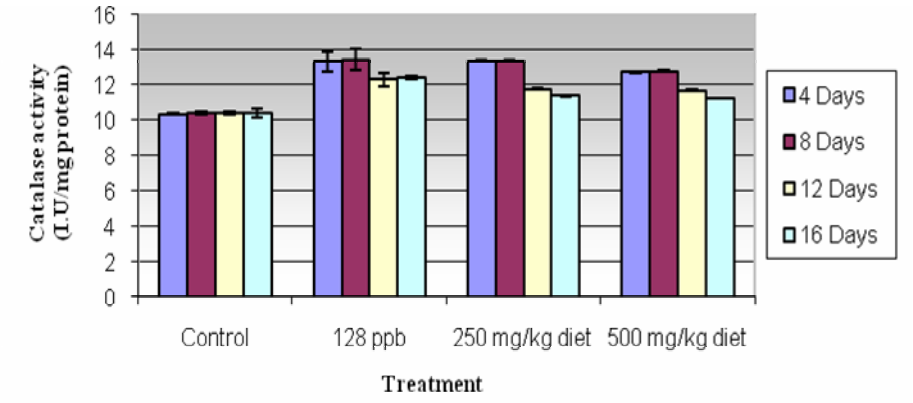


Fig. 5.3.1.3. Variation in catalse activity in the gill of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.1.2 a). Changes in the activity level of superoxide dismutase in the tissues copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation.

Tissue	Concentration	Duration of Exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	0.992± 0.0075	0.998± 0.0371	1.023± 0.1544	1.192± 0.3432
	128 ppb (copper intoxicated)	0.880± 0.0228	0.852± 0.1485	0.908± 0.1007	1.005± 0.1106
	250 mg vit C/kg diet	0.905± 0.0513	0.880± 0.0237	0.922± 0.0117	1.008± 0.0075
	500 mg vit C/kg diet	1.013± 0.0163	1.003± 0.0207	1.020± 0.0110	1.015± 0.0164
Kidney	Control	0.320± 0.0443	0.342± 0.0578	0.310± 0.0587	0.358± 0.0763
	128 ppb (copper intoxicated)	0.352± 0.0041	0.360± 0.0089	0.415± 0.0084	0.328± 0.0133
	250 mg vit C/kg diet	0.340± 0.0000	0.333± 0.0082	0.330± 0.0000	0.320± 0.0000
	500 mg vit C/kg diet	0.317± 0.0052	0.318± 0.0041	0.313± 0.0121	0.322± 0.0075
Gills	Control	1.168± 0.0483	1.130± 0.1380	1.117± 0.1553	1.193± 0.1795
	128 ppb (copper intoxicated)	1.252± 0.1027	1.505± 0.0524	1.282± 0.0835	1.650± 0.3927
	250 mg vit C/kg diet	0.832± 0.5087	1.142± 0.0041	1.042± 0.0075	1.052± 0.0075
	500 mg vit C/kg diet	1.185± 0.0055	1.167± 0.0052	1.133± 0.0082	1.130± 0.0310

Table 5.3.1.2 b). ANOVA for superoxide dismutase activity in the tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	28.947	2	14.473	160.754	.000
Duration	1.266	3	.422	4.687	.004
Concentration	1.082	1	1.082	12.013	.001
Error	12.335	137	.090		
Total	43.630	143			

Table 5.3.1.2 c). ANOVA for superoxide dismutase activity in the tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation (comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	27.386	2	13.693	207.357	.000
Day	1.040	3	.347	5.249	.002
Concentration	2.071	1	2.071	31.365	.000
Error	9.047	137	.066		
Total	39.544	143			

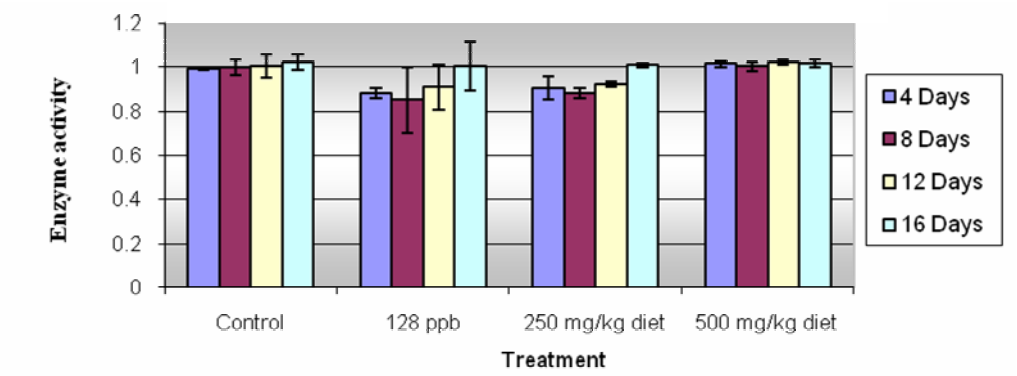


Fig. 5.3.1.4 Variation in Superoxide dismutase in the liver of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

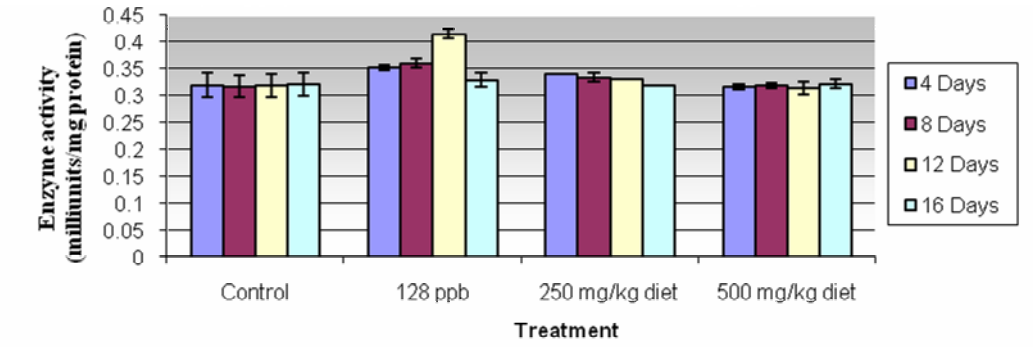


Fig. 5.3.1.5 Variation in Superoxide dismutase in the kidney of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

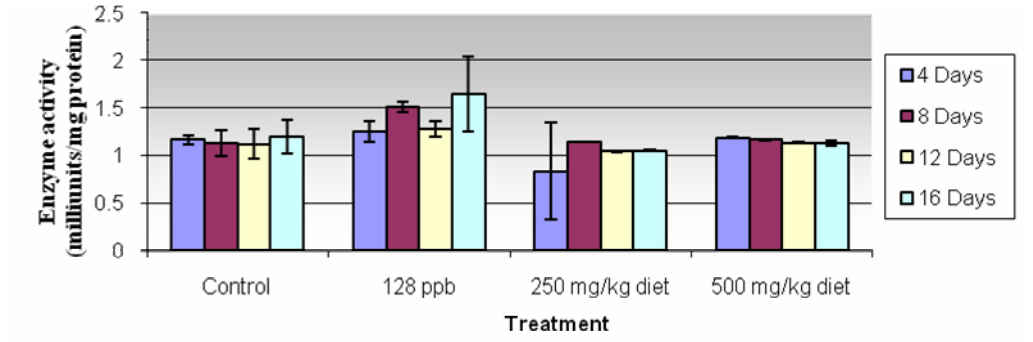


Fig. 5.3.1.6 Variation in Superoxide dismutase in the gill of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.1.3 a). Changes in the activity level of glutathione peroxidase in the tissues copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation.

Tissue	Concentration	Duration of Exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	8.532± 0.5709	8.502± 0.6548	8.485± 0.3626	8.550± 0.5337
	128 ppb (copper intoxicated)	22.49± 0.2873	20.36± 0.2172	14.62± 0.3720	11.52± 0.2785
	250 mg vit C/kg diet	21.51± 0.3285	21.63± 0.0646	18.07± 0.0999	18.17± 0.0463
	500 mg vit C/kg diet	21.56± 0.0741	21.19± 0.1729	16.25± 0.1161	16.55± 0.2584
Kidney	Control	5.232± 0.5198	5.207± 0.5438	5.182± 0.5877	5.347± 0.6036
	128 ppb (copper intoxicated)	9.307± 0.2181	11.57± 0.3665	13.35± 0.6243	10.74± 0.7128
	250 mg vit C/kg diet	9.440± 0.0822	10.69± 0.0573	10.27± 0.0566	10.19± 0.0331
	500 mg vit C/kg diet	9.445± 0.2435	10.48± 0.0372	10.19± 0.0362	9.658± 0.2403
Gills	Control	12.71± 0.4834	12.80± 0.4667	12.70± 0.9438	12.74± 0.6785
	128 ppb (copper intoxicated)	17.88± 0.4705	16.99± 0.5699	16.48± 0.3790	13.48± 0.6102
	250 mg vit C/kg diet	17.88± 0.4705	15.25± 0.0475	13.28± 0.1661	13.16± 0.0343
	500 mg vit C/kg diet	14.13± 0.0940	13.67± 0.0573	13.18± 0.0403	13.12± 0.1501

Table 5.3.1.3 b). ANOVA for glutathione peroxidase activity in the tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	986.468	2	493.234	85.049	.042
Duration	381.211	3	127.070	21.911	.030
Concentration	115.616	1	115.616	19.936	.001
Error	794.515	137	5.799		
Total	2277.810	143			

Table 5.3.1.3 c). ANOVA for glutathione peroxidase activity in the tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation (comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	823.121	2	411.561	70.936	.010
Day	101.824	3	33.941	5.850	.001
Concentration	777.434	1	777.434	133.998	.001
Error	794.854	137	5.802		
Total	2497.233	143			

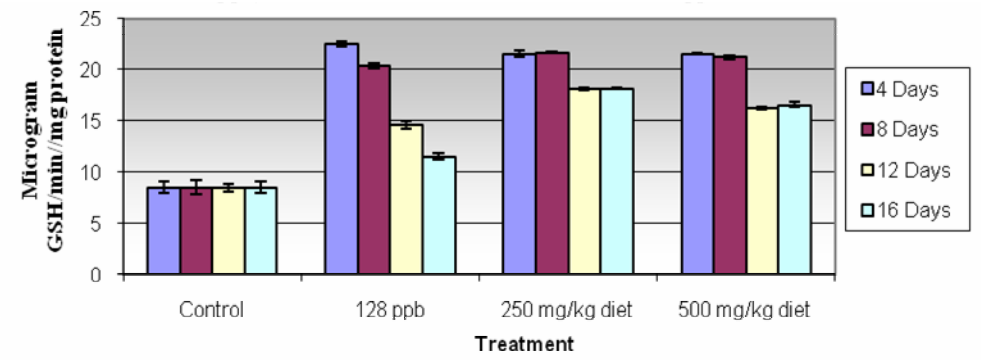


Fig.5.3.1.7. Variation in Glutatione peroxidase in the liver of copper intoxicated (128 ppb) *Oreochromis massambicus* after vitamin C supplementation

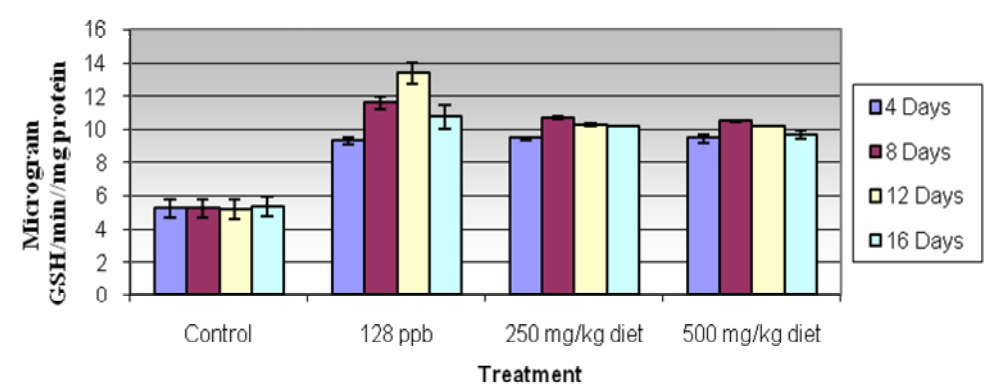


Fig.5.3.1.8. Variation in Glutatione peroxidase in the kidney of copper intoxicated (128 ppb) *Oreochromis massambicus* after vitamin C supplementation

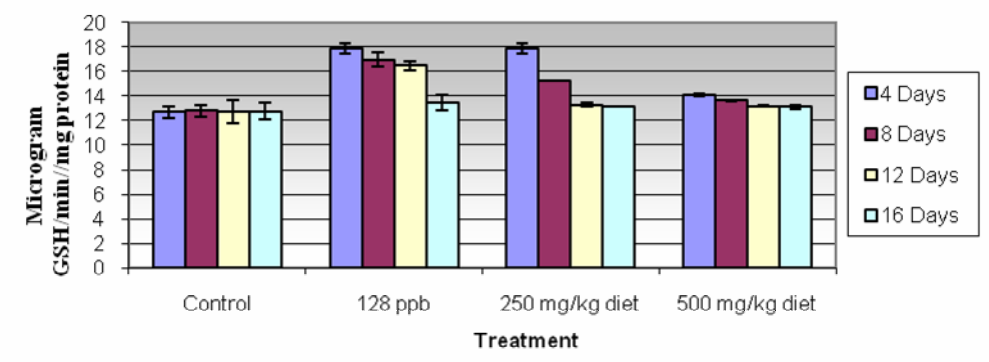


Fig.5.3.1.9. Variation in Glutatione peroxidase in the gill of copper intoxicated

Table 5.3.1.4 a). Changes in the activity level of glutathione S transferase activity in tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation.

Tissue	Concentration	Duration of Exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	23.84± 1.2199	23.68± 1.5092	23.61± 1.1620	23.81± 1.0566
	128 ppb (copper intoxicated)	30.45± 0.6993	37.08± 1.2060	43.64± 0.6256	53.48± 0.6146
	250 mg vit C/kg diet	26.14± 0.1506	26.35± 0.1058	25.43± 0.2027	25.14± 0.1021
	500 mg vit C/kg diet	25.46± 0.2069	25.34± 0.1827	25.34± 0.2062	25.19± 0.1129
Kidney	Control	12.44± 0.3030	12.40± 0.4940	12.36± 0.3894	12.40± 0.3178
	128 ppb (copper intoxicated)	13.71± 0.1528	21.46± 0.4234	28.42± 0.5654	36.19± 0.9381
Gills	250 mg vit C/kg diet	13.31± 0.2587	16.32± 0.0940	16.32± 0.0704	17.20± 0.0319
	500 mg vit C/kg diet	12.86± 0.1210	17.20± 0.0824	16.20± 0.1095	16.60± 0.0812
	Control	14.30± 0.7535	14.30± 0.7076	14.33± 0.5113	14.30± 0.7365
	128 ppb (copper intoxicated)	17.41± 0.3095	22.93± 1.3272	29.93± 0.6176	34.73± 0.2103
	250 mg vit C/kg diet	18.23± 0.0847	19.14± 0.2924	16.25± 0.1511	14.40± 0.2905
	500 mg vit C/kg diet	18.31± 0.1279	17.86± 0.0674	15.34± 0.0698	14.47± 0.1291

Table 5.3.1.4 b). ANOVA for glutathione S transferase activity in the tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	5762.321	2	2881.160	194.213	.001
Duration	3024.974	3	1008.325	67.969	.043
Concentration	3728.934	1	3728.934	251.360	.001
Error	2032.402	137	14.835		
Total	14548.631	143			

Table 5.3.1.4 c). ANOVA for glutathione S transferase activity in the tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation (comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	3751.534	2	1875.767	586.621	.001
Day	67.497	3	22.499	7.036	.023
Concentration	518.777	1	518.777	162.240	.001
Error	438.068	137	3.198		
Total	4775.876	143			

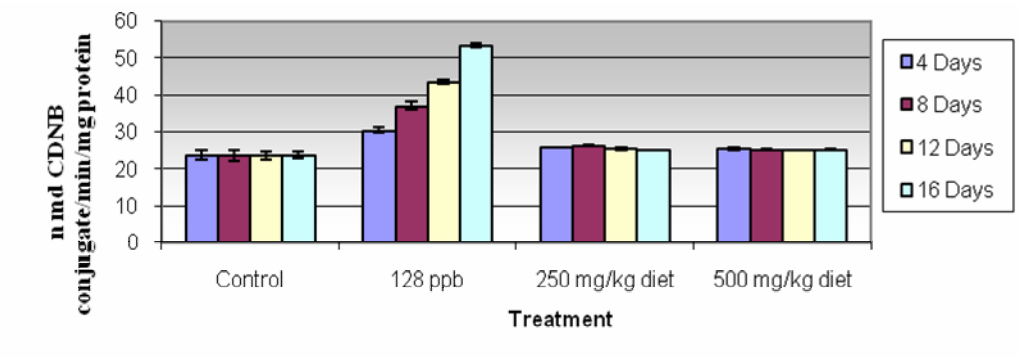


Fig.5.3.1.10. Variation in Glutathione S transferase in the liver of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

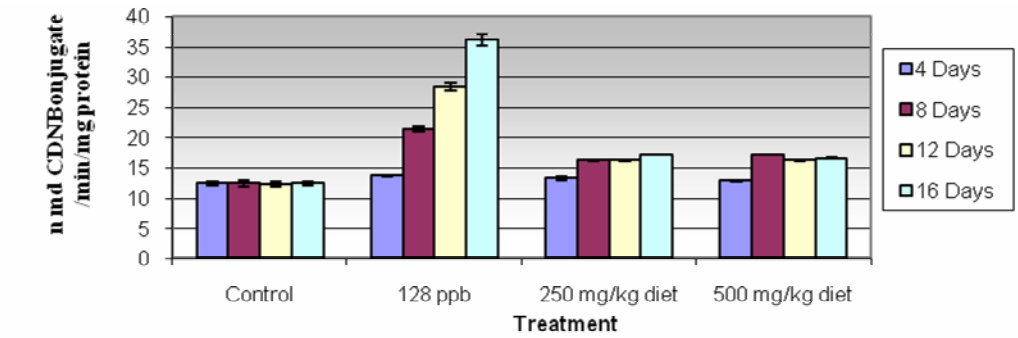


Fig.5.3.1.11. Variation in Glutathione S transferase in the kidney of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

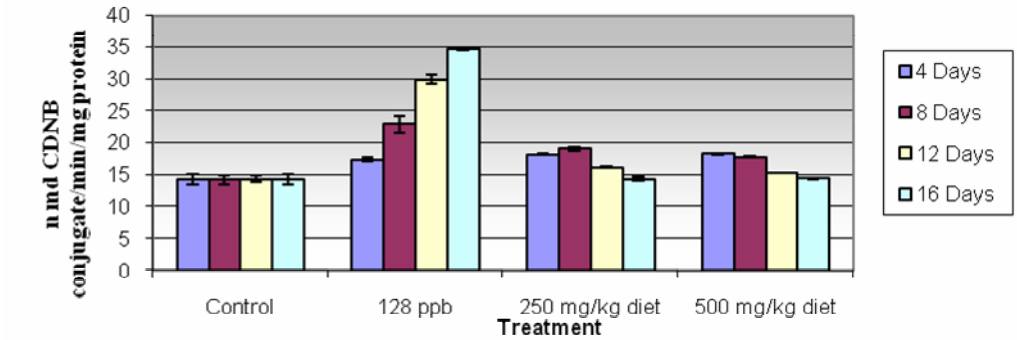


Fig.5.3.1.12. Variation in Glutathione S transferase in the gills of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.1.5 a). Changes in the activity of glutathione reductase activity in tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation.

Tissue	Concentration	Duration of Exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	3.648± 0.4492	3.628± 0.4216	3.610± 0.7435	3.602± 0.7697
	128 ppb (copper intoxicated)	11.38± 0.3728	11.74± 0.6277	12.76± 0.2108	17.21± 0.1392
	250 mg vit C/kg diet	7.292± 0.0656	7.233± 0.0493	7.130± 0.0537	7.373± 0.0612
	500 mg vit C/kg diet	7.433± 0.0493	8.050± 0.1982	7.877± 0.0582	7.872± 0.0366
Kidney	Control	1.873± 0.3598	1.898± 0.5011	1.943± 0.6556	1.878± 0.5227
	128 ppb (copper intoxicated)	9.460± 0.8103	8.937± 3.7650	3.415± 1.3728	1.338± 0.2406
	250 mg vit C/kg diet	8.945± 0.1372	9.285± 0.1519	8.768± 0.1125	5.097± 0.0468
	500 mg vit C/kg diet	9.175± 0.0609	9.613± 0.3722	9.213± 0.1189	7.123± 0.1314
Gills	Control	2.675± 0.4622	2.645± 0.6197	2.632± 0.4567	2.685± 0.4805
	128 ppb (copper intoxicated)	7.123± 0.1314	2.190± 0.1265	1.780± 0.5757	1.368± 0.5772
	250 mg vit C/kg diet	1.868± 0.0804	2.190± 0.1265	1.590± 0.0724	1.135± 0.0513
	500 mg vit C/kg diet	1.932± 0.0515	2.082± 0.1076	1.758± 0.0674	1.242± 0.0422

Table 5.3.1.5 b). ANOVA for glutathione reductase activity in the tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	2105.946	2	1052.973	176.957	.041
Duration	91.176	3	30.392	5.108	.002
Concentration	22.562	1	22.562	3.792	.054
Error	815.211	137	5.950		
Total	3034.895	143			

Table 5.3.1.5 c). ANOVA for glutathione reductase activity in the tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation (comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	409.407	2	204.703	52.542	.000
Day	34.798	3	11.599	2.977	.034
Concentration	492.211	1	492.211	126.338	.001
Error	533.750	137	3.896		
	1470.166	143			

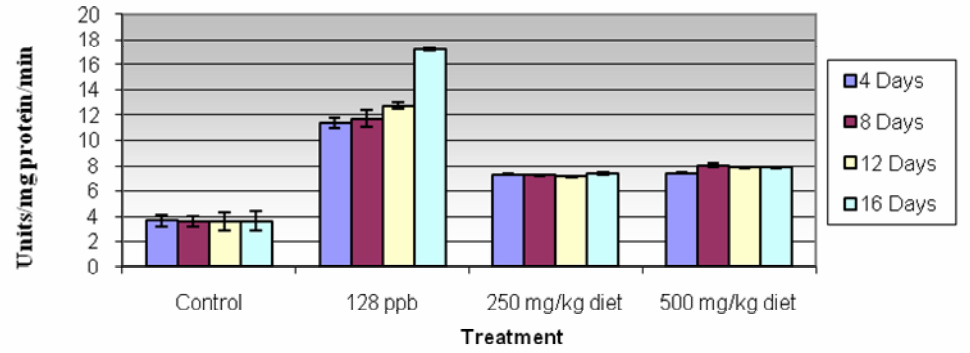


Fig.5.3.1.13. Variation in Glutathione reductase in the liver of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

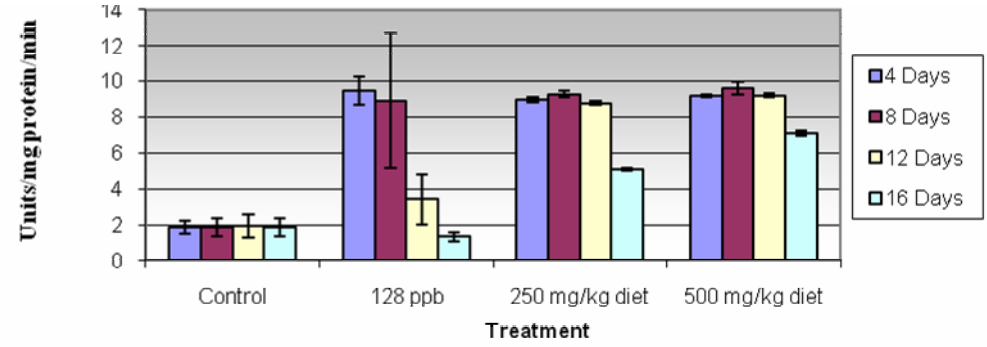


Fig.5.3.1.14. Variation in Glutathione reductase in the kidney of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

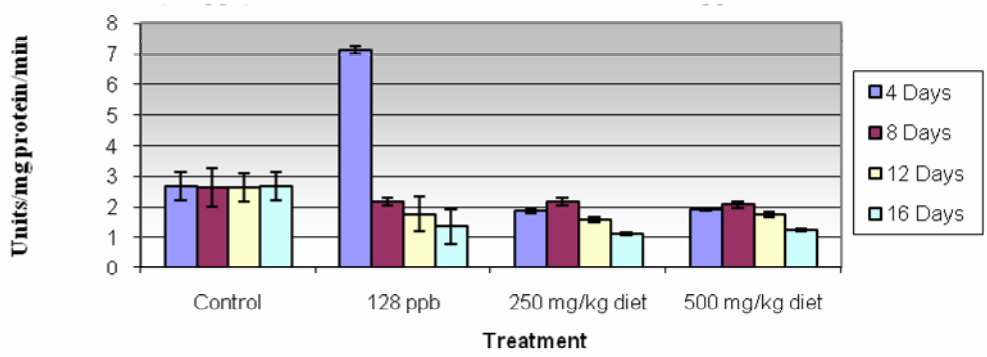


Fig.5.3.1.15. Variation in Glutathione reductase in the gill of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.1.6 a). Changes in lipid peroxidation level in tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation.

Tissue	Concentration	Duration of Exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	0.737± 0.0789	0.730± 0.1041	0.763± 0.1046	0.770± 0.0633
	128 ppb (copper intoxicated)	2.295± 0.2170	2.568± 0.0880	3.145± 0.1931	7.608± 0.2333
	250 mg vit C/kg diet	2.453± 0.0418	2.135± 0.0138	1.280± 0.0179	0.803± 0.0308
	500 mg vit C/kg diet	2.472± 0.0467	2.030± 0.0390	1.072± 0.0194	0.765± 0.0055
Kidney	Control	0.362± 0.0500	0.347± 0.0766	0.348± 0.0778	0.340± 0.1002
	128 ppb (copper intoxicated)	1.233± 0.1882	2.342± 0.1362	6.305± 0.1893	9.227± 0.2884
	250 mg vit C/kg diet	1.135± 0.0084	2.648± 0.0325	4.288± 0.0483	5.297± 0.0845
	500 mg vit C/kg diet	1.253± 0.0052	2.505± 0.0418	4.288± 0.0483	5.297± 0.0845
Gills	Control	0.642± 0.1042	0.618± 0.1301	0.682± 0.1245	0.668± 0.1116
	128 ppb (copper intoxicated)	0.748± 0.0098	1.472± 0.2559	3.122± 0.1818	4.223± 0.1145
	250 mg vit C/kg diet	0.785± 0.0198	1.523± 0.0350	1.472± 0.0293	1.737± 0.0301
	500 mg vit C/kg diet	0.837± 0.0234	1.493± 0.0350	2.152± 0.0483	1.762± 0.0813

Table 5.3.1.6 b). ANOVA for lipid peroxidation level in tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	130.118	2	65.059	45.947	.003
Duration	286.056	3	95.352	67.342	.015
Concentration	64.494	1	64.494	45.549	.001
Error	193.985	137	1.416		
Total	674.653	143			

Table 5.3.1.6 c). ANOVA for lipid peroxidation level in the tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation (comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	24.976	2	12.488	17.534	.001
Day	17.837	3	5.946	8.348	.000
Concentration	112.572	1	112.572	158.055	.021
Error	97.576	137	.712		
Total	252.961	143			

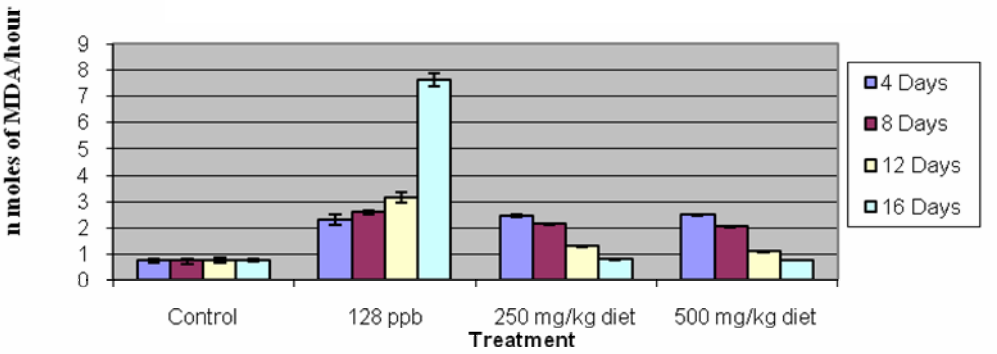


Fig.5.3.1.16. Variation in Lipid peroxidation levels in the liver of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

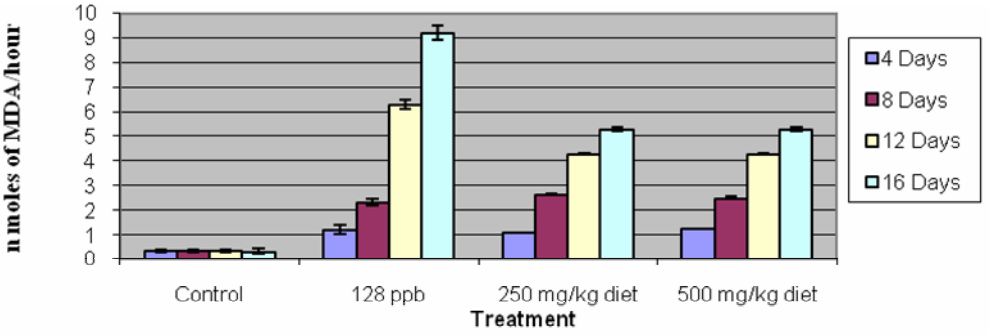


Fig.5.3.1.17. Variation in Lipid peroxidation levels in the kidney of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

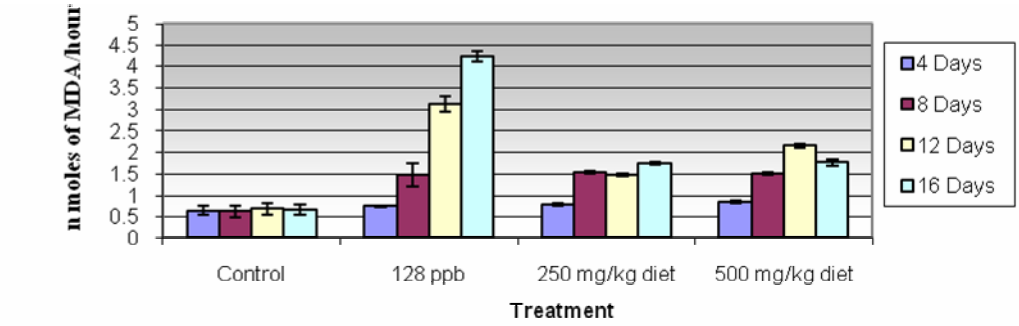


Fig.5.3.1.18. Variation in Lipid peroxidation levels in the gill of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.1.7 a). Changes in reduced glutathione level in tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation.

Tissue	Concentration	Duration of Exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	1748.7± 16.488	1749± 33.130	1750.8± 20.990	1749.5± 33.279
	128 ppb (copper intoxicated)	1324.5± 1.5166	1318.8± 1.1691	1251.7± 0.8165	1229.2± 1.8349
	250 mg vit C/kg diet	1375.2± 1.4720	1386± 1.2649	1212.3± 3.7771	1169.3± 3.7771
	500 mg vit C/kg diet	1415.5± 2.2583	1476.2± 3.1885	1225.2± 3.2506	1186.3± 2.8048
Kidney	Control	1016.8± 1.7568	1017.4± 1.3545	1016.7± 1.3129	1017± 1.0540
	128 ppb (copper intoxicated)	927.2± 2.0412	913.2± 1.9408	914.2± 2.4833	913.5± 1.0488
	250 mg vit C/kg diet	925.8± 4.3551	933.7± 6.7429	937.7± 7.6070	941.8± 2.6394
	500 mg vit C/kg diet	943.0± 6.9857	952.8± 2.3166	956.7± 7.6333	957.5± 6.7157
Gills	Control	1412.5± 0.7838	1412.6± 0.9663	1412.7± 0.8704	1412.4± 0.9934
	128 ppb (copper intoxicated)	1133.8± 0.7528	1139± 1.2649	1074.7± 0.5164	1084.7± 1.0328
	250 mg vit C/kg diet	1144.8± 3.5450	1147.3± 3.9833	1116.8± 2.9269	1080.5± 6.6558
	500 mg vit C/kg diet	1151.7± 3.6148	1168.2± 1.3292	1149.3± 5.0067	1114± 3.2863

Table 5.3.1.7 b). ANOVA for in reduced glutathione level in tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	4.267	2	2.133	658.116	.028
Duration	.007	3	.002	.737	.532
Concentration	.510	1	.510	157.232	.001
Error	.444	137	.003		
Total	5.228	143			

Table 5.3.1.7 c). ANOVA for reduced glutathione level in the tissues of copper intoxicated *Oreochromis mossambicus* after vitamin C supplementation (comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	6.655	2	3.328	2180.501	.012
Day	.034	3	.011	7.488	.001
Concentration	.361	1	.361	236.325	.001
Error	.209	137	.002		
Total	7.259	143			

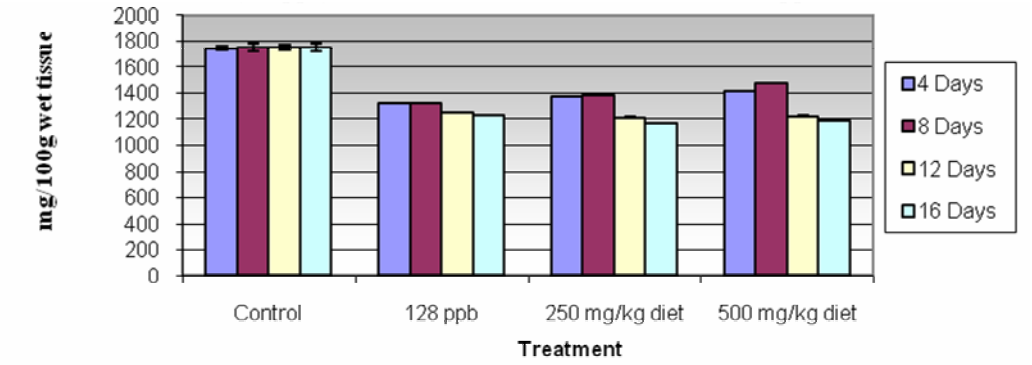


Fig.5.3.1.19. Variation in Reduced glutathione in the liver of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

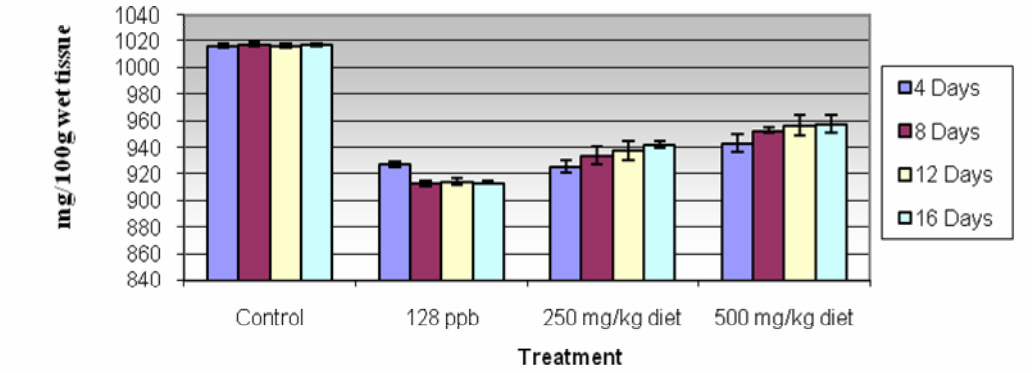


Fig.5.3.1.20. Variation in Reduced glutathione in the kidney of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

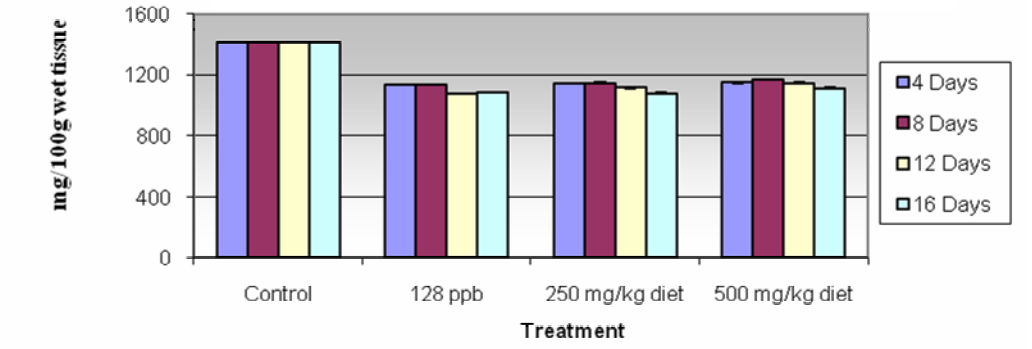


Fig.5.3.1.21. Variation in Reduced glutathione in the gill of copper intoxicated (128 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.1.8. Multiple Comparison Test for Enzyme Activities in Vitamin C supplemented *Oreochromis mossambicus* (comparison with control group).

	Groups	Catalase	Superoxide dismutase	Glutathione peroxidase	Glutathione S transferase	Glutathione reductase	Lipid peroxidation	Reduced glutathione
Tissue	Liver & Kidney	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.699 [d]	0.000 [a]
	Liver & Gills	0.901 [d]	0.116 [d]	0.035 [c]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	Kidney & Gills	0.000 [a]	0.000 [a]	0.000 [a]	0.807 [d]	0.000 [a]	0.000 [a]	0.000 [a]
Day	4 Days & 8 Days	0.669 [d]	0.653 [d]	0.728 [d]	0.325 [d]	1.000 [d]	0.306 [d]	0.437 [d]
	4 Days & 12 Days	0.327 [d]	0.075 [d]	0.188 [d]	0.039 [c]	0.963 [d]	0.001 [a]	0.018 [c]
	4 Days & 16 Days	0.619 [d]	0.001 [a]	0.001 [a]	0.000 [a]	0.053 [d]	0.000 [a]	0.000 [a]
	8 Days & 12 Days	0.942 [d]	0.580 [d]	0.762 [d]	0.754 [d]	0.971 [d]	0.134 [d]	0.458 [d]
	8 Days & 16 Days	1.000 [d]	0.050 [c]	0.018 [c]	0.031 [c]	0.059 [d]	0.041 [c]	0.017 [c]
	12 Days & 16 Days	0.962 [d]	0.551 [d]	0.192 [d]	0.283 [d]	0.161 [d]	0.960 [d]	0.423 [d]
Concentration (mg Vit. C/kg diet)	Control & 250 mg	0.000 [a]	0.000 [a]	0.000 [a]	0.001 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	Control & 500 mg	0.000 [a]	0.002 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	250 mg & 500 mg	0.718 [d]	0.736 [d]	0.960 [d]	0.459 [d]	0.633 [d]	0.967 [d]	0.937 [d]

The values are significant at a=P<.001, b=P<.01 and c=P<.05 and not significant at d.

Table 5.3.1.9. Multiple Comparison Test for Enzyme Activities in Vitamin C supplemented *Oreochromis mossambicus* (comparison with copper intoxicated group).

	Groups	Catalase	Superoxide dismutase	Glutathione peroxidase	Glutathione S transferase	Glutathione reductase	Lipid peroxidation	Reduced glutathione
Tissue	Liver & Kidney	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.005 [b]	0.000 [a]
	Liver & Gills	0.346 [d]	0.230 [d]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	Kidney & Gills	0.000 [a]	0.000 [a]	0.000 [a]	0.995 [d]	0.000 [a]	0.000 [a]	0.000 [a]
Day	4 Days & 8 Days	0.103 [d]	0.419 [d]	0.861 [d]	0.000 [a]	0.854 [d]	0.062 [d]	0.798 [d]
	4 Days & 12 Days	0.020 [c]	0.054 [d]	0.003 [b]	0.000 [a]	0.546 [d]	0.000 [a]	0.963 [d]
	4 Days & 16 Days	0.006 [b]	0.003 [b]	0.000 [a]	0.000 [a]	0.026 [c]	0.000 [a]	0.487 [d]
	8 Days & 12 Days	0.920 [d]	0.730 [d]	0.035 [c]	0.000 [a]	0.149 [d]	0.000 [a]	0.973 [d]
	8 Days & 16 Days	0.723 [d]	0.175 [d]	0.000 [a]	0.000 [a]	0.002 [b]	0.000 [a]	0.957 [d]
	12 Days & 16 Days	0.977 [d]	0.740 [d]	0.001 [a]	0.000 [a]	0.430 [d]	0.000 [a]	0.783 [d]
Concentration (ppb Cu in water and mg Vit. C/kg diet)	128 ppb & 250 mg	0.000 [a]	0.297 [d]	0.000 [a]	0.002 [b]	0.000 [a]	0.000 [a]	0.573 [d]
	128 ppb & 500 mg	0.000 [a]	0.001 [a]	0.000 [a]	0.222 [d]	0.000 [a]	0.000 [a]	0.196 [d]
	250 mg & 500 mg	0.911 [d]	0.069 [d]	0.236 [d]	0.164 [d]	0.230 [d]	0.422 [d]	0.650 [d]

The values are significant at a=P<.001, b=P<.01 and c=P<.05 and not significant at d.

The p value of catalase activity in the case of different tissues, duration of exposure and the concentration of copper are less than 0.5. This indicates that the catalase activity vary significantly in the tissues as a function of duration and toxicity. Among the three tissues tested, gills showed maximum changes in catalase activity followed by liver and kidney. Between concentrations and duration, the effects were significant at lower concentrations in the case of kidney and liver. However, the change observed in gills was more or less comparable under all the concentrations employed (Fig. 5.3.1.1 to 5.3.1.3).

In the case of superoxide dismutase activity, the variations were significant. The actual changes in activity rates were found to remain more or less comparable. Further, the activity rates showed variations in the lowest concentration more significantly than in the higher concentration. There was a distinct reduction in the case of fishes given higher concentration of vitamin C (Fig. 5.3.1.4 to 5.3.1.6).

In the case of fishes intoxicated with copper, administration of vitamin C through diet, the glutathione peroxidase activity varied significantly with reference to tissue and duration. The enzyme activity was found to significantly vary in liver. The activity of this enzyme in gills was found to be less when administered with higher vitamin C levels (Fig. 5.3.1.9).

Glutathione S transferase activity was comparable to control animals where vitamin C was administered. The increase in the activity showed by the intoxicated animal tissues decreased considerably and became comparable to that of the control animals (Table 5.3.1.4 a & b and Fig. 5.3.1.10 to 5.3.1.12).

Glutathione reductase activity showed distinct variations between tissues both in response to toxicity and vitamin administration. Clear cut increase in activity was registered in the liver of the fish due to toxicity and this was found to reduce considerably after vitamin supplementation. Increased activity in kidney was found to remain unchanged during the period 4 to 12 days. However, slight reduction occurred after 16 days. The activity levels were very much above that of the control animals (Fig. 5.3.1.14). The enzyme did not depict any conceivable change in general although copper intoxication resulted in a sprout of activity after four days in the gills of the fishes. Largely, administration of vitamin C through diet did not show any clear-cut change in 250 mg /kg diet and 500 mg/ kg doses (Fig. 5.3.1.15).

Lipid peroxidation levels were clearly influenced by vitamin supplementation. The increased level peroxidation in liver, kidney and gills due to 128 ppb of copper was drastically reduced and was found to reach comparable levels with those of control animals (Fig. 5.3.1.16 to 5.3.1.18).

Reduced glutathione levels recorded in the liver of copper intoxicated fishes was found to increase only marginally, whereas decrease noticed in the kidney due to intoxication did not show any precise recovery after vitamin supplementation (Fig. 5.3.1.20).

In the case of gill tissue, the value of reduced glutathione was more or less comparable to copper intoxicated and vitamin administered fishes. These were always lower to those recorded in the gills of control animals (fig. 5.3.1.21).

Effects of Vitamin C treatment on copper induced histopathology

A study of microphotographs of the liver, kidney and gills both microscopically and by graphic methods showed very interesting features.

The liver of the fishes which suffered serious damage due to copper intoxication showed considerable recovery after maintaining the fishes in a diet containing vitamin C (Fig. 5.3.1.22, Table 5.3.1.10 and Fig. 5.3.1.23). This indicates that recovery is possible by the supply of vitamin C or the presence of the vitamin in the diet may considerably reduce the damage of the liver. On the other hand, the recovery from damage in the case of kidney was confined to only in reducing the rate of pycnosis. Hyalinisation of glomeruli, Bowman's spaces and tubules more or less remained the same after 16 days of vitamin supplementation (Fig. 5.3.1.24, 25 and Table 5.3.1.9). In the case of gills of the fishes, treated with vitamin C containing diet, there was considerable repair of the damages. The lamellar fusion, epithelial lifting, hyperplasia, squamous metaplasia and balloon degeneration underwent repair although there was not perceivable changes in the secondary lamellar curling (Fig. 5.3.26&27; Table4.31.9).

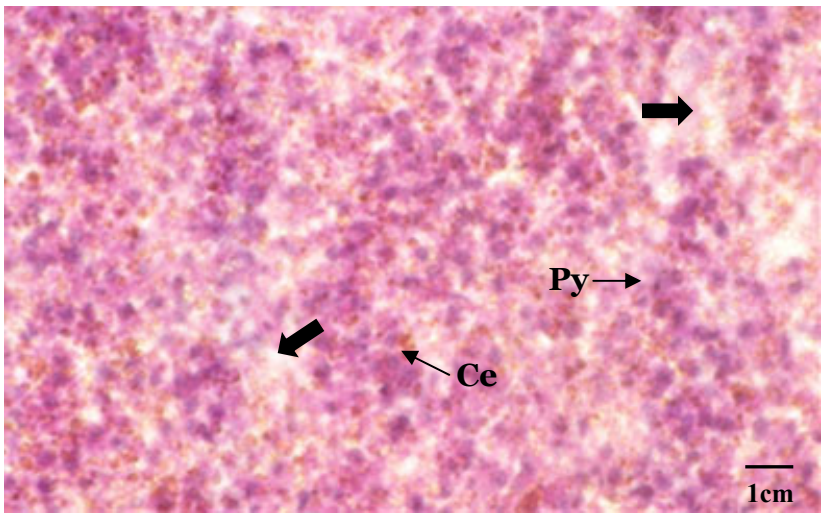


Fig. 5.3.1.22. Microphotograph of the liver of copper intoxicated *Oreochromis mossambicus* after supplementing with vitamin C (500 mg vit C/kg diet) depicting necrosis (block arrows), pycnotic nuclei (Py) and ceroid pigments (Ce). H & E, 20x.

Table 5.3.1.10. Histological changes in the liver of copper intoxicated *Oreochromis mossambicus* after supplementing with vitamin C (500 mg vit C/kg diet).

Treatment	Percentage damage	
	Pycnosis	**Necrosis**
Normal	0	0
16th day after copper exposure (128 ppb)	70.5	61
16th day after vitamin C supplementation.	24.68	20.51

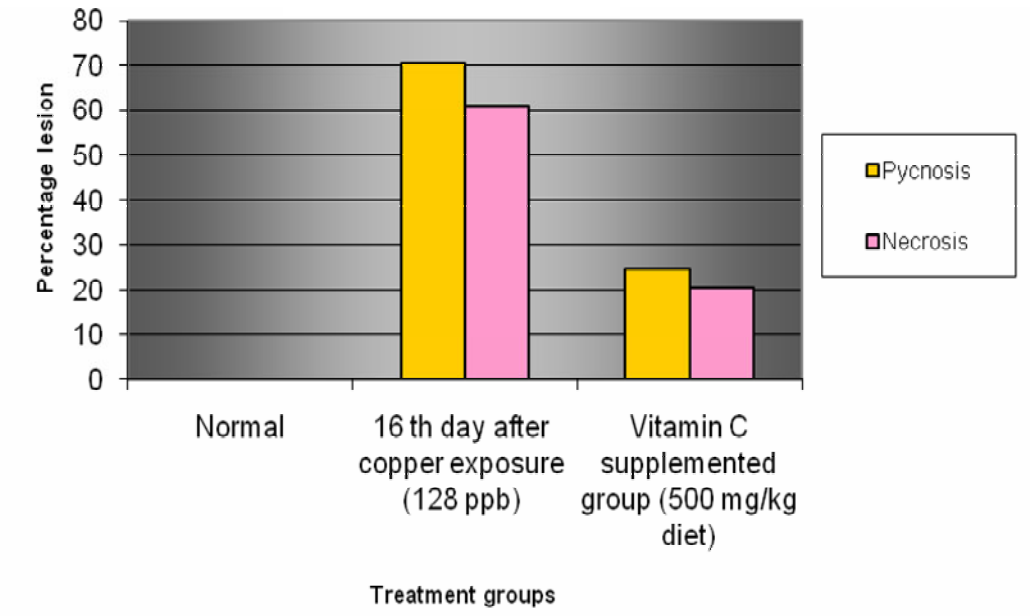


Fig. 5.3.1.23. Histological alterations in the liver of copper intoxicated Oreochromis mossambicus after vitamin C supplementation

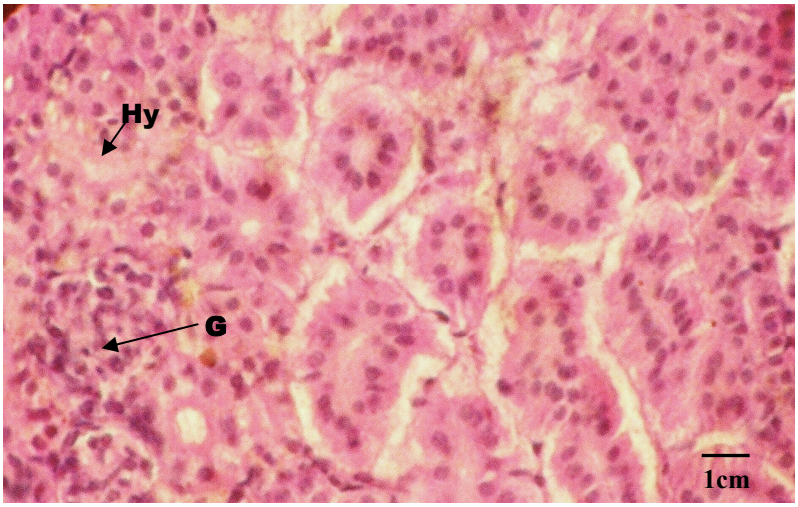


Fig. 5.3.1.24. Microphotograph of the kidney of copper intoxicated *Oreochromis mossambicus* after supplementing with vitamin C (500 mg vit C/kg diet) illustrating reduction in hyalinisation (Hy) of tubules and absence of tubular casts. The glomeruli (G) retain the swollen condition. H & E, 40x.

Table 5.3.1.11. Histological changes in the kidney of copper intoxicated *Oreochromis mossambicus* after supplementing with vitamin C (500 mg vit C/kg diet).

Treatment	Percentage damage				
	Hyalinisation	**Casts**	**Pycnosis**	**Desquamation**	**Number of swollen glomeruli and Bowman's capsules**
Normal	0.00	0.00	24.00	0.00	0.00
16th day after copper treatment.	66.70	9.30	83.60	10.00	3.00
16th day after vitamin C supplementation.	63.18	0.00	26.52	0.00	3.00

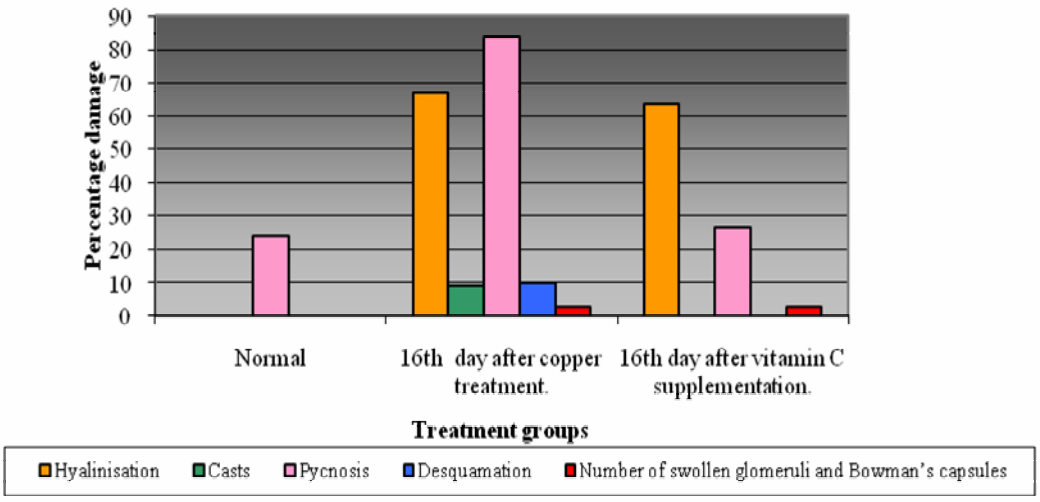


Fig. 5.3.1.25. Histological changes in the kidney of copper intoxicated *Oreochromis mossambicus* after supplementing with vitamin C

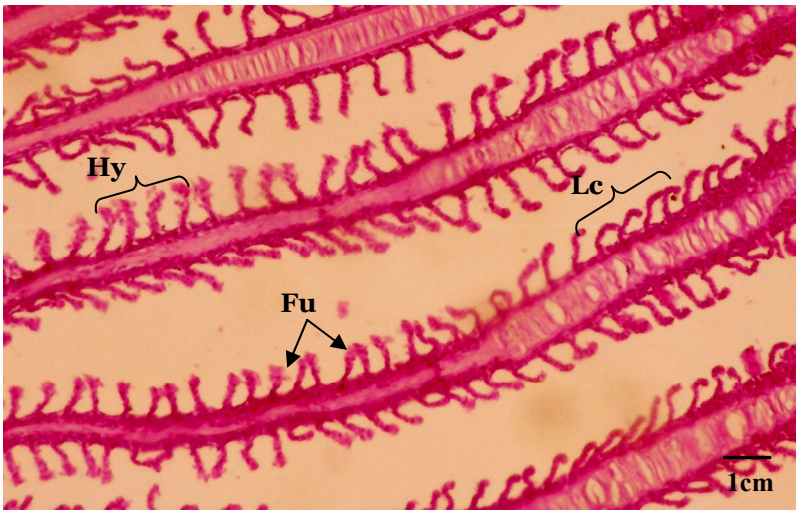


Fig. 5.3.1.26. Microphotograph of the gill of *Oreochromis mossambicus* (exposed to copper stress) after supplementing with vitamin C @ 500 mg vit C/kg diet. Considerable reduction in lamellar fusion (Fu) and hyperplasia (Hy) were apparent when compared to the group subjected to copper alone. There is slight reduction in secondary lamellar curling (Lc). H & E, 20x.

Table 4.3.1.12. Histopathology of Gill after exposure to 128 ppb of copper.

Treatment	Percentage damage					
	Lamellar curling	Lamellar fusion	Epithelial lifting	Hyperplasia	Squamous metaplasia	Balloon degeneration
Normal	0.00	0.00	0.00	1.50	0.00	0.00
16th day after copper treatment.	56.00	74.00	100.00	86.00	65.38	0.90
16th day after Vitamin C supplementation.	48.71	7.69	0.00	34.18	0.00	0.00

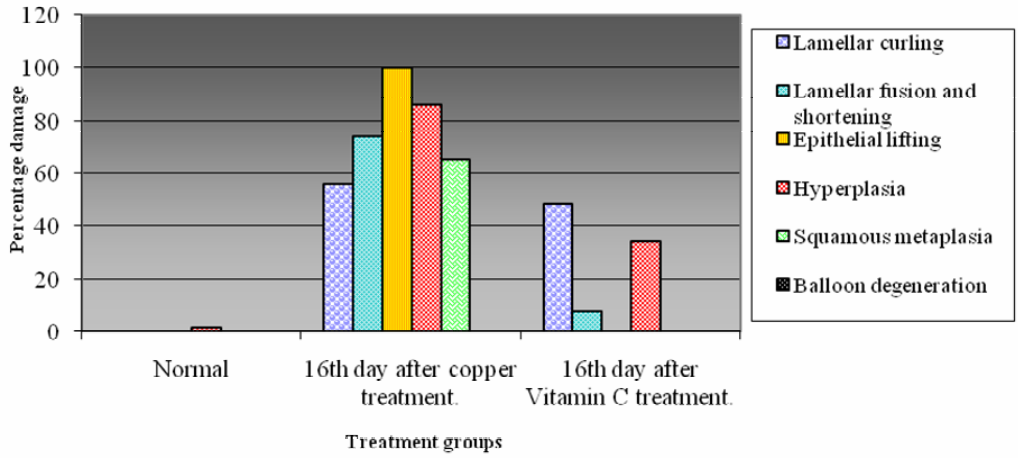


Fig. 5.3.1.27. Histolofical changes in the gills of copper intoxicated *Oreochromis mossambicus* after supplementing with vitamin C

5.3.2. Effect of Vitamin C on Malachite green toxicity.

Exposure to malachite green results in changes in the antioxidant machinery and histology of cardinal tissues of *Oreochromis mossambicus*. The influence of vitamin C administered through diet, its ameliorative role in controlling and repairing the toxic effects were studied and the results are presented here.

Presence of vitamin C was found to impart an increase, in general, the catalase activity in liver of the fishes intoxicated with malachite green. Duration dependent enhancement was observed in both the concentrations tested. In the case of kidney, there were changes, which indicated this enzyme's activity is increasing marginally and showed a trend of nearing the values recorded in the control animals. On the contrary, the changes in activity recorded in the gills in intoxicated and treated fishes were more or less comparable (Fig. 5.3.2.1 to 5.3.2.3).

With reference to superoxide dismutase, the activity was more in all the treatments when compared with that of the control animals' liver. Elevated activity was noticed after 12 and 16 days of supplementation with vitamin C through the diet (Fig. 5.3.2.4).

In the case of kidney, the declension in activity recorded during exposure to malachite green was found to reach levels above that of control animals after 12 and 16 days (Fig.5.3.2.5).

The gills reacted in a totally different fashion. Thus while the activity was high in the control animals, a reduction in activity occurred in the intoxicated and vitamin treated fishes. However, maintenance in the fortified diet from 12 to 16 days did not result in enhancement of activity although the values recorded were higher than those recorded for the intoxicated animals (Fig. 5.3.2.6).

Elevation of activity of glutathione peroxidase occurred both in the intoxicated and treated fishes in the case of liver. Continued treatment with fortified feed did not result in reducing the activity even after 16 days (Fig. 5.3.2.7).

In the kidney, the activity of this enzyme remained lower than that of the control after 16 days of treatment. However the organ of the animals

responded with considerably higher activity during the whole period of treatment in comparison with that of the fish exposed to 25 ppb of malachite green (Fig. 5.3.2.8).

Activity of glutathione peroxidase in the gills was found to be elevated than that of both control and malachite green exposed fishes from the very beginning of administration of vitamin fortified diet (Fig. 5.3.2.9).

Duration dependent declension in the activity of glutathione S transferase activity was noticed in the liver of *Oreochromis mossambicus.* The rate of reduction recorded after 12 and 16 days was very similar to levels that were noticed in the control animals after 16 days (Fig. 5.3.2.10).

An increase in the activity of the enzyme (glutathione S transferase) in the kidney soon after exposure (4 days) in malachite green exposed fishes kept with fortified food was found to decrease after 8 days. Although the activity was minimal after 16 days in the intoxicated fishes, this was found to be elevated in the kidneys of fishes that received both doses of vitamin fortified diet (Fig. 5.3.2.11).

No definite variation was recorded in the case of the activity of glutathione S transferase in both the vitamin supplemented group and the control animals which indicated signs of recovery (Fig. 5.3.2.12).

The liver of control fishes recorded lower glutathione reductase activity. The high activity shown in the intoxicated fishes during the early half was not depicted in the treated animals. The levels remained unchanged even after 16 days. These levels were higher than those recorded both in the control and the intoxicated fishes. The picture derived from the kidney samplings was different. Time dependent

reduction in activity occurred in both the sets of treated fish. Very low level in activity was reached in both the groups after 16 days (Fig. 5.3.2.14). In the case of gills the activity was high in fishes maintained with fortified food. A reduction in activity was recorded after 16 days in these groups and the levels were comparable to those recorded for the control animals (Fig. 5.3.2.15).

The level of lipid peroxidation in the liver of vitamin supplemented group of fishes approached those of the control animals (Fig. 5.3.2.16).Treatment with vitamin fortified diet did not clearly alter lipid peroxidation in the kidney of the fishes. The levels recorded were similar to those obtained for the malachite green exposed fishes. The levels in the intoxicated and treated fishes were much above those recorded for the control animals. It is clear that the assessed level of lipid peroxidation remained unchanged even on administration of fortified food (Fig. 5.3.2.17).

Higher level of lipid peroxidation resulted from exposure to malachite green was found to decline in the gills of fishes supplied with fortified food (Fig. 5.3.2.18).

The reduction in the reduced glutathione levels due to toxicity of malachite green in the liver of intoxicated fish was slightly elevated when the fish were provided with fortified diet. However, the level reached were far below those recorded in control animals (Fig. 5.3.2.19).

The level of reduced glutathione in kidney was higher than that recorded for the intoxicated fish. Vitamin C evidently increased the level of GSH in kidney of *Oreochromis mossambicus* (Fig. 5.3.2.20).

Maintaining in supplementary diet supplied condition did not considerably alter the GSH levels in the gills of the fishes (Fig. 5.3. 2.21).

Table 5.3.2.1 a). Changes in the activity of catalase in tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation.

Tissue	Concentration	Duration of exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	9.688± 0.4401	9.653± 0.6755	9.690± 0.7740	9.682± 0.4237
	25 ppb (Malachite green stressed)	13.74± 0.2466	14.18± 0.1086	18.23± 0.1176	11.22± 0.1769
	250 mg vit C/kg diet	15.02± 0.0882	15.27± 0.0331	17.20± 0.0685	17.34± 0.0578
	500 mg vit C/kg diet	15.02± 0.1545	15.65± 0.0606	18.34± 0.0937	17.94± 0.1515
Kidney	Control	2.967± 0.5075	2.913± 0.6369	2.910± 0.3378	2.940± 0.3457
	25 ppb (Malachite green stressed)	2.392± 0.0717	2.130± 0.0228	2.018± 0.0467	1.692± 0.0739
	250 mg vit C/kg diet	2.290± 0.0544	2.393± 0.0234	2.032± 0.0440	3.102± 0.0264
	500 mg vit C/kg diet	2.352± 0.0337	2.318± 0.0286	2.137± 0.0266	3.162± 0.0349
Gills	Control	15.01± 0.9889	14.97± 0.5447	15.09± 0.6228	14.99± 0.6446
	25 ppb (Malachite green stressed)	17.46± 0.0194	20.94± 0.0718	17.12± 0.2845	13.34± 0.1275
	250 mg vit C/kg diet	16.90± 0.0853	18.31± 0.1532	18.62± 0.0665	14.05± 0.1027
	500 mg vit C/kg diet	17.31± 0.0647	18.31± 0.0900	18.52± 0.6375	13.96± 0.0674

Table 5.3.2.1 b). ANOVA for changes in catalase activity in tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	9579.063	2	4789.532	2024.794	.001
Duration	129.978	3	43.326	18.316	.000
Concentration	30.307	2	15.153	6.406	.002
Error	492.012	208	2.365		
Total	10231.360	215			

Table 5.3.2.1 c). ANOVA for catalase activity in the tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation. (Comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	7838.103	2	3919.051	1201.779	.001
Day	26.426	3	8.809	2.701	.047
Concentration	370.326	2	185.163	56.780	.001
Error	678.297	208	3.261		
Total	8913.152	215			

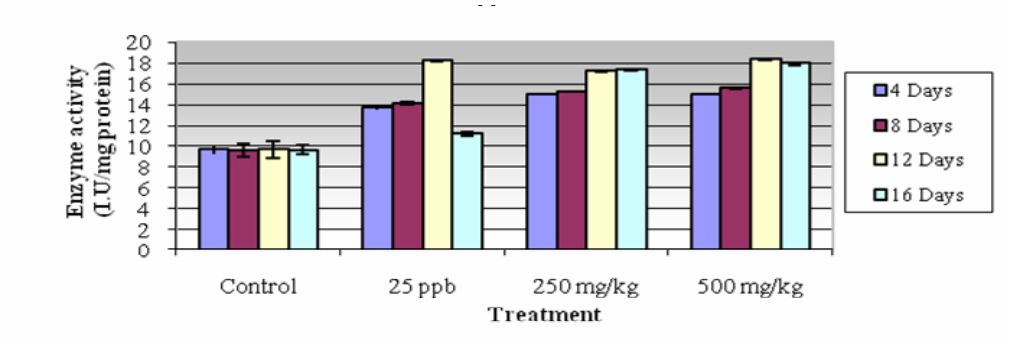


Fig 5.3.2.1. Variation in catalase activity in the liver of Malachite green intoxicated *Oreochromis mossambicus* (25 ppb) after vitamin C supplementation

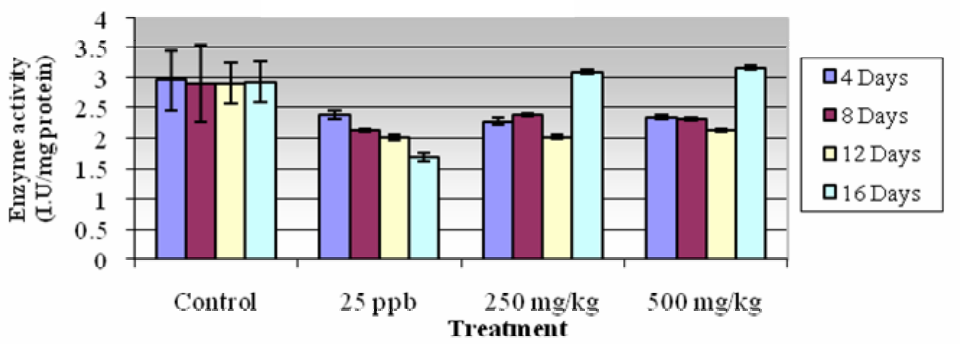


Fig 5.3.2.2. Variation in catalase activity in the kidney of Malachite green intoxicated *Oreochromis mossambicus* (25 ppb) after vitamin C supplementation

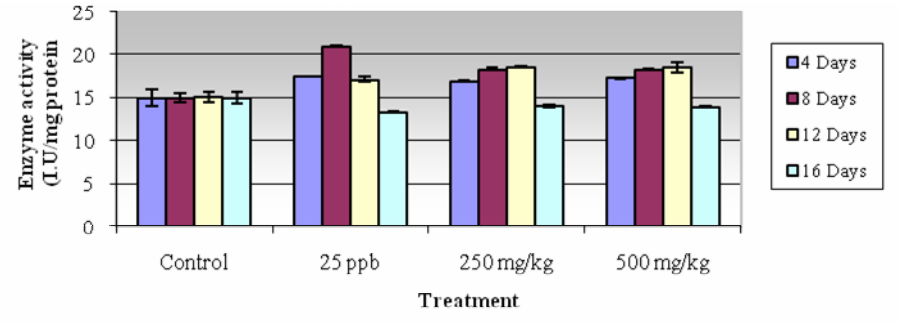


Fig 5.3.2.3. Variation in catalase activity in the gills of Malachite green intoxicated *Oreochromis mossambicus* (25 ppb) after vitamin C supplementation

Table 5.3.2.2 a). Changes in the activity of superoxide dismutase in tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation.

Tissue	Concentration	Duration of exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	0.980± 0.2118	0.982± 0.2922	0.937± 0.0817	0.952± 0.2020
	25 ppb (Malachite green stressed)	1.320± 0.0261	4.123± 0.0362	9.332± 0.0496	3.048± 0.0656
	250 mg vit C/kg diet	1.253± 0.0327	2.135± 1.6090	5.167± 0.0388	5.155± 0.0485
	500 mg vit C/kg diet	1.610± 0.0562	3.735± 0.0187	4.620± 0.0228	5.150± 0.1882
Kidney	Control	0.323± 0.0582	0.347± 0.0582	0.308± 0.0739	0.330± 0.0901
	25 ppb (Malachite green stressed)	0.042± 0.0041	0.220± 0.3234	0.040± 0.0063	0.310± 0.0219
	250 mg vit C/kg diet	0.085± 0.0084	0.227± 0.0052	0.433± 0.0052	0.440± 0.0110
	500 mg vit C/kg diet	0.132± 0.0041	0.142± 0.0041	0.425± 0.0055	0.463± 0.0314
Gills	Control	1.023± 0.3239	1.058± 0.2899	1.097± 0.3444	1.080± 0.4071
	25 ppb (Malachite green stressed)	0.692± 0.0564	0.317± 0.0234	0.223± 0.0103	0.120± 0.0237
	250 mg vit C/kg diet	0.777± 0.0516	0.638± 0.0223	0.415± 0.0055	0.422± 0.0075
	500 mg vit C/kg diet	0.783± 0.0052	0.647± 0.0225	0.433± 0.0103	0.430± 0.0089

Table 5.3.2.2 b). ANOVA for changes in superoxide dismutase activity in tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	596.358	2	298.179	214.213	.021
Duration	72.825	3	24.275	17.439	.001
Concentration	1.746	2	.873	.627	.535
Error	289.531	208	1.392		
Total	960.460	215			

Table 5.3.2.2 c). ANOVA for superoxide dismutase activity in the tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation. (Comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	239.754	2	119.877	121.544	.024
Day	24.587	3	8.196	8.310	.001
Concentration	24.260	2	12.130	12.299	.001
Error	205.146	208	.986		
Total	493.747	215			

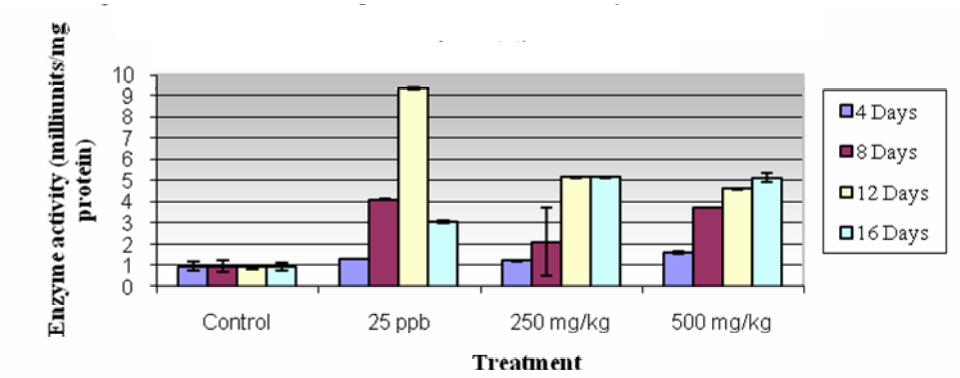


Fig. 5.3.2.4 Variation in superoxide dismutase activity in the liver of Malachite green intoxicated *Oreochromis mossambicus* (25ppb) after vitamin C supplementaion

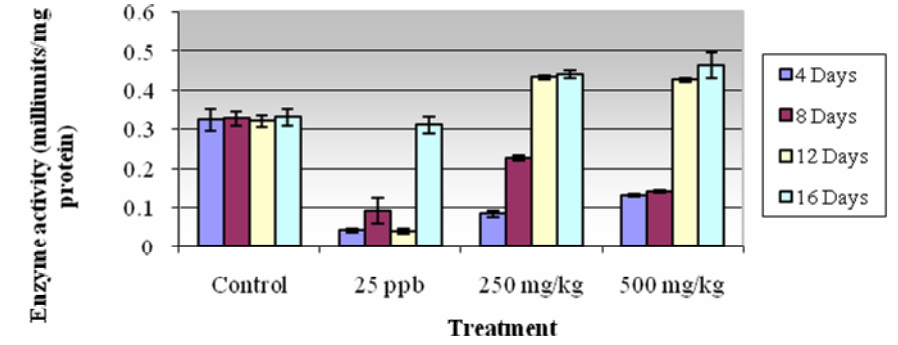


Fig. 5.3.2.5 Variation in superoxide dismutase activity in the kidney of Malachite green intoxicated *Oreochromis mossambicus* (25ppb) after vitamin C supplementaion

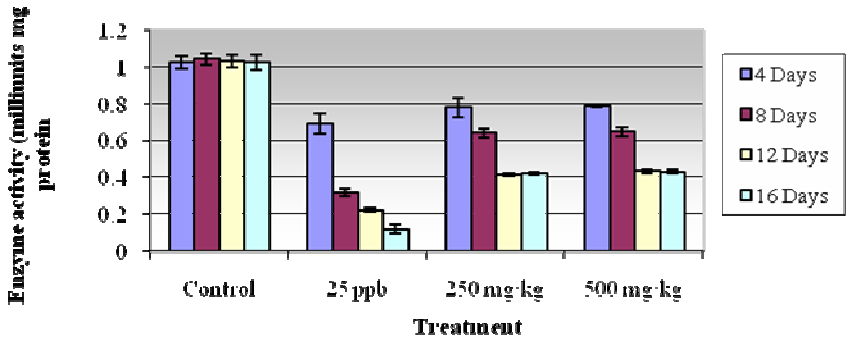


Fig. 5.3.2.5 Variation in superoxide dismutase activity in the gills of Malachite green intoxicated *Oreochromis mossambicus* (25ppb) after vitamin C supplementaion

Table 5.3.2.3 a). Changes in the activity of glutathione peroxidase in tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation.

Tissue	Concentration	Duration of exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	7.572± 0.5428	7.635± 0.5622	7.650± 0.5673	7.663± 0.4117
	25 ppb (Malachite green stressed)	15.65± 0.3792	18.19± 0.2282	23.26± 0.4298	16.05± 0.1660
	250 mg vit C/kg diet	15.80± 0.1011	18.22± 0.1717	14.34± 0.0892	14.64± 0.0716
	500 mg vit C/kg diet	14.96± 0.1401	17.97± 0.1443	13.99± 0.3565	13.82± 0.5570
Kidney	Control	4.145± 0.6195	4.112± 0.7795	4.138± 0.4838	4.157± 0.7241
	25 ppb (Malachite green stressed)	2.112± 0.0436	1.185± 0.0599	1.878± 0.0387	0.908± 0.0417
	250 mg vit C/kg diet	3.688± 0.0870	2.810± 0.1097	3.315± 0.0327	3.128± 0.0564
	500 mg vit C/kg diet	3.795± 0.0373	3.495± 0.0869	3.452± 0.2580	3.368± 0.0920
Gills	Control	12.26± 0.8823	12.23± 0.8324	12.19± 0.3242	12.23± 0.4331
	25 ppb (Malachite green stressed)	11.62± 0.1231	10.11± 0.1056	11.30± 0.1089	9.097± 0.0520
	250 mg vit C/kg diet	12.16± 0.0598	13.11± 0.0573	13.16± 0.0927	12.67± 0.0645
	500 mg vit C/kg diet	12.46± 0.0540	13.27± 0.0424	12.74± 0.0683	12.78± 0.0985

Table 5.3.2.3 b). ANOVA for changes in glutathione peroxidase activity in tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	6992.529	2	3496.265	1237.665	.001
Duration	60.059	3	20.020	7.087	.000
Concentration	9.320	2	4.660	1.650	.195
Error	587.577	208	2.825		
Total	7649.485	215			

Table 5.3.2.3 c). ANOVA for glutathione peroxidase activity in the tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation. (Comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	3973.855	2	1986.927	505.062	.000
Day	29.704	3	9.901	2.517	.059
Concentration	312.033	2	156.017	39.658	.025
Error	818.278	208	3.934		
Total	5133.870	215			

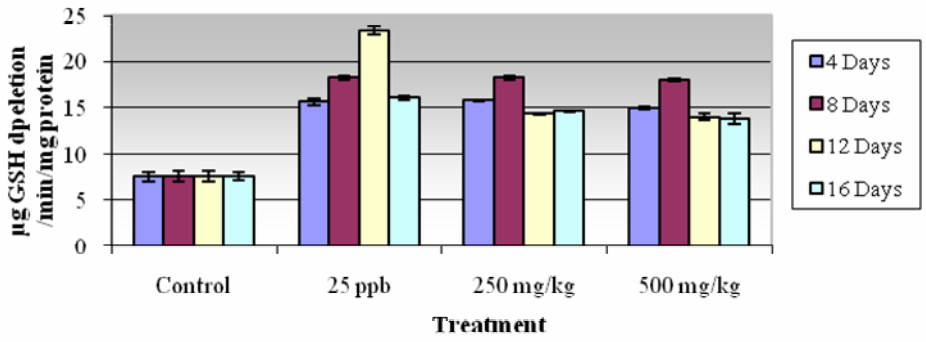


Fig. 5.3.2.7. Variation in glutathione peroxidase activity in the liver of Malachite green intodicated (25 ppb) *Oreochromis mossambicus* after vitamin C supplementation

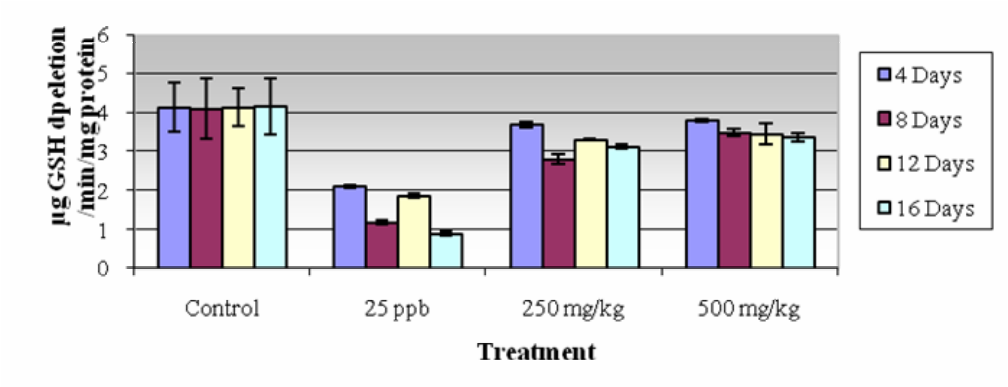


Fig. 5.3.2.8. Variation in glutathione peroxidase activity in the kindney of Malachite green intodicated (25 ppb) *Oreochromis mossambicus* after vitamin C supplementation

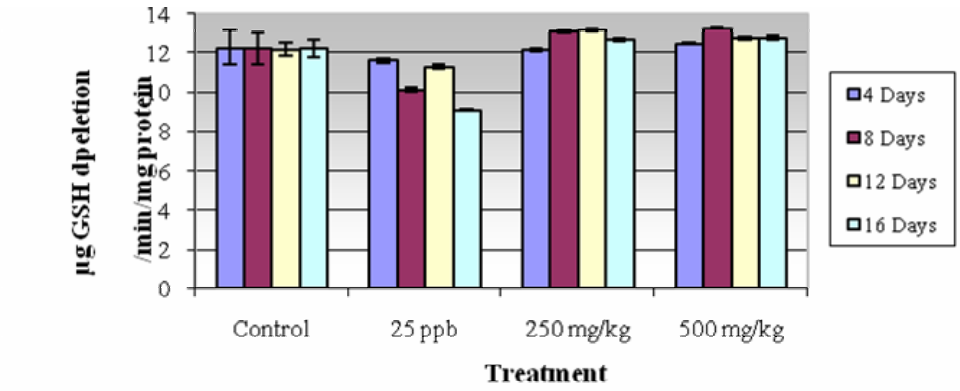


Fig. 5.3.2.9. Variation in glutathione peroxidase activity in the gills of Malachite green intodicated (25 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.2.4 a). Changes in the activity of glutathione S transferase in tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation.

Tissue	Concentration	Duration of exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	22.52± 0.6035	22.67± 1.4309	22.72± 1.4762	22.69± 2.0945
	25 ppb (Malachite green stressed)	40.90± 0.5224	35.36± 0.4862	27.39± 0.3984	20.71± 0.2354
	250 mg vit C/kg diet	36.99± 0.3969	35.11± 0.2190	26.07± 0.1595	22.79± 0.1555
	500 mg vit C/kg diet	36.96± 0.3661	35.98± 0.2402	25.54± 0.1668	22.82± 0.0864
Kidney	Control	12.43± 1.0201	12.45± 0.9576	13.07± 1.2525	12.47± 1.0062
	25 ppb (Malachite green stressed)	13.97± 0.3090	10.91± 0.2313	7.485± 0.1310	2.425± 0.0709
	250 mg vit C/kg diet	15.08± 0.1889	11.19± 0.1650	8.500± 0.0724	7.637± 0.3893
	500 mg vit C/kg diet	14.92± 0.3126	11.75± 0.1760	8.132± 0.2194	7.805± 0.1371
Gills	Control	14.75± 0.7467	14.79± 0.4351	14.77± 0.6252	14.76± 0.5501
	25 ppb (Malachite green stressed)	15.48± 0.1323	17.98± 0.2349	11.44± 0.1143	9.903± 0.3084
	250 mg vit C/kg diet	14.84± 0.4564	18.43± 0.4696	15.25± 0.1223	15.27± 0.1548
	500 mg vit C/kg diet	15.50± 0.5057	17.93± 0.2200	15.38± 0.0750	15.28± 0.2561

Table 5.3.2.4 b). ANOVA for changes in glutathione S transferase activity in tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	16447.657	2	8223.829	1132.944	.001
Duration	2967.902	3	989.301	136.290	.001
Concentration	61.793	2	30.896	4.256	.015
Error	1509.833	208	7.259		
Total	20987.185	215			

Table 5.3.2.4 c). ANOVA for glutathione S transferase activity in the tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation. (Comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	10484.218	2	5242.109	496.071	.010
Day	921.846	3	307.282	29.079	.005
Concentration	252.123	2	126.061	11.929	.001
Error	2197.989	208	10.567		
Total	13856.176	215			

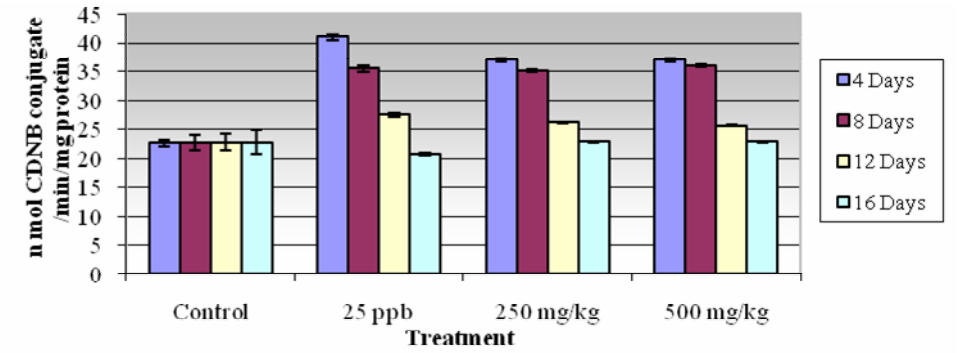


Fig. 5.3.2.10. Variation in glutathione S transferase activity in the liver of Malachite green intoxicated (25ppb) *Oreochromis mossambicus* after vitamin C supplementation

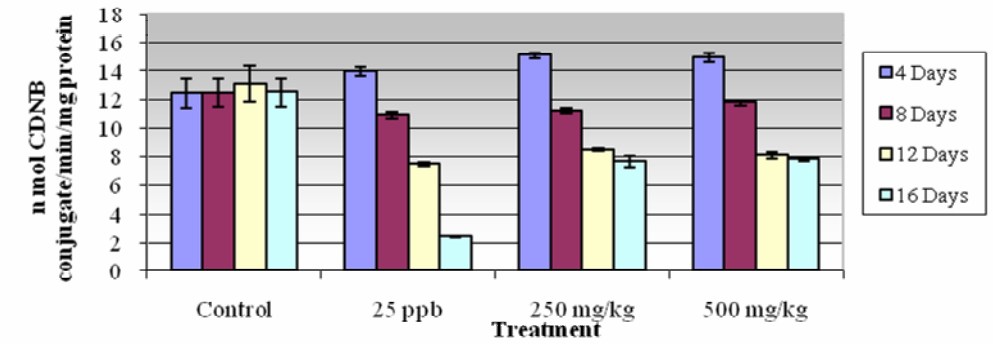


Fig. 5.3.2.11. Variation in glutathione S transferase activity in the kidney of Malachite green intoxicated (25ppb) *Oreochromis mossambicus* after vitamin C supplementation

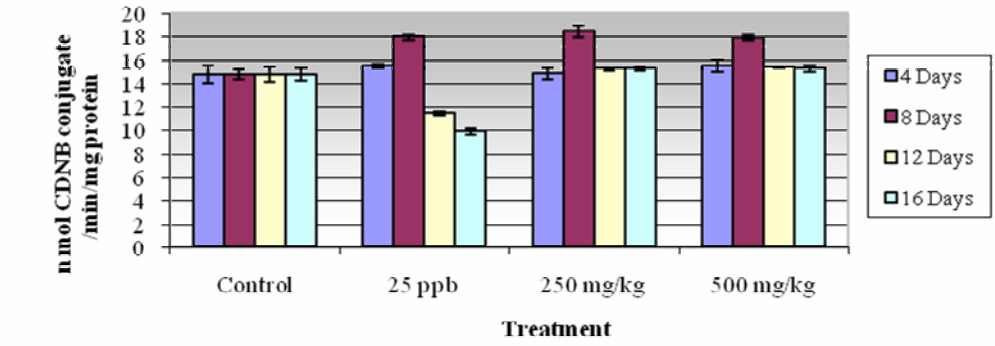


Fig. 5.3.2.11. Variation in glutathione S transferase activity in the gills of Malachite green intoxicated (25ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.2.5 a). Changes in the activity of glutathione reductase in tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation.

Tissue	Concentration	Duration of exposure			
		4 Days	**8 Days**	**12 Days**	**16 Days**
Liver	**Control**	4.067± 0.8254	4.240± 0.5645	4.063± 0.6369	4.165± 0.3137
	25 ppb (Malachite green stressed)	9.677± 0.2774	13.27± 0.2878	6.170± 0.1135	2.223± 0.1737
	250 mg vit C/kg diet	6.410± 0.0452	6.535± 0.3035	6.360± 0.0663	6.508± 0.0884
	500 mg vit C/kg diet	6.375± 0.1045	6.753± 0.2457	6.442± 0.0833	6.527± 0.0441
Kidney	**Control**	2.452± 0.7743	2.513± 0.6170	2.480± 0.5016	2.467± 0.6863
	25 ppb (Malachite green stressed)	3.247± 0.2202	1.647± 0.0771	0.435± 0.0327	0.098± 0.0075
	250 mg vit C/kg diet	3.342± 0.0991	2.180± 0.0400	1.247± 0.0782	0.633± 0.0683
	500 mg vit C/kg diet	3.448± 0.0637	2.362± 0.0483	1.482± 0.0431	0.640± 0.0429
Gills	**Control**	2.675± 0.4622	2.645± 0.6197	2.632± 0.4567	2.685± 0.4805
	25 ppb (Malachite green stressed)	7.390± 0.1035	8.347± 2.0438	3.762± 1.0193	1.450± 0.3574
	250 mg vit C/kg diet	6.805± 0.0864	5.025± 0.0769	3.445± 0.0715	3.165± 0.0356
	500 mg vit C/kg diet	7.367± 0.1127	4.940± 0.1677	3.533± 0.1419	3.455± 0.0809

Table 5.3.2.5 b). ANOVA for changes in glutathione reductase activity in tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	991.068	2	495.534	232.156	.001
Duration	401.985	3	133.995	62.776	.001
Concentration	9.793	2	4.897	2.294	.103
Error	443.973	208	2.134		
Total	1846.819	215			

Table 5.3.2.5 c). ANOVA for glutathione reductase activity in the tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation. (Comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	467.337	2	233.668	241.828	.005
Day	66.935	3	22.312	23.091	.001
Concentration	79.809	2	39.905	41.298	.022
Error	200.981	208	.966		
Total	815.062	215			

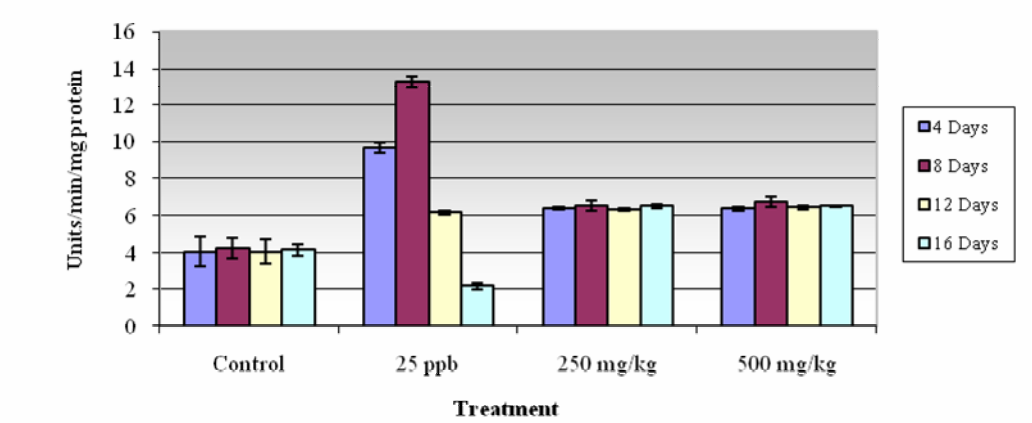


Fig. 5.3.2.13. Variation in glutathionereductase in the liver of Malachite green intoxicated (25 ppb) *Oreochromis mossambicus* after vitamin C supplementation

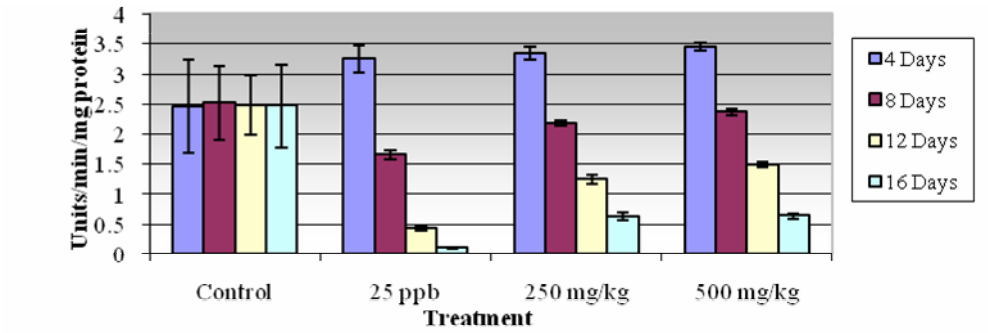


Fig. 5.3.2.14. Variation in glutathionereductase in the kidney of Malachite green intoxicated (25 ppb) *Oreochromis mossambicus* after vitamin C supplementation

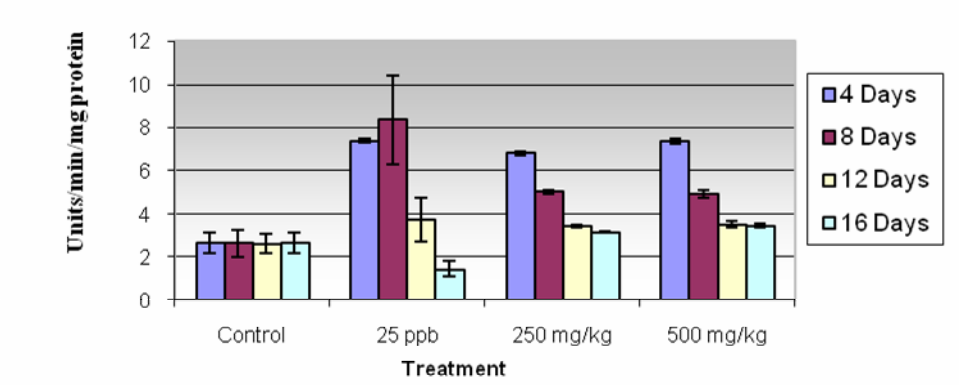


Fig. 5.3.2.15. Variation in glutathionereductase in the gills of Malachite green intoxicated (25 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.2.6 a). Changes in lipid peroxidation in tissues of malachite green affected *Oreochromis* mossambicus after vitamin C supplementation.

Tissue	Concentration	Duration of exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	0.922± 0.2633	0.868± 0.1483	0.915± 0.2389	0.865± 0.0846
	25 ppb (Malachite green stressed)	0.918± 0.0075	1.153± 0.0242	3.632± 0.0809	7.112± 0.3265
	250 mg vit C/kg diet	1.013± 0.1169	0.895± 0.0718	1.143± 0.1226	1.268± 0.0789
	500 mg vit C/kg diet	1.022± 0.1419	0.922± 0.1184	1.203± 0.0878	1.273± 0.0882
Kidney	Control	0.362± 0.0500	0.347± 0.0766	0.348± 0.0778	0.340± 0.1002
	25 ppb (Malachite green stressed)	0.948± 0.0271	2.278± 0.0880	4.583± 0.2545	5.185± 0.0476
	250 mg vit C/kg diet	1.098± 0.0637	1.777± 0.0674	3.997± 0.1266	4.620± 0.0934
	500 mg vit C/kg diet	1.252± 0.1826	1.783± 0.1203	3.528± 0.3102	4.662± 0.1146
Gills	Control	1.030± 0.1318	0.983± 0.2033	1.003± 0.2438	1.057± 0.2790
	25 ppb (Malachite green stressed)	4.430± 0.0645	12.52± 0.1429	19.26± 0.4062	19.36± 0.0634
	250 mg vit C/kg diet	4.677± 0.1159	6.923± 0.2213	7.208± 0.2370	4.463± 0.1063
	500 mg vit C/kg diet	4.338± 0.4178	6.668± 0.2676	7.507± 0.1496	4.318± 0.1614

Table 5.3.2.6 b). ANOVA for lipid peroxidation in tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation. (comparison with high concentration of the toxicant).

Source	Sum of Squares	Df	Mean Square	F	P-value
Tissue	1828.059	2	914.029	141.203	.005
Duration	489.061	3	163.020	25.184	.001
Concentration	604.823	2	302.411	46.718	.001
Error	1346.419	208	6.473		
Total	4268.362	215			

Table 5.3.2.6 c). ANOVA for lipid peroxidation in the tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation. (Comparison with control).

Source	Sum of Squares	Df	Mean Square	F	P-value
Tissue	375.417	2	187.708	125.486	.001
Day	42.688	3	14.229	9.513	.001
Concentration	294.915	2	147.457	98.578	.025
Error	311.136	208	1.496		
Total	1024.156	215			

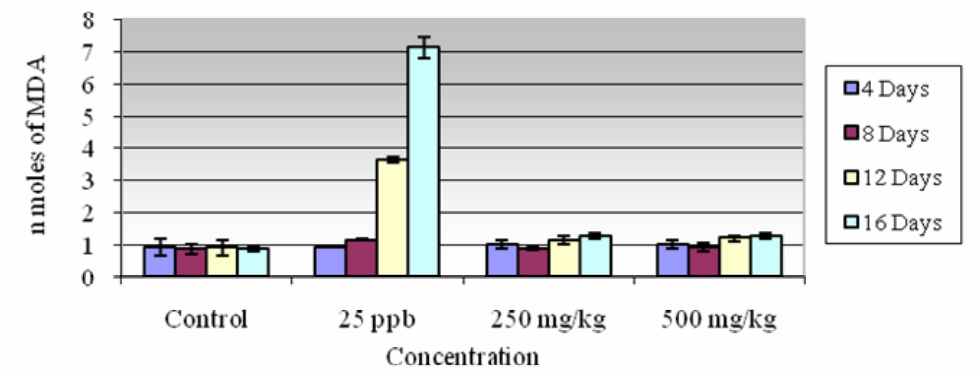


Fig. 5.3.2.16. Variation in lipid peroxidation in the liver of Malachite green intoxicated (25 ppb) *Oreochromis mossambicus* after vitamin C supplementation

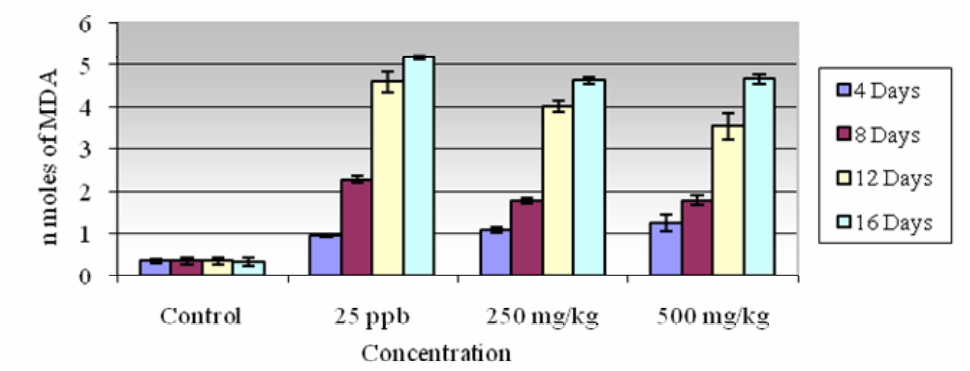


Fig. 5.3.2.17. Variation in lipid peroxidation in the kidney of Malachite green intoxicated

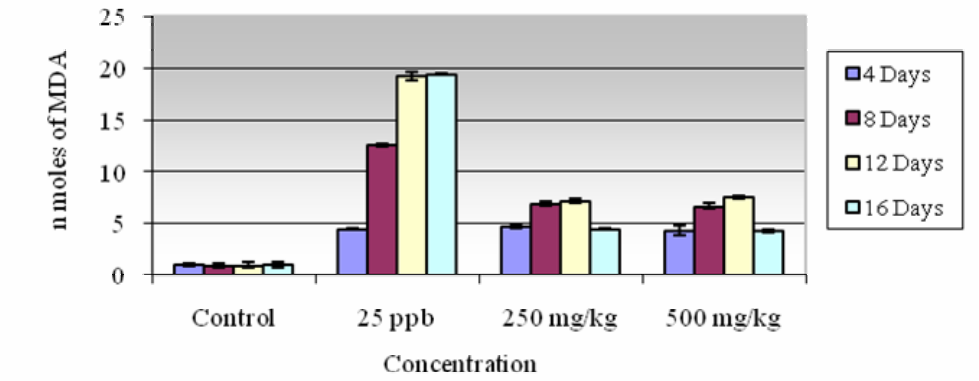


Fig. 5.3.2.18. Variation in lipid peroxidation in the gills of Malachite green intoxicated (25 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.2.7 a) Changes in reduced glutathione content in tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation.

Tissue	Concentration	Duration of exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	1748± 9.7193	1746.7± 14.445	1746.8± 14.035	1748± 12.116
	25 ppb (Malachite green stressed)	1213.7± 1.0328	964.6± 0.6802	937.5± 1.0078	670.9± 0.2535
	250 mg vit C/kg diet	1166.5± 47.086	974.2± 6.5549	915.5± 20.695	1073.2± 63.745
	500 mg vit C/kg diet	1175.2± 90.319	974.3± 14.081	1056.5± 55.028	1124.5± 70.149
Kidney	Control	1018.9± 1.7286	1018.4± 3.3752	1018.9± 2.2228	1018.9± 2.2871
	25 ppb (Malachite green stressed)	1018.9± 0.4454	1074.1± 0.2101	975.2± 0.2422	969.0± 0.2637
	250 mg vit C/kg diet	1112.4± 21.474	1089.6± 59.708	1051.2± 61.246	984.6± 21.143
	500 mg vit C/kg diet	1081± 76.149	1082.6± 51.832	1039.8± 48.462	1018.8± 11.273
Gills	Control	1313.2± 93.681	1313.2± 55.982	1325.6± 65.789	1312.9± 63.980
	25 ppb (Malachite green stressed)	1212.9± 0.5288	1199.4± 1.4460	1027.9± 0.8271	1025.8± 1.1465
	250 mg vit C/kg diet	1322.9± 34.454	1066± 64.267	1041.5± 63.858	1299.7± 61.360
	500 mg vit C/kg diet	1296.2± 37.430	1003.8± 65.242	991.8± 55.460	1219.3± 107.22

Table 5.3.2.7 b). ANOVA for changes in reduced glutathione content in tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	.538	2	.269	28.749	.003
Duration	.793	3	.264	28.272	.023
Concentration	.220	2	.110	11.746	.000
Error	1.945	208	.009		
Total	3.496	215			

Table 5.3.2.7 c). ANOVA for reduced glutathione in the tissues of malachite green affected *Oreochromis mossambicus* after vitamin C supplementation. (Comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	1.261	2	.630	37.327	.015
Day	.385	3	.128	7.590	.001
Concentration	1.975	2	.988	58.476	.001
Error	3.513	208	.017		
Total	7.134	215			

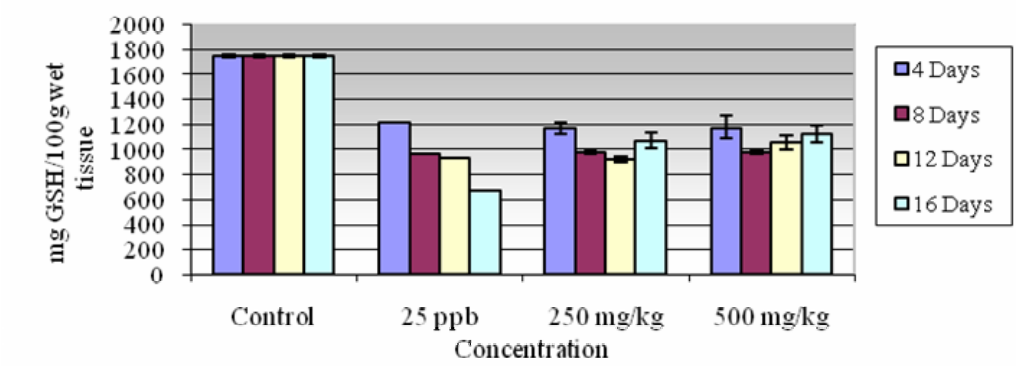


Fig. 5.3.2.19. Variation reduced glutathionelevel in the liver of Malachite green intoxicated (25 ppb) *Oreochromis mossambicus* after vitamin C supplementation

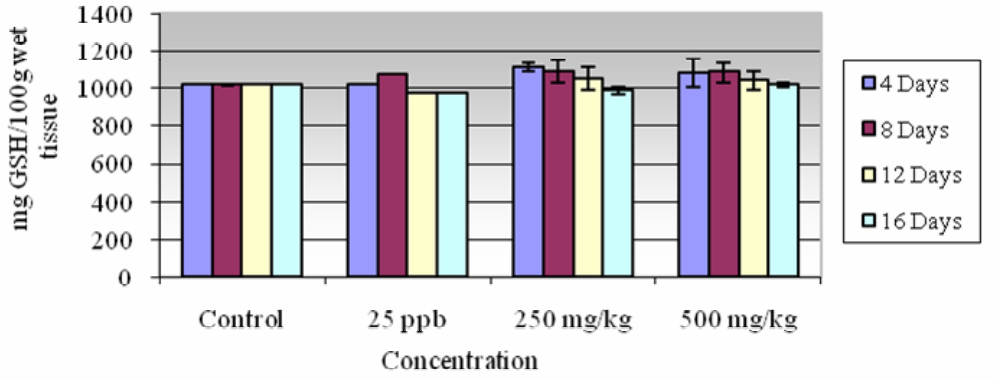


Fig. 5.3.2.20. Variation reduced glutathionelevel in the kidney of Malachite green intoxicated (25 ppb) *Oreochromis mossambicus* after vitamin C supplementation

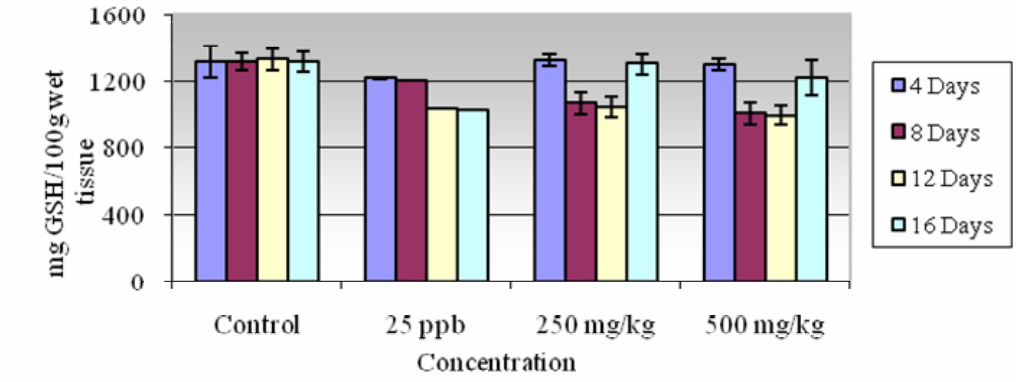


Fig. 5.3.2.21. Variation reduced glutathionelevel in the gills of Malachite green intoxicated (25 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.2.8. Multiple Comparison Test for Enzyme Activities in Vitamin C supplemented *Oreochromis mossambicus* (comparison with control group).

	Groups	Catalase	Superoxide dismutase	Glutathione peroxidase	Glutathione S transferase	Glutathione reductase	Lipid peroxidation	Reduced glutathione
Tissue	Liver & Kidney	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	Liver & Gills	0.000 [a]	0.000 [a]	0.730 [d]	0.000 [a]	0.000 [a]	0.000 [a]	0.183 [d]
	Kidney & Gills	0.000 [a]	0.027 [c]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
Day	4 Days & 8 Days	0.730 [d]	0.320 [d]	0.300 [d]	0.913 [d]	0.005 [b]	0.052 [d]	0.001 [a]
	4 Days & 12 Days	0.055 [d]	0.001 [a]	0.947 [d]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	4 Days & 16 Days	0.997 [d]	0.000 [a]	0.900 [d]	0.000 [a]	0.000 [a]	0.005 [b]	0.313 [d]
	8 Days & 12 Days	0.426 [d]	0.106 [d]	0.102 [d]	0.000 [a]	0.008 [b]	0.039 [c]	0.982 [d]
	8 Days & 16 Days	0.838 [d]	0.046 [c]	0.072 [d]	0.000 [a]	0.000 [a]	0.853 [d]	0.161 [d]
	12 Days & 16 Days	0.089 [d]	0.986 [d]	0.999 [d]	0.496 [d]	0.833 [d]	0.239 [d]	0.070 [d]
Concentration (mg Vit. C/kg diet)	Control & 250 mg	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	Control & 500 mg	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	250 mg & 500 mg	0.768 [d]	0.754 [d]	0.970 [d]	0.991 [d]	0.673 [d]	0.967 [d]	0.998 [d]

The values are significant at a=P<.001, b=P<.01 and c=P<.05 and not significant at d.

Table 5.3.2.9. Multiple Comparison Test for Enzyme Activities in Vitamin C supplemented *Oreochromis mossambicus* (comparison with malachite green intoxicated group).

	Groups	Catalase	Superoxide dismutase	Glutathione peroxidase	Glutathione S transferase	Glutathione reductase	Lipid peroxidation	Reduced glutathione
Tissue	Liver & Kidney	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.016 [c]	0.146 [d]
	Liver & Gills	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	Kidney & Gills	0.000 [a]	0.428 [d]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
Day	4 Days & 8 Days	0.044 [c]	0.039 [c]	0.156 [d]	0.143 [d]	0.636 [d]	0.004 [b]	0.000 [a]
	4 Days & 12 Days	0.000 [a]	0.000 [a]	0.285 [d]	0.000 [a]	0.000 [a]	0.001 [a]	0.000 [a]
	4 Days & 16 Days	0.063 [d]	0.000 [a]	0.197 [d]	0.000 [a]	0.000 [a]	0.001 [a]	0.000 [a]
	8 Days & 12 Days	0.289 [d]	0.000 [a]	0.989 [d]	0.000 [a]	0.000 [a]	0.000 [a]	0.120 [d]
	8 Days & 16 Days	0.000 [a]	0.358 [d]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.715 [d]
	12 Days & 16 Days	0.000 [a]	0.036 [c]	0.001 [a]	0.000 [a]	0.008 [b]	1.000 [d]	0.559 [d]
Concentration(ppb mal. green in water and mg Vit.C/kg diet)	25 ppb & 250 mg vit C/kg diet	0.026 [c]	0.504 [d]	0.210 [d]	0.040 [c]	0.098 [d]	0.000 [a]	0.000 [a]
	25 ppb& 500 mg	0.002 [b]	0.864 [d]	0.335 [d]	0.027 [c]	0.292 [d]	0.000 [a]	0.000 [a]
	250 mg & 500 mg	0.695 [d]	0.819 [d]	0.958 [d]	0.987 [d]	0.836 [d]	0.992 [d]	0.997 [d]

The values are significant at a=P<.001, b=P<.01 and c=P<.05 and not significant at d.

Effects of Vitamin C treatment on malachite green - induced histopathology:

Considerable reduction in necrosis and regaining of architecture were the effect of supply of vitamin C along with the diet in the case of the damaged liver of malachite green intoxicated fishes. Appearance of ceroid pigments in the liver, an aspect not noticed in the intoxicated fishes is significant. The vacuolation and pycnosis; important histopathological changes that occurred due to the toxicity of malachite green were not repaired noticeably by feeding on fortified diet (Fig. 5.3.2.22 & 5.3.2.23). Both constrictions of tubular lumen and occurrence of pycnotic nuclei were found to reduce due to vitamin C supply. Similarly, the number of swollen glomeruli and Bowman's capsules was considerably declined showing a possibility of repair of the damage due to vitamin C supplementation. However, no clear-cut change was evident in the percentage of narrowing of space of Bowman's capsules and hyalinization of tubules (Fig. 5.3.2.24 and 5.3.2.25).

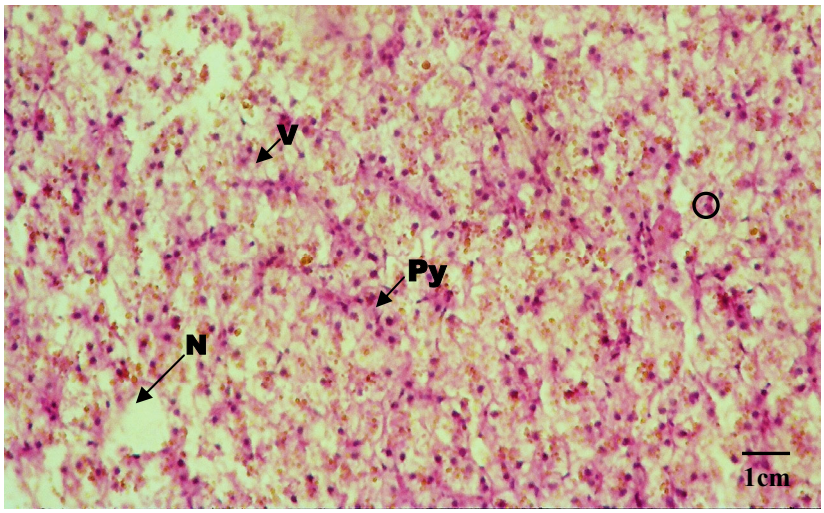


Fig.5.3.2.22. Microphotograph showing the histological changes in the liver of Malachite green stressed *Oreochromis mossambicus* after supplementation with vitamin C @ 500 mg vit C/kg diet. An insignificant reduction in non-fatty vacuolation (V) and pycnotic nuclei (Py) is evident. There occurs a turn back in necrosis and architectural loss (N). H & E, 20x.

Lamellar curling was considerably controlled by supplying vitamin C in the diet of the fishes which had damaged gills due to malachite green toxicity. Curiously, enough increased hyperplasia occurred in the gills of fishes fed with vitamin C fortified food (Fig. 5.3.2.26 and 5.3.2.27).

Table 5.3.2.10. Histological changes in the liver of Malachite green stressed *Oreochromis mossambicus* after supplementing with vitamin C.

Treatment	Pycnosis	Necrosis and/or architectural loss	Vacuolation (non fatty)
Normal	0.00	0.00	0.00
16th day after exposure to Malachite green	92.60	87.75	81.30
16th day after supplementation with vitamin C	86.30	41.00	79.00

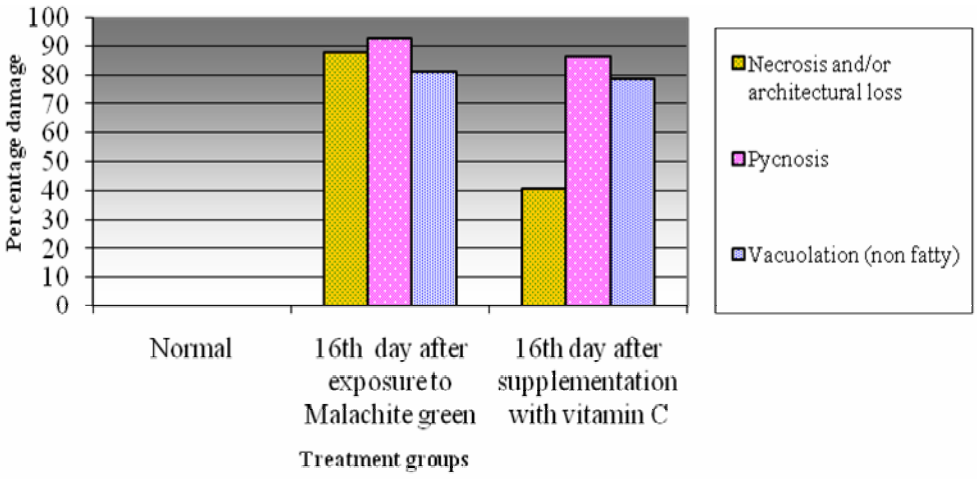


Fig. 5.3.2.23. Histological changes in the liver of Malachite green intoxicated *Oreochromis mossambicus* after supplementing with vitamin C

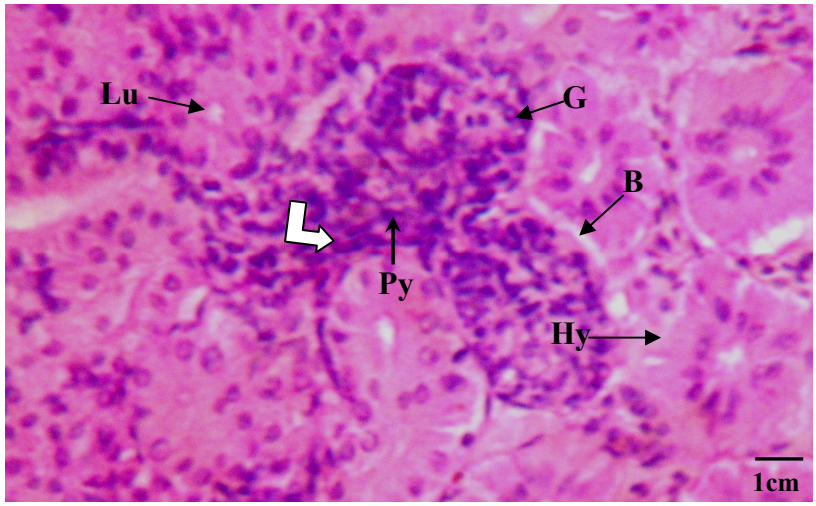


Fig. 5.3.2.24. Microphotograph of the kidney of malachite green stressed *Oreochromis mossambicus* after supplementation with vitamin C. There is reduction in pycnotic nuclei (Py) and constriction of tubular lumen (Lu). The other features like glomerular swelling (G), glomerular leakage (white block arrow), narrowing of Bowman's space (B) and hyalinisation of tubules (Hy) remain nearly the same as in malachite green treatment. H & E, 40x.

Table. 5.3.2.11. Morphological changes in the kidney of Malachite green stressed *Oreochromis mossambicus* after supplementation with vitamin C

Treatment	Percentage damage				
	Constriction of tubular lumen	Hyalinisation	Pycnosis	Glomerular leakage	Number of swollen glomeruli and Bowman's capsules
Control	0.00	0.00	24.00	0.00	0.00
16th day after Malachite green exposure (25 ppb)	69.00	73.00	100.00	1.00	4.27
16th day after vitamin C supplementation.	11.11	76.43	29.05	3.00	2.00

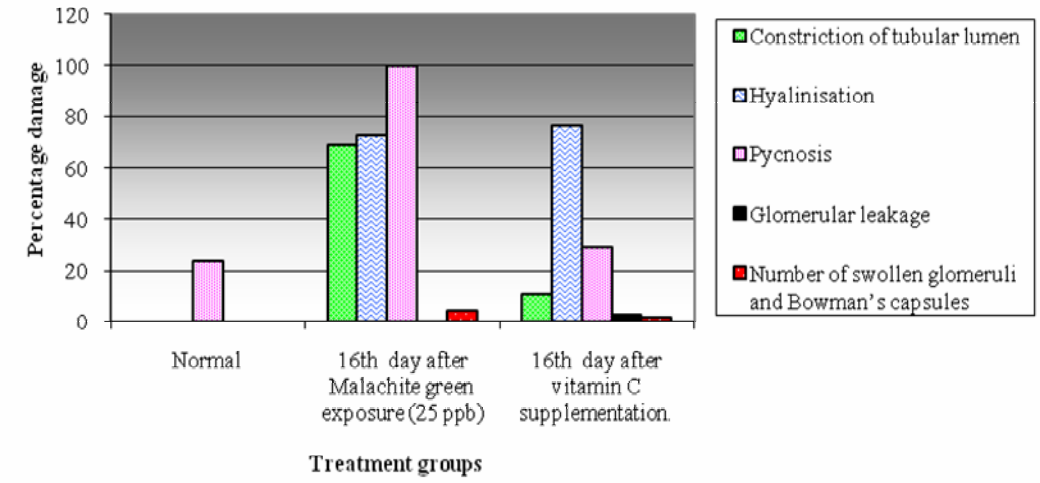


Fig. 5.3.2.25. Changes in renal morphology of malachite green stressed *Oreochromis mossambicus* after supplementation with vitamin C

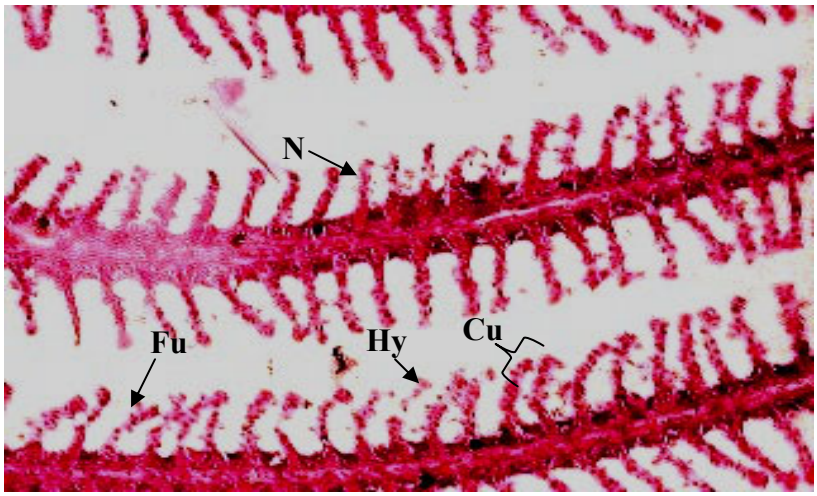


Fig. 5.3.2.26. Microphotograph of gill of malachite green stressed *Oreochromis mossambicus* illustrating changes after treatment with vitamin C. There comes up considerable decline in secondary lamellar curling (Cu) and fusion (Fu) and exfoliation. However, necrosis (N) and hyperplasia (Hy) persist. H& E, 20x.

Table 5.3.2.12. Histological changes in the gill of malachite green stressed *Oreochromis mossambicus* after supplementing with vitamin C.

Treatment	Percentage damage			
	Lamellar curling	Lamellar fusion	Hyperplasia	Exfoliation
Normal	0.00	0.00	1.50	0.00
16th day after malachite green exposure	43.60	16.43	17.85	14.06
16th day after vitamin C treatment.	11.11	11.00	29.92	0.00

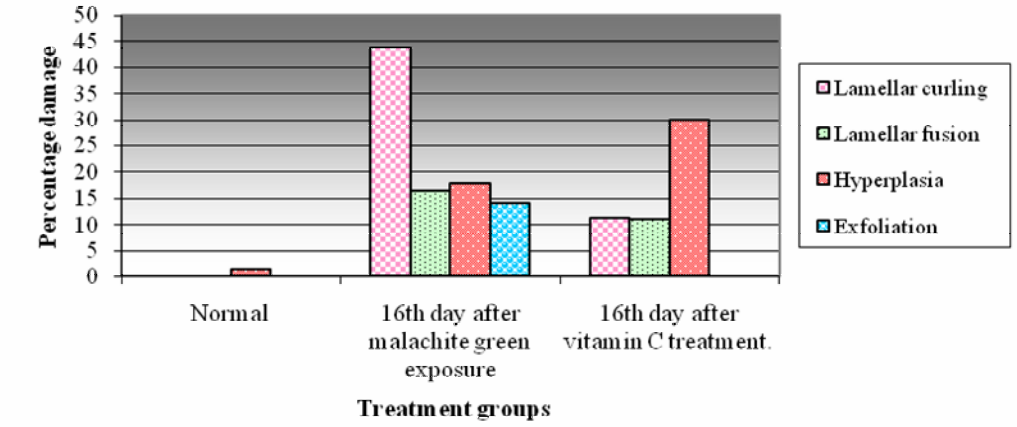


Fig. 5.3.2.27. Morphology of the gill in malachite green stressed *Oreochromis mossambicus* after treatment with vitamin C

5.3.3 Effect of Vitamin C on toxicity of Metacid 50.

The anti oxidative mechanisms and histology of liver, kidney and gills of the experimental fish remain adversely affected by the organophosphorous pesticide, metacid 50. As in the case of copper and malachite green, the ameliorative effect of vitamin C was tested for the pesticide by analyzing the biochemical and histological parameters of the test organs.

Enhanced catalase activity in the liver of *Oreochromis mossambicus* exposed to Metacid 50 was found to regain normalcy very comparable to that of the control animals after 12 to 16 days treatment with vitamin-

fortified diet. On the other hand, there was recovery in this rate function in kidney. Normalcy was not made good in the fishes fed on vitamin-fortified food. No clear-cut enhancement in catalase activity was noticed in the gills. This rate function remained more or less comparable to that of control animals (Fig. 5.3.3.1 to 5.3.3.3).

Superoxide dismutase activity in the liver was considerably reduced when the fishes were exposed to Metacid 50. This reduction was found to get evened out when the animals were fed on vitamin fortified food. Interestingly, the liver functioned in a fashion very similar to that of control animals. Superoxide dismutase activity in the kidney also showed recovery on treatment with vitamin supplementation. The activity was found to increase slightly after 12 days when the animals with diet loaded with vitamin C (Fig. 5.3.3.4 to 5.3. 3.6).

The increased glutathione peroxide activity of fishes exposed to Metacid was found to get reduced on fortified diet treatment. However, this rate function did not regain the rates similar to that of control animals. Drastic reduction that occurred in this rate function in the kidney on exposure to Metacid was found to get back to normalcy after 12 to 16 days treatment with vitamin C fortified diet. However, glutathione peroxidase activity of the gills remained elevated as in the early phase of intoxicated fishes, in the case of those fed with fortified diet (Fig. 5.3.3.37 to 5.3.3.9).

In the liver, variation in glutathione S transferase activity was found to regain normalcy on treatment of the intoxicated fishes with vitamin C enriched diet. The values obtained after 12 to 16 days were more or less similar to that obtained for the control animals. This was the condition in the kidney also: A very comparable result was obtained with reference to

gill tissue of the fishes treated. The activity of this enzyme seems to be really influenced by the presence of vitamin C in the diet (Fig. 5.3.3.10 to 5.3.3.12).

The results obtained for the glutathione reductase activity of the liver showed that the imbalance that resulted from intoxication not recovered during the early phase of the experiments. After maintenance in fortified diet for 16 days, there were signs of recovery wherein the activity of glutathione reductase was found to reach the levels nearer to that of control animals. On the contrary no detectable revival was noticed in the kidney of the fishes treated with vitamin C fortified diet. The activity of the enzyme remained very similar to that depicted by the intoxicated fishes. However, the situation of the gills was totally different. This rate function recovered to normal levels after 16 days of treatment with the fortified diet (Fig. 5.3.3.13 to 5.3.3.15).

Variations noticed in the lipid peroxidation in the liver of fishes exposed to Metacid 50 were very significant. This change was found to regain near normalcy soon after treatment with vitamin C fortified diet. Though the levels did not reach those of control animals, the reduction in levels that occurred in comparison to that of intoxicated fishes were very significant. It is clear that a similar trend was noticed in the kidney also. Although the levels reached after treatment for 16 days were not equal to that of control, the trend did not indicate that vitamin C can reduce the bad effect of toxicity. In the case of gills, the fishes did not recover totally and the extent of lipid peroxidation showed a picture very similar to that of control animals after 16 days treatment with vitamin C fortified diet (Fig. 5.3.3.16 to 5.3.3.18).

Variation in reduced glutathione levels of liver of Metacid exposed fishes was found to reach levels comparable to control animals after 16

days treatment with vitamin C fortified diet, the GSH levels reached near to that of control animals. However, the gills did not respond in a similar fashion. The variation in reduced glutathione levels were very less than those depicted by the gills of control fishes (Fig. 5.3.3.19 to 5.3.3.21).

Table 5.3.3.1.a). Changes in catalase activity in tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after supplementing with vitamin C.

Tissue	Concentration	Duration of exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	7.367± 0.4985	7.392± 0.6060	7.425± 0.6624	7.420± 0.6453
	1.24 ppb	15.30± 0.1714	15.03± 0.2764	15.45± 1.0629	20.34± 0.6084
	250 mg vit C/kg diet	13.58± 0.1218	11.16± 0.3022	8.097± 0.3904	8.111± 0.3238
	500 mg vit C/kg diet	13.14± 0.1816	10.51± 0.2849	8.215± 0.2447	8.248± 0.3483
Kidney	Control	4.278± 0.3489	4.257± 0.3173	4.298± 0.6093	4.310± 0.5586
	1.24 ppb	10.25± 0.1253	10.95± 0.3525	11.99± 0.6518	7.063± 0.2823
	250 mg vit C/kg diet	10.01± 0.4358	10.00± 0.4493	9.457± 0.2326	10.02± 0.3222
	500 mg vit C/kg diet	9.655± 0.5310	10.32± 0.2349	9.602± 0.3716	10.07± 0.3534
Gills	Control	13.75± 0.7253	13.85± 0.7818	13.84± 0.6032	13.82± 0.3637
	1.24 ppb	15.99± 0.4727	17.67± 0.2707	25.60± 0.1029	14.99± 0.7215
	250 mg vit C/kg diet	14.05± 0.5282	14.21± 0.3681	13.42± 0.2021	13.32± 0.1634
	500 mg vit C/kg diet	14.37± 0.3308	13.74± 0.1926	13.38± 0.1958	13.36± 0.3017

Table 5.3.3.1.b). ANOVA for changes in catalase activity in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after supplementing with vitamin C. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	1052.638	2	526.319	93.757	.001
Duration	47.879	3	15.960	2.843	.039
Concentration	693.595	2	346.797	61.778	.000
Error	1167.637	208	5.614		
Total	2961.749	215			

Table 5.3.3.1.c). ANOVA for catalase activity in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after vitamin C supplementation. (Comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	1317.051	2	658.525	298.587	.001
Day	69.442	3	23.147	10.495	.025
Concentration	363.501	2	181.750	82.409	.013
Error	458.738	208	2.205		
Total	2208.732	215			

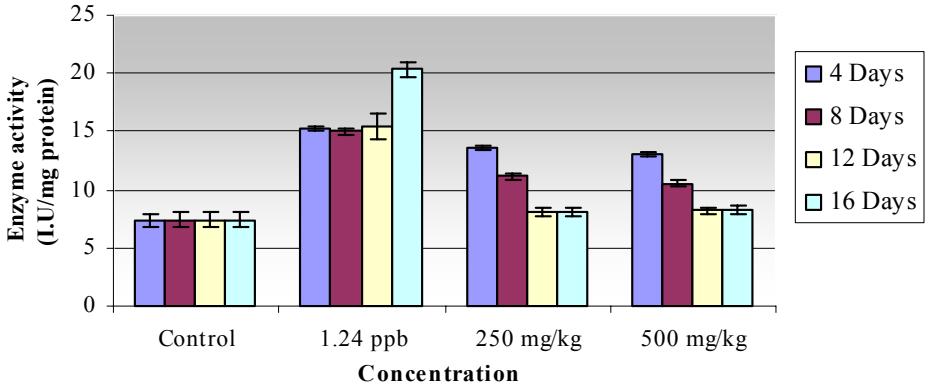


Fig. 5.3.3.1. Variation in catalase activity in the liver of Metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after vitamin C supplementation

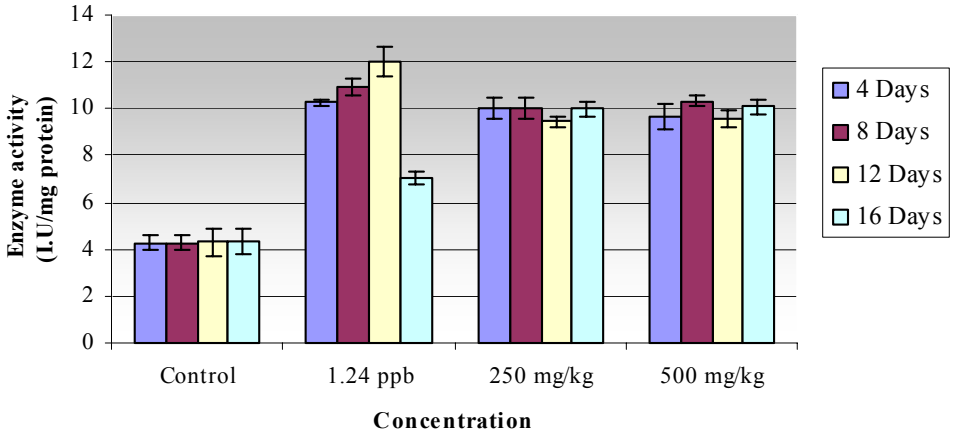


Fig. 5.3.3.2. Variation in catalase activity in the kidney of Metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after vitamin C supplementation

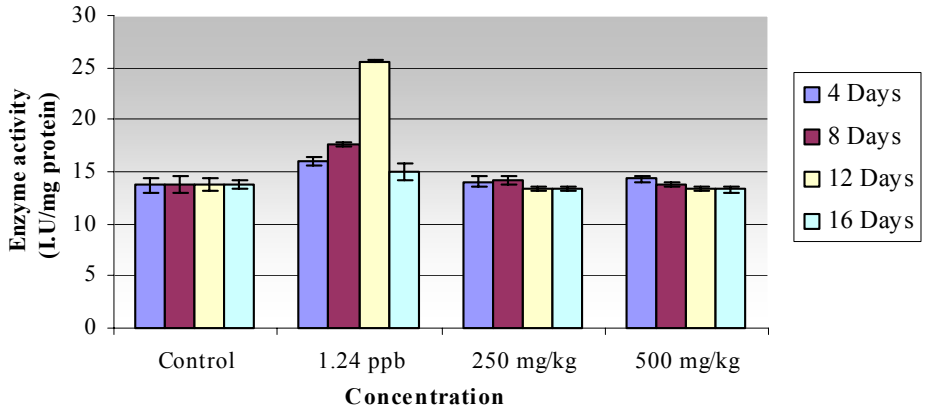


Fig. 5.3.3.3. Variation in catalase activity in the gills of Metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.3.2.a). Changes in superoxide dismutase activity in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after supplementing with vitamin C.

Tissue	Concentration	Duration of exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	1.362± 0.0854	1.368± 0.3956	1.355± 0.1817	1.362± 0.1511
	1.24 ppb	0.887± 0.0216	0.447± 0.0383	0.242± 0.0147	0.577± 0.0266
	250 mg vit C/kg diet	1.337± 0.0493	1.330± 0.0856	1.355± 0.0259	1.368± 0.0611
	500 mg vit C/kg diet	1.538± 0.0771	1.390± 0.0155	1.427± 0.0887	1.380± 0.0930
Kidney	Control	0.413± 0.0758	0.398± 0.0804	0.388± 0.0789	0.387± 0.0625
	1.24 ppb	0.225± 0.0138	0.180± 0.0063	0.175± 0.0084	0.082± 0.0075
	250 mg vit C/kg diet	0.337± 0.0314	0.435± 0.0547	0.437± 0.0513	0.412± 0.0847
	500 mg vit C/kg diet	0.353± 0.0175	0.407± 0.0476	0.562± 0.0611	0.428± 0.0731
Gills	Control	2.398± 0.3064	2.418± 0.2829	2.415± 0.1058	2.407± 0.0720
	1.24 ppb	2.525± 0.0476	3.132± 0.0471	3.135± 0.0123	1.168± 0.0436
	250 mg vit C/kg diet	2.448± 0.0581	2.782± 0.0431	2.443± 0.1009	2.388± 0.0646
	500 mg vit C/kg diet	2.635± 0.0896	2.628± 0.0331	2.420± 0.0597	2.500± 0.0600

Table 5.3.3.2 b). ANOVA for changes in superoxide dismutase activity in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after supplementing with vitamin C. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	176.180	2	88.090	840.248	.023
Duration	2.314	3	.771	7.359	.015
Concentration	7.132	2	3.566	34.015	.001
Error	21.806	208	.105		
Total	207.432	215			

Table 5.3.3.2 c). ANOVA for superoxide dismutase activity in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after vitamin C supplementation. (Comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	155.575	2	77.787	4330.249	.000
Day	.097	3	.032	1.791	.150
Concentration	.252	2	.126	7.002	.001
Error	3.736	208	.018		
Total	159.660	215			

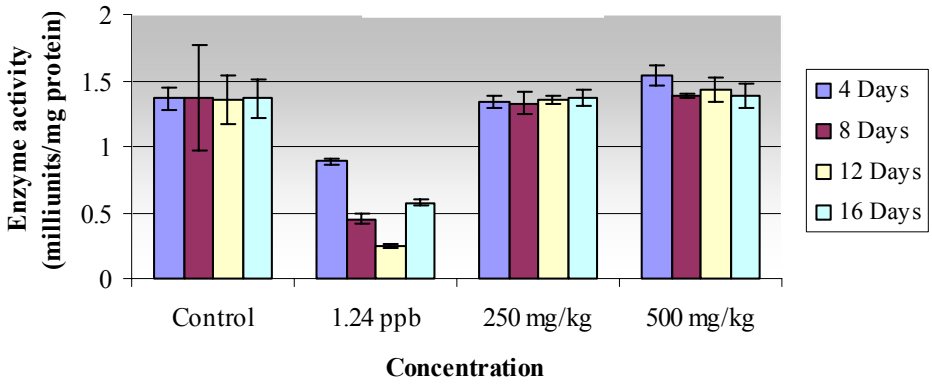


Fig. 5.3.3.4. Variation sup eroxide dismutase activity in the liver of Metacid 50 (1.24 ppb) intoxicated *Oreochromis mossabicus* after vitamin C supplementation

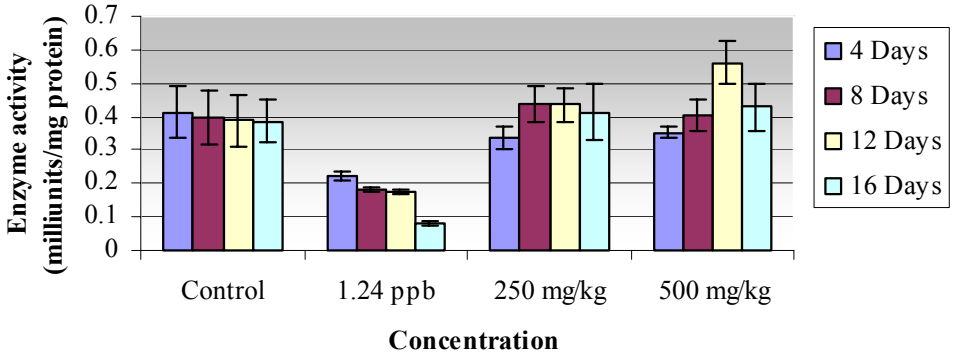


Fig. 5.3.3.5. Variation sup eroxide dismutase activity in the kidney of Metacid 50 (1.24 ppb) intoxicated *Oreochromis mossabicus* after vitamin C supplementation

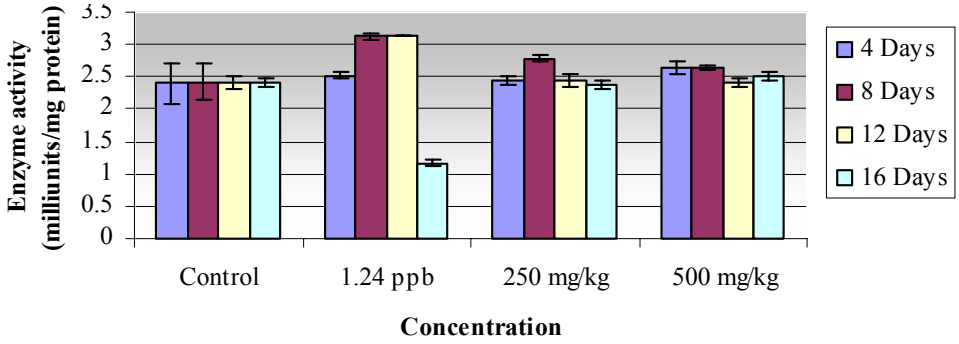


Fig. 5.3.3.6. Variation sup eroxide dismutase activity in the gills of Metacid 50 (1.24 ppb) intoxicated *Oreochromis mossabicus* after vitamin C supplementation

Table 5.3.3.3 a). Changes in glutathione peroxidase activity in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after supplementing with vitamin C.

Tissue	Concentration	Duration of exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	7.572± 0.5807	7.550± 0.4313	7.567± 0.2963	7.555± 0.4663
	1.24 ppb	11.43± 0.1336	13.21± 0.1487	15.26± 0.1247	15.82± 0.1009
	250 mg vit C/kg diet	11.45± 0.0872	13.39± 0.0862	9.047± 0.2158	9.215± 0.2877
	500 mg vit C/kg diet	11.48± 0.0579	11.52± 0.1067	8.605± 0.3854	8.440± 0.0696
Kidney	Control	4.897± 0.5452	4.865± 0.2807	4.918± 0.4551	4.888± 0.3933
	1.24 ppb	1.763± 0.2015	1.217± 0.0186	0.977± 0.0572	0.327± 0.0103
	250 mg vit C/kg diet	2.357± 0.3239	3.758± 0.1630	4.380± 0.5278	4.628± 0.1139
	500 mg vit C/kg diet	2.552± 0.1687	4.045± 0.1488	4.982± 0.2783	4.773± 0.2840
Gills	Control	12.47± 0.5175	12.47± 0.3003	12.53± 0.5696	12.52± 0.3590
	1.24 ppb	17.63± 0.1957	19.68± 0.4864	7.088± 0.0392	5.548± 0.2368
	250 mg vit C/kg diet	17.00± 0.4499	16.17± 0.6171	15.52± 0.1878	14.72± 0.9625
	500 mg vit C/kg diet	16.84± 0.5952	15.66± 0.3357	15.42± 0.1648	15.07± 0.4853

Table 5.3.3.3 b). ANOVA for changes in glutathione peroxidase activity in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after supplementing with vitamin C. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	5300.136	2	2650.068	361.207	.002
Duration	178.526	3	59.509	8.111	.001
Concentration	38.359	2	19.179	2.614	.076
Error	1526.034	208	7.337		
Total	7043.055	215			

Table 5.3.3.3 c). ANOVA for glutathione peroxidase activity in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after vitamin C supplementation. (Comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	3927.462	2	1963.731	1067.617	.001
Day	24.156	3	8.052	4.378	.005
Concentration	144.243	2	72.122	39.210	.023
Error	382.587	208	1.839		
Total	4478.448	215			

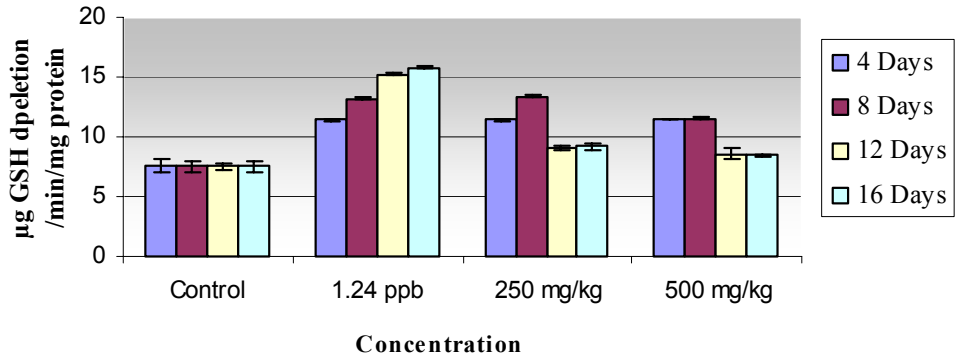


Fig. 5.3.3.7. Variation in glutathione peroxidase activity in the liver of Metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after vitamin C supplementation

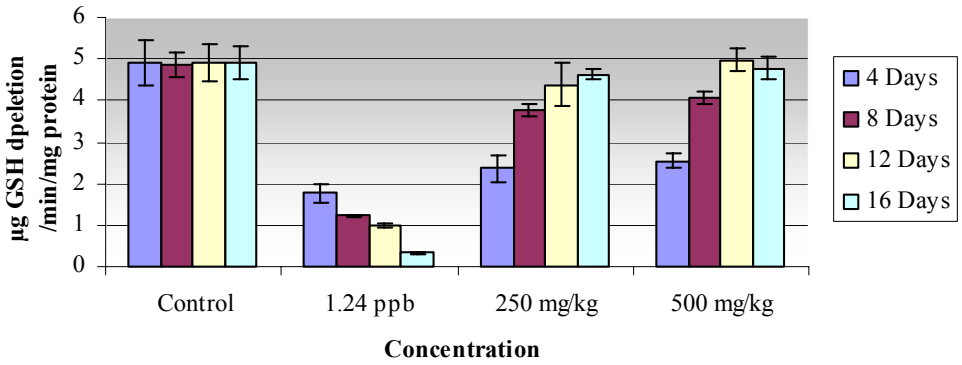


Fig. 5.3.3.8. Variation in glutathione peroxidase activity in the kidney of Metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after vitamin C supplementation

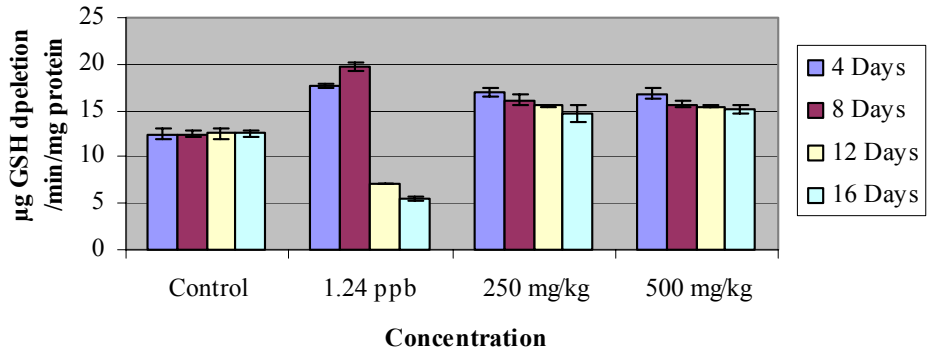


Fig. 5.3.3.9. Variation in glutathione peroxidase activity in the gills of Metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.3.4 a). Changes in glutathione S transferase activity in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after supplementing with vitamin C.

Tissue	Concentration	Duration of exposure			
		4 Days	**8 Days**	**12 Days**	**16 Days**
Liver	**Control**	20.68± 0.8443	20.59± 1.1275	20.48± 0.6142	20.49± 1.1394
	1.24 ppb	25.36± 0.1317	33.14± 0.0388	33.50± 0.0896	34.07± 0.0398
	250 mg vit C/kg diet	23.98± 0.3217	22.21± 0.1678	19.65± 0.3164	19.51± 0.5394
	500 mg vit C/kg diet	23.61± 0.3857	21.54± 0.5926	19.93± 0.3014	20.04± 0.2718
Kidney	**Control**	11.32± 0.6112	11.36± 0.4626	11.37± 0.4874	11.39± 0.6214
	1.24 ppb	12.07± 0.0388	12.92± 0.4814	16.38± 0.0226	18.28± 0.0493
	250 mg vit C/kg diet	13.24± 0.1686	12.85± 0.4973	11.57± 0.0971	11.51± 0.1306
	500 mg vit C/kg diet	11.86± 0.1443	11.52± 0.1871	11.32± 0.0637	11.46± 0.1423
Gills	**Control**	13.17± 0.4418	13.07± 0.8293	12.80± 0.8822	13.09± 0.9673
	1.24 ppb	11.21± 0.0449	9.308± 0.1556	6.440± 0.0127	5.225± 0.0666
	250 mg vit C/kg diet	12.06± 0.1352	13.11± 0.2320	13.52± 0.3448	13.34± 0.2121
	500 mg vit C/kg diet	12.07± 0.3223	13.13± 0.3742	13.25± 0.1385	13.27± 0.2160

Table 5.3.3.4 b). ANOVA for changes in glutathione S transferase activity in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after supplementing with vitamin C. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	7698.761	2	3849.380	308.153	.011
Duration	7.899	3	2.633	.211	.889
Concentration	369.012	2	184.506	14.770	.001
Error	2598.289	208	12.492		
Total	10673.961	215			

Table 5.3.3.4 c). ANOVA for glutathione S transferase activity in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after vitamin C supplementation. (Comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	3689.341	2	1844.670	2005.807	.001
Day	32.209	3	10.736	11.674	.001
Concentration	11.293	2	5.647	6.140	.003
Error	191.290	208	.920		
Total	3924.133	215			

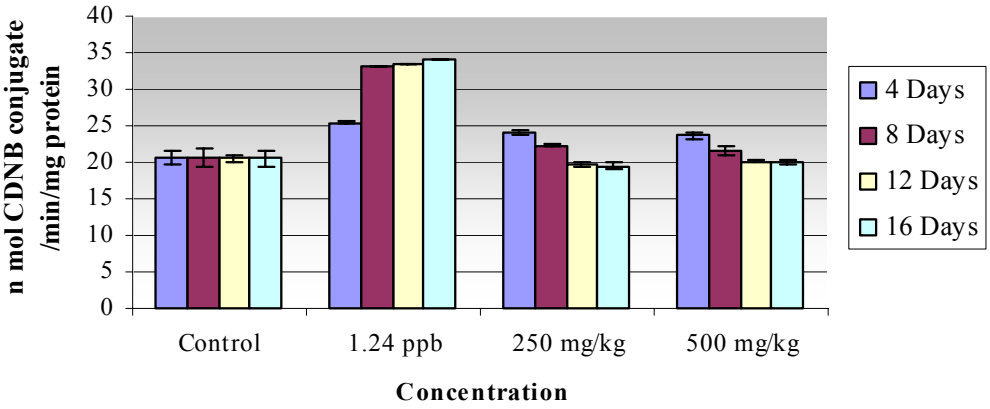


Fig. 5.3.3.10. Variation in glutathione S transferase activity in the liver of Metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after vitamin C supplementation

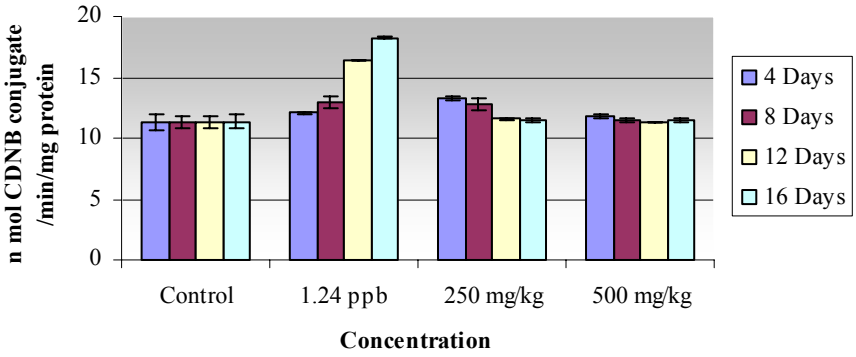


Fig. 5.3.3.11. Variation in glutathione S transferase activity in the kidney of Metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after vitamin C supplementation

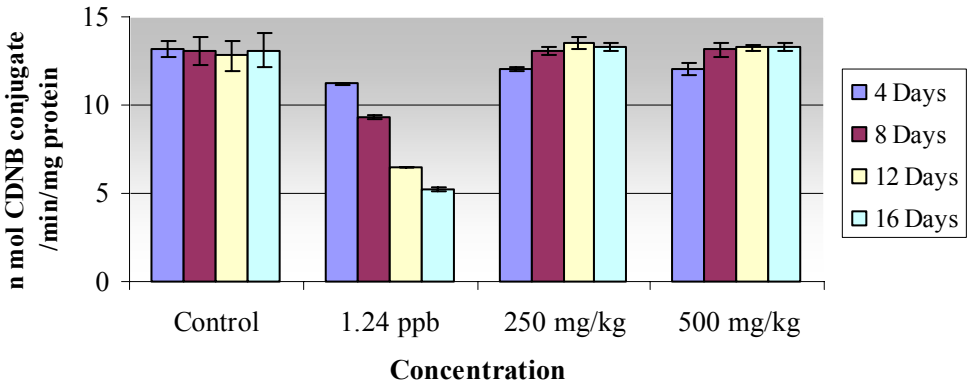


Fig. 5.3.3.12. Variation in glutathione S transferase activity in the gills of Metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.3.5 a). Changes in glutathione reductase activity in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after supplementing with vitamin C.

Tissue	Concentration	Duration of exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	2.983± 0.2340	2.997± 0.2013	3.097± 0.2212	3.017± 0.2777
	1.24 ppb	10.22± 0.0207	10.79± 0.1050	13.47± 0.0590	13.75± 0.0417
	250 mg vit C/kg diet	12.14± 0.1510	12.07± 0.2800	10.05± 0.3677	6.037± 0.2836
	500 mg vit C/kg diet	12.00± 0.5418	11.93± 0.6073	9.085± 0.4697	5.815± 0.3815
Kidney	Control	1.570± 0.1361	1.598± 0.2665	1.635± 0.0950	1.625± 0.1723
	1.24 ppb	5.447± 0.1075	7.543± 0.0520	9.467± 0.0250	11.15± 0.0302
	250 mg vit C/kg diet	5.442± 0.0945	6.348± 0.1905	8.277± 0.2059	9.517± 0.4845
	500 mg vit C/kg diet	5.107± 0.1219	6.453± 0.1044	7.692± 0.2854	9.113± 0.3310
Gills	Control	2.377± 0.1134	2.432± 0.3826	2.408± 0.3657	2.407± 0.2535
	1.24 ppb	5.200± 0.0283	8.345± 0.0339	10.28± 0.0635	3.030± 0.1731
	250 mg vit C/kg diet	3.328± 0.1745	3.560± 0.1898	3.983± 0.2084	3.375± 0.0979
	500 mg vit C/kg diet	2.867± 0.2536	2.438± 0.1046	2.92± 0.3893	2.483± 0.1037

Table 5.3.3.5 b). ANOVA for changes in glutathione reductase activity in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after supplementing with vitamin C. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	1427.995	2	713.997	194.414	.041
Duration	71.342	3	23.781	6.475	.001
Concentration	264.862	2	132.431	36.060	.025
Error	763.892	208	3.673		
Total	2528.091	215			

Table 5.3.3.5 c). ANOVA for glutathione reductase activity in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after vitamin C supplementation. (Comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	802.610	2	401.305	110.137	.001
Day	16.737	3	5.579	1.531	.207
Concentration	941.401	2	470.700	129.182	.001
Error	757.888	208	3.644		
Total	2518.636	215			

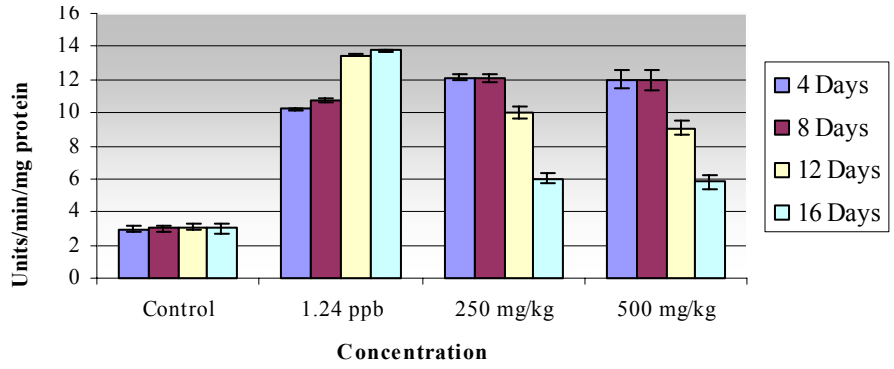


Fig. 5.3.3.13. Variation in glutathione reductase activity in the liver of Metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after vitamin C supplementation

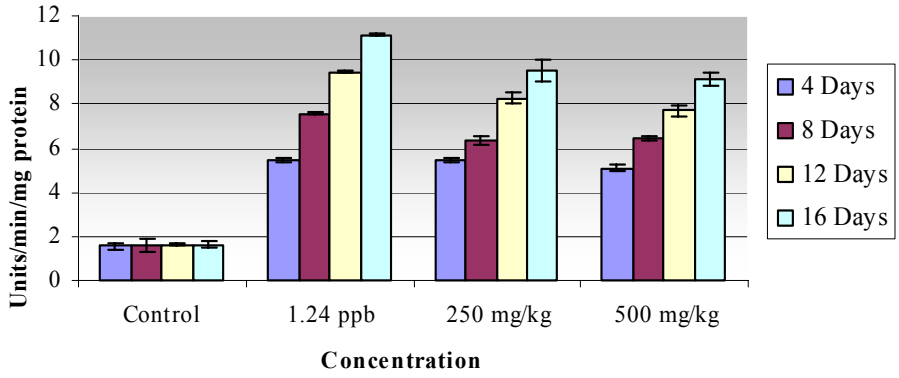


Fig. 5.3.3.14. Variation in glutathione reductase activity in the kidney of Metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after vitamin C supplementation

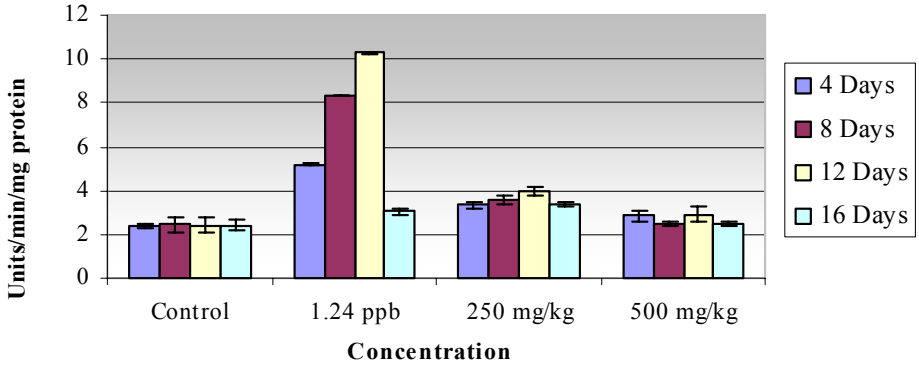


Fig. 5.3.3.15. Variation in glutathione reductase activity in the gills of Metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.3.6 a). Changes in lipid peroxidation in tissues *Oreochromis mossambicus* (intoxicated with Metacid 50) after vitamin C supplementation.

Tissue	Concentration	Duration of exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	0.718± 0.1251	0.697± 0.0826	0.672± 0.1067	0.685± 0.1314
	1.24 ppb	2.458± 0.0319	5.108± 0.0194	5.498± 0.0884	7.848± 0.8190
	250 mg vit C/kg diet	2.400± 0.0452	2.902± 0.3438	2.393± 0.1706	2.288± 0.1473
	500 mg vit C/kg diet	2.335± 0.0695	3.077± 0.4335	2.237± 0.1363	2.283± 0.1612
Kidney	Control	0.365± 0.0720	0.353± 0.0547	0.365± 0.0731	0.368± 0.0605
	1.24 ppb	1.398± 0.0720	2.683± 0.0557	3.152± 0.0264	4.055± 0.1043
	250 mg vit C/kg diet	1.247± 0.2199	1.525± 0.1142	1.287± 0.0671	0.548± 0.1216
	500 mg vit C/kg diet	1.258± 0.1005	1.467± 0.1041	1.240± 0.0699	0.483± 0.1903
Gills	Control	0.618± 0.1123	0.580± 0.0982	0.617± 0.0937	0.608± 0.0731
	1.24 ppb	1.793± 0.1311	2.717± 0.0509	4.238± 0.2424	6.367± 0.1875
	250 mg vit C/kg diet	1.245± 0.1541	0.813± 0.1252	0.633± 0.2609	0.587± 0.2075
	500 mg vit C/kg diet	1.077± 0.1962	0.717± 0.1178	0.593± 0.2340	0.578± 0.2039

Table 5.3.3.6 b). ANOVA for lipid peroxidation in tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after vitamin C supplementation. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	133.291	2	66.646	74.606	.001
Duration	32.898	3	10.966	12.276	.023
Concentration	294.297	2	147.148	164.724	.017
Error	185.807	208	.893		
Total	646.293	215			

Table 5.3.3.6 c). ANOVA for lipid peroxidation in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after vitamin C supplementation. (Comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	58.050	2	29.025	174.777	.001
Day	5.156	3	1.719	10.349	.001
Concentration	40.109	2	20.055	120.760	.018
Error	34.542	208	.166		
Total	137.857	215			

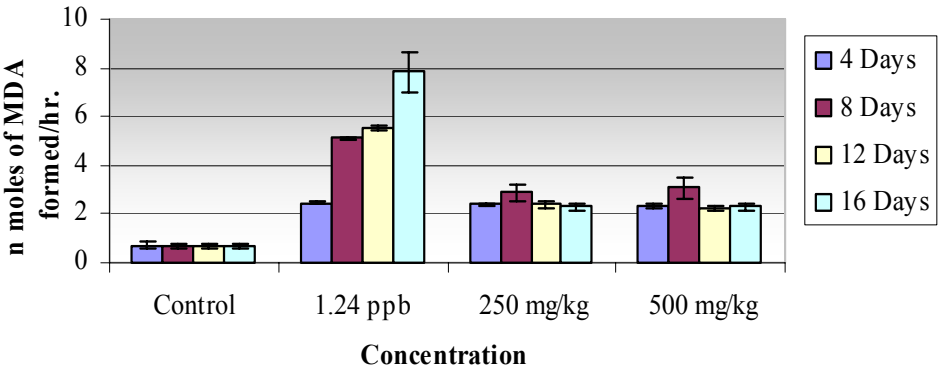


Fig. 5.3.3.16. Variation in lipid peroxidation in the liver of Metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after vitamin C supplementation

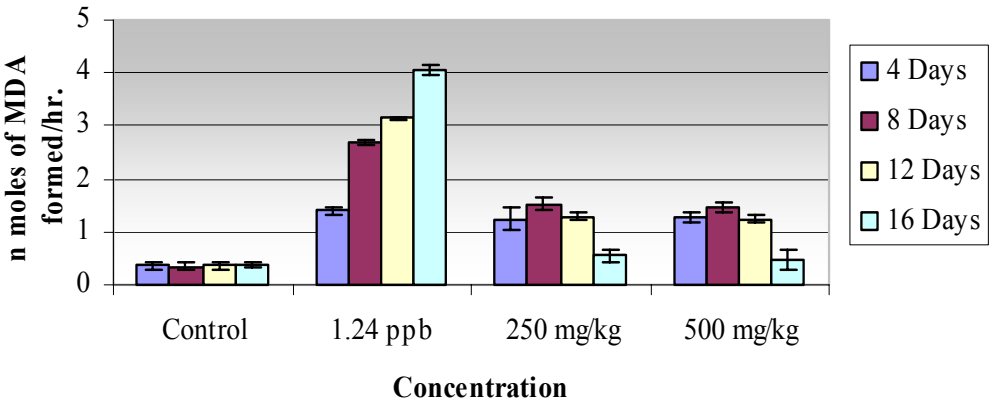


Fig. 5.3.3.17. Variation in lipid peroxidation in the kidney of Metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after vitamin C supplementation

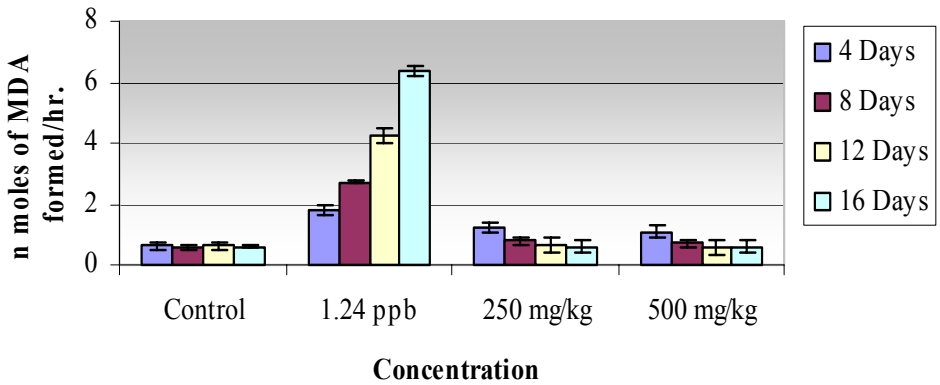


Fig. 5.3.3.18. Variation in lipid peroxidation in the gills of Metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Table 5.3.3.7 a). Changes in reduced glutathione content in tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after vitamin C supplementation.

Tissue	Concentration	Duration of exposure			
		4 Days	8 Days	12 Days	16 Days
Liver	Control	1760.2± 78.211	1760.9± 48.536	1761.3± 40.864	1766.4± 60.418
	1.24 ppb	1453.5± 3.6742	1379.2± 5.4924	1227.5± 1.3784	1196.3± 1.2111
	250 mg vit C/kg diet	1429± 12.116	1569.7± 10.539	1633.3± 4.6762	1699.2± 11.409
	500 mg vit C/kg diet	1445± 18.396	1586.2± 7.627	1631.5± 37.039	1713± 12.345
Kidney	Control	1010.2± 18.691	1009.6± 16.401	1009.8± 33.988	1009± 9.2561
	1.24 ppb	2106± 3.2863	2099± 2.3664	2070± 8.5323	2017.2± 0.7528
	250 mg vit C/kg diet	1973.3± 7.7632	1419.7± 11.501	1286.7± 7.1461	1175.3± 5.2789
	500 mg vit C/kg diet	1981.2± 8.6120	1383± 6.9282	1265.2± 4.2622	1148.5± 13.576
Gills	Control	1262.3± 62.456	1261.4± 54.955	1262.3± 45.779	1286.3± 54.520
	1.24 ppb	962.2± 1.7224	863.0± 1.5492	1281.8± 1.7224	1272.7± 1.6330
	250 mg vit C/kg diet	1017.2± 9.6626	1125.8± 6.9976	770.5± 13.751	754.3± 8.5010
	500 mg vit C/kg diet	1034.3± 8.7102	1141.2± 8.2321	691.2± 7.1949	640.5± 7.2595

Table 5.3.3.7 b). ANOVA for changes in reduced glutathione content in tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after vitamin C supplementation. (comparison with high concentration of the toxicant).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	12.262	2	6.131	180.151	.002
Duration	.895	3	.298	8.764	.022
Concentration	.842	2	.421	12.376	.001
Error	7.079	208	.034		
Total	21.078	215			

Table 5.3.3.7 c). ANOVA for reduced glutathione content in the tissues of *Oreochromis mossambicus* (intoxicated with Metacid 50) after vitamin C supplementation. (Comparison with control).

Source	Sum of Squares	df	Mean Square	F	P-value
Tissue	9.181	2	4.590	126.570	.001
Day	1.062	3	.354	9.762	.001
Concentration	.110	2	.055	1.513	.223
Error	7.544	208	.036		
Total	17.897	215			

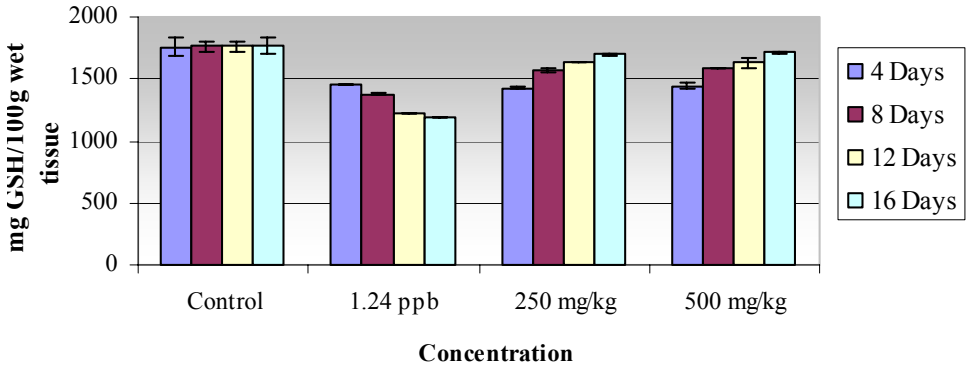


Fig. 5.3.3.19. Variation in reduced glutathione level in the liver of metacid 50 intoxicated (124 ppb) *Oreochromis mossambicus* after vitamin C supplementation

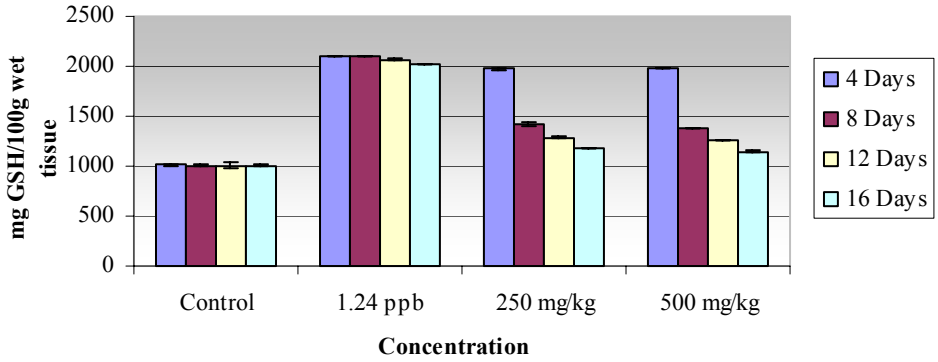


Fig. 5.3.3.20. Variation in reduced glutathione level in the kidney of metacid 50 intoxicated (124 ppb) *Oreochromis mossambicus* after vitamin C supplementation

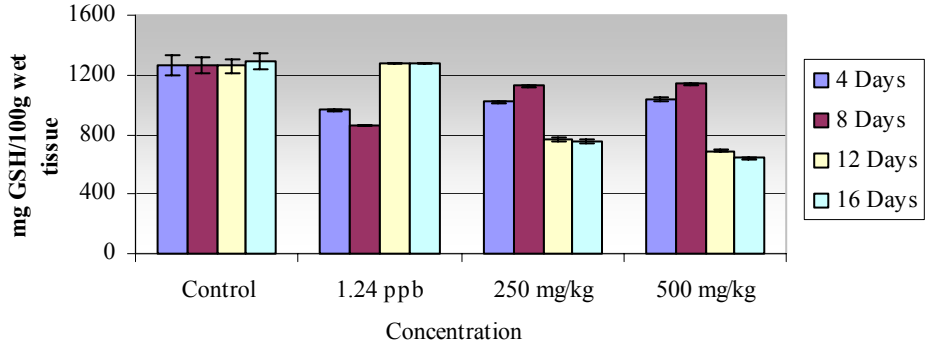


Fig. 5.3.3.21. Variation in reduced glutathione level in the gills of metacid 50 intoxicated (124 ppb) *Oreochromis mossambicus* after vitamin C supplementation

Fig. 5.3.3.8. Multiple Comparison Test for Enzyme Activities in Vitamin C supplemented *Oreochromis mossambicus* (comparison with control group).

	Groups	Catalase	Superoxide dismutase	Glutathione peroxidase	Glutathione S transferase	Glutathione reductase	Lipid peroxidation	Reduced glutathione
Tissue	Liver & Kidney	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	Liver & Gills	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	Kidney & Gills	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.064 [d]	0.000 [a]
Day	4 Days & 8 Days	0.252 [d]	0.474 [d]	0.631 [d]	0.396 [d]	0.930 [d]	0.610 [d]	0.797 [d]
	4 Days & 12 Days	0.000 [a]	1.000 [d]	0.411 [d]	0.000 [a]	0.977 [d]	0.307 [d]	0.001 [a]
	4 Days & 16 Days	0.000 [a]	0.846 [d]	0.175 [d]	0.000 [a]	0.540 [d]	0.000 [a]	0.000 [a]
	8 Days & 12 Days	0.016 [c]	0.422 [d]	0.033 [c]	0.006 [b]	0.997 [d]	0.018 [c]	0.015 [c]
	8 Days & 16 Days	0.045 [c]	0.111 [d]	0.008 [b]	0.009 [b]	0.212 [d]	0.000 [a]	0.003 [b]
	12 Days & 16 Days	0.983 [d]	0.884 [d]	0.960 [d]	0.999 [d]	0.304 [d]	0.107 [d]	0.951 [d]
Concentration (mg Vit. C/kg diet)	Control & 250 mg	0.000 [a]	0.297 [d]	0.000 [a]	0.002 [b]	0.000 [a]	0.000 [a]	0.573 [d]
	Control & 500 mg	0.000 [a]	0.001 [a]	0.000 [a]	0.222 [d]	0.000 [a]	0.000 [a]	0.196 [d]
	250 mg & 500 mg	0.958 [d]	0.069 [d]	0.686 [d]	0.160 [d]	0.234 [d]	0.797 [d]	0.750 [d]

The values are significant at a=P<.001, b=P<.01 and c=P<.05 and not significant at d.

Table 5.3.3.9. Multiple Comparison Test for Enzyme Activities in Vitamin C supplemented *Oreochromis mossambicus* (comparison with Metacid 50 intoxicated group)

	Groups	Catalase	Superoxide dismutase	Glutathione peroxidase	Glutathione S transferase	Glutathione reductase	Lipid peroxidation	Reduced glutathione
Tissue	Liver & Kidney	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.024 [c]
	Liver & Gills	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	Kidney & Gills	0.000 [a]	0.000 [a]	0.000 [a]	0.021 [c]	0.000 [a]	0.853 [d]	0.000 [a]
Day	4 Days & 8 Days	0.908 [d]	0.857 [d]	0.558 [d]	0.899 [d]	0.095 [d]	0.003 [b]	0.464 [d]
	4 Days & 12 Days	0.993 [d]	0.999 [d]	0.082 [d]	1.000 [d]	0.000 [a]	0.002 [b]	0.001 [a]
	4 Days & 16 Days	0.044 [c]	0.003 [b]	0.017 [c]	0.997 [d]	0.872 [d]	0.000 [a]	0.000 [a]
	8 Days & 12 Days	0.979 [d]	0.776 [d]	0.002 [b]	0.905 [d]	0.310 [d]	0.999 [d]	0.101 [d]
	8 Days & 16 Days	0.205 [d]	0.000 [a]	0.000 [a]	0.960 [d]	0.399 [d]	0.069 [d]	0.012 [c]
	12 Days & 16 Days	0.088 [d]	0.005 [b]	0.936 [d]	0.998 [d]	0.006 [b]	0.101 [d]	0.855 [d]
Concentration (ppb Metacid in water and mg Vit. C/kg diet)	1.24 ppb & 250 mg	0.000 [a]	0.000 [a]	0.082 [d]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	1.24 ppb & 500 mg	0.000 [a]	0.000 [a]	0.193 [d]	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]
	250 mg & 500 mg	0.983 [d]	0.627 [d]	0.909 [d]	0.872 [d]	0.237 [d]	0.959 [d]	0.736 [d]

The values are significant at a=P<.001, b=P<.01 and c=P<.05 and not significant at d.

Effects of Vitamin C treatment on Metacid 50-induced histopathology:

Metacid 50, being was found to be capable of imparting undesirable alterations in the hepatic, renal, and branchial morphology of tilapia. After sublethal exposure at 1.24 ppb, the pesticide has done several damages in these tissues, some of them reversible. When the fishes were supplemented with vitamin C in two different concentrations (see section 5.2) in the diet, certain biologically favourable changes manifested in the test organs. Illustrations and descriptions to this effect follow.

Hepatic tissue damaged in such a fashion that the biliary hyperplasia, pycnosis and fatty vacuolation occurred. On treatment of the affected fishes with vitamin fortified diet, resulted in considerable reduction in the percentage damage of the liver tissue. The repair of damage occurred in pycnosis and fatty vacuolation. A marginal increase in biliary hyperplasia was noticed. As indicated in the figures 5.3.3.22 and 5.3.3.23 there occurs almost 93 % reduction in fatty vacuolation and 66 % decrease in pycnosis. Liver being the site of detoxification, the recovery is commendable. There were no macrophage aggregates. Slight enhancement in biliary hyperplasia seemed to be insignificant.

Repair of the renal tissue was confined to reduction in tubular degeneration, hyalinization and tubular casts.

Gills were extensively damaged in *Oreochromis mossambicus* as a result of exposure to Metacid. Noticeable lamellar curling, lamellar fusion, necrosis and squamous metaplasia were rampant after 16 days of toxic insult. Treatment for recovery by feeding the fishes with vitamin supplemented diet resulted in drastic reduction of the above damages occurred due to toxicity although lamellar curling increased. It is

significant that necrosis, squamous metaplasia and exfoliation disappeared completely (Fig.5.3.3.25 and 5.3.3.27).

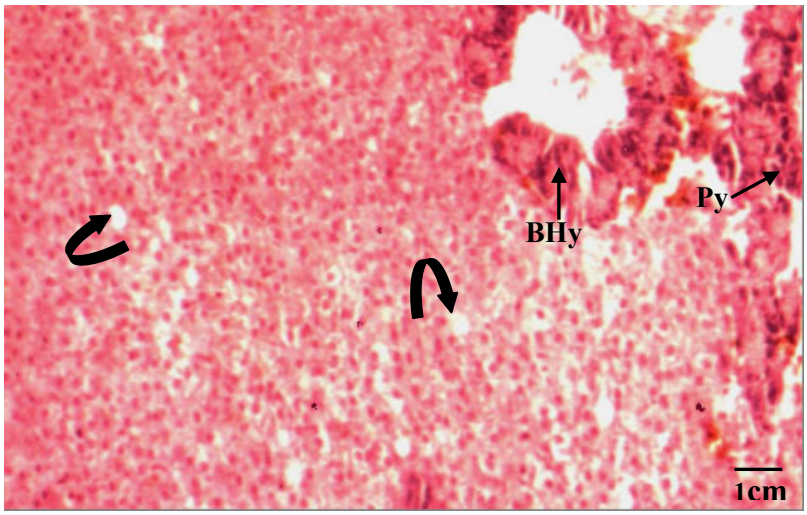


Fig. 5.3.3.22. Microphotograph of the liver in metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after supplementation with vitamin C. Note the apparent reduction in fatty vacuolation (curved block arrows) and number of pycnotic nuclei (Py). Slight increase in biliary hyperplasia (BHy). H & E, 20x.

Table 5.3.3.10. Histological changes in the liver of metacid 50 intoxicated *Oreochromis mossambicus* after supplementation with vitamin C.

Treatment	Percentage damage			
	Biliary hyperplasia	**Macrophage aggregates**	**Pycnosis**	**Fatty vacuolation**
Normal	**0.00**	**0.00**	**0.00**	**0.00**
16^{th} day after exposure to metacid 50 (1.24 ppb)	18.00	0.00	63.27	94.8
16^{th} day after vitamin C supplementation (500 mg vit C/kg diet)	19.65	0.00	21.36	5.98

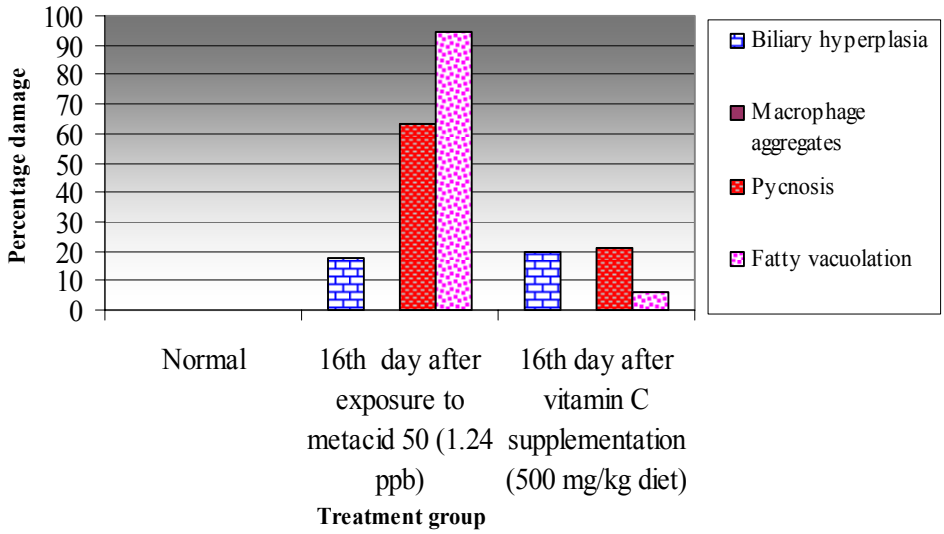


Fig. 5.3.3.23. Histological changes in the liver metacid 50 intoxicated *Oreochromis mossambicus* after treatment with vitamin C

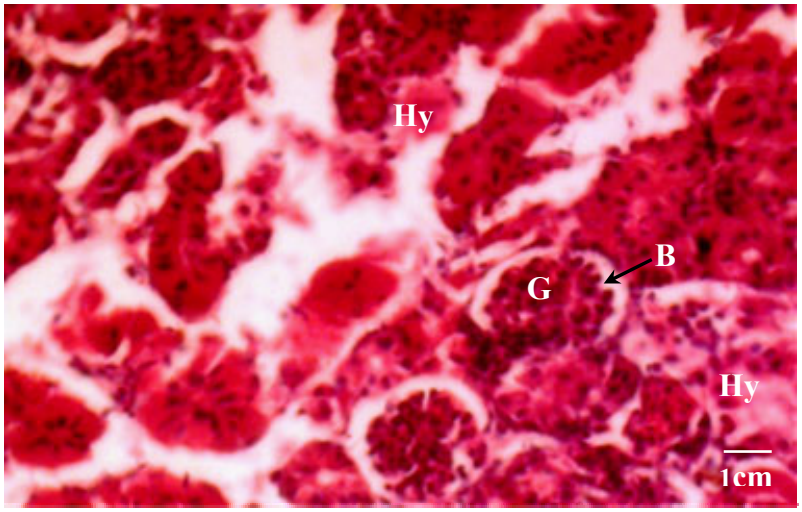


Fig. 5.3.3.24. Microphotograph of the kidney in metacid 50 intoxicated (1.24 ppb) *Oreochromis mossambicus* after supplementation with vitamin C. Glomeruli become swollen (G), Bowman's space narrows (B) and hyalinisation and degeneration of tubules (Hy) persist. However, hyalinisation decreases and tubular casts disappear. H & E. 40x.

Table 5.3.3.11. Histological changes in the kidney of metacid 50 intoxicated *Oreochromis mossambicus* after supplementation with vitamin C.

Treatment	Percentage damage						
	Glomerular shrinkage.	Glomerular swelling	Thickening of Bowman's space	Narrowing of Bowman's space	Tubular degeneration	Hyalinization.	Tubular casts.
Normal	0.00	0.00	0.00	0.00	0.00	0.00	0.00
16th day after exposure to metacid 50 (1.24ppb)	2.56	0.00	6.83	0.00	27.35	76.15	2.53
16th day after vit. C supplementation (500 mg vit C/kg diet)	0.00	5.12	0.00	6.23	24.20	49.00	0.00

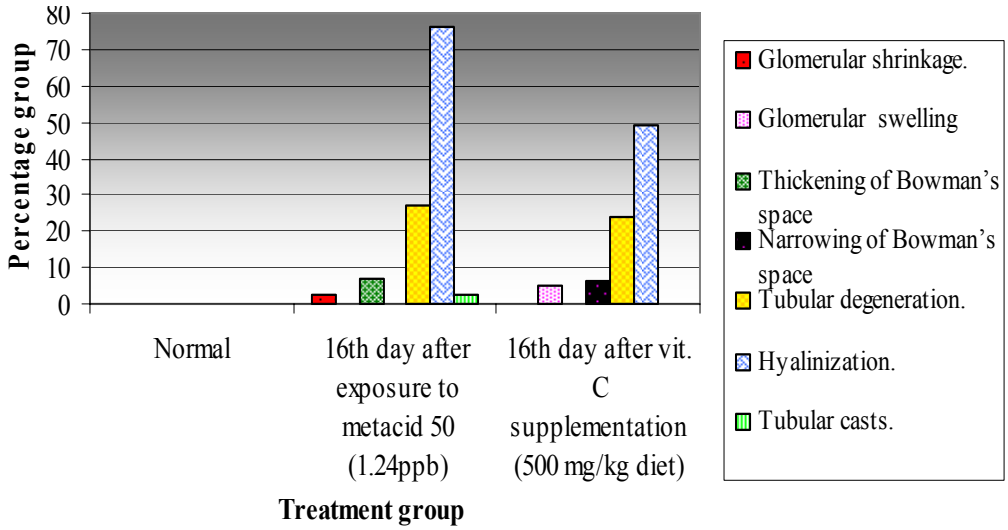
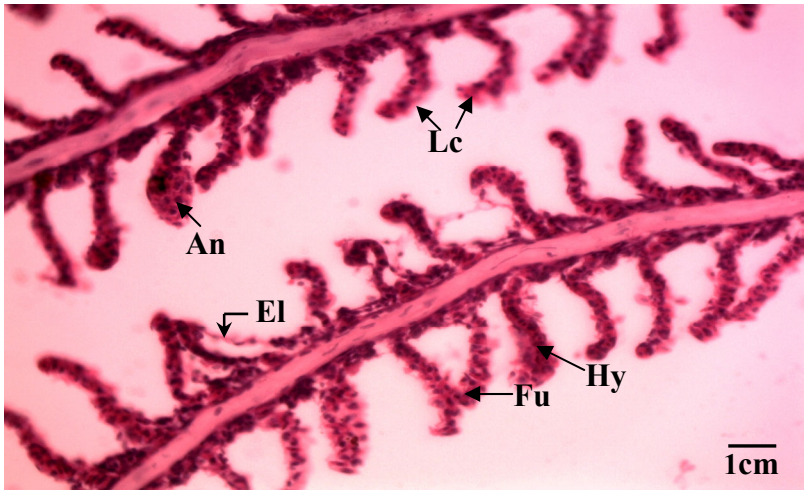


Fig. 5.3.25. Renal morphology of metacid 50 intoxicated *Oreochromis mossambicus* after supplementation with vitamin C

(a)



(b)

Fig. 5.3.3.26. a) Microphotograph of gill in metacid 50 intoxicated *Oreochromis mossambicus* after supplementing with vitamin C. H & E,20x. **b)** The same. H & E, 40x. Even though there is enhancement in secondary lamellar curling (Lc), there occurs reduction in hyperplasia (Hy), aneurism (An), lamellar fusion (Fu) and epithelial lifting (El). There is recovery from squamous metaplasia and exfoliation.

Table 5.3.3.12. Histological changes in the gills of metacid 50 intoxicated *Oreochromis mossambicus* after supplementation with vitamin C.

Treatment	Percentage damage							
	Lamellar curling	Lamellar fusion	Hyperplasia	Aneurism	Epithelial lifting	Necrosis	Squamous metaplasia	Exfoliation
Normal	0.00	0.00	1.50	0.00	0.00	0.00	0.00	0.00
16th day after metacid 50 exposure (1.24 ppb)	5.12	68.00	62.17	6.83	7.10	72.35	14.50	7.00
16th day after vitamin C supplementation (500mg/kg diet)	53.00	3.41	11.11	3.24	6.00	0.00	0.00	0.00

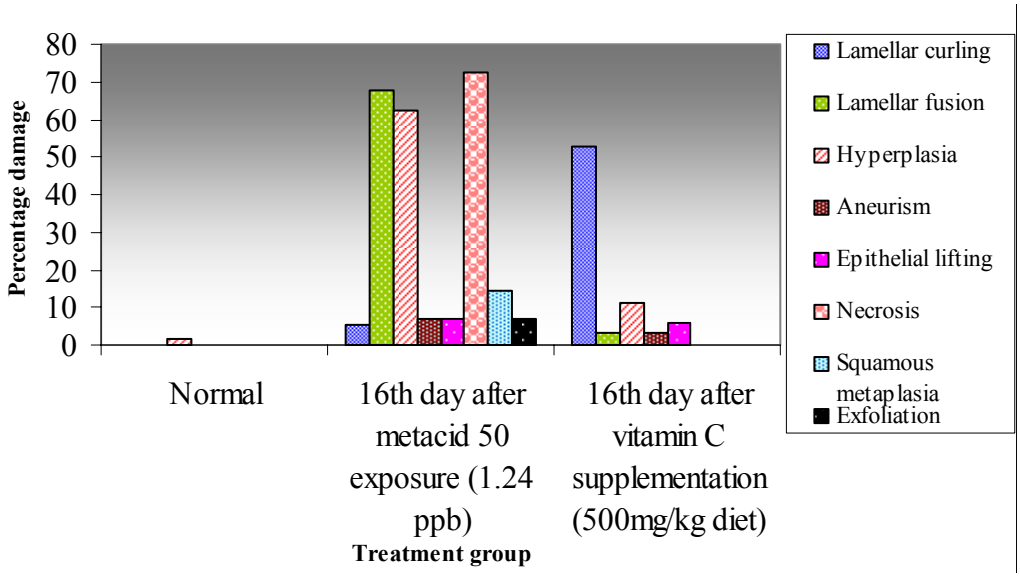


Fig. 5.3.3.27. Gill morphology of metacid 50 intoxicated *Oreohromis mossambicus* after supplementation with vitamin C

5.4 Discussion

Vitamin C supplementation restored the activities of catalase, superoxide dismutase, glutathione S transferase in exposed animals to near normal concentrations. Vitamin C reduces damage to cells by neutralizing toxic oxygen radicals thereby protecting the body against contaminants, aging and some diseases (Brody, 1999).

A close examination of the results of activities of different antioxidant enzyme assays after supplementing the diet of copper intoxicated *Oreochromis mossambicus* with vitamin C reveals some amelioration of toxicity in liver, kidney and gills. Decreased lipid peroxidation and enhancement of glutathione levels when compared to that shown by intoxicated group is an indicator in this direction. Moreover, the histology of the target organs showed repair of lesions to some extent thereby supporting such an assumption.

Liver and kidney of *Oreochromis mossambicus* revealing a near control level activity of catalase after providing them with vitamin C enriched diet is a hint of the tissue undergoing some repair process. Interestingly, the gills of the fish showed no significant variation in catalase activity even under high concentration of the vitamin. Furthermore, the branchial tissue showed clear indications of recovery from copper stress, as there occurred considerable reduction in lamellar curling, hyperplasia of respiratory epithelium and lamellar fusion and therefore it serves the justification of changes in antioxidant enzyme activity.

It was observed in general that in liver, kidney and gills, lipid peroxidation status rarely reached near normal activity but the vitamin

supplementation helped to reduce severity of the process; a fact further supported by the satisfactory level of glutathione.

The activity data of superoxide dismutase in test tissues after feeding with vitamin C – enriched diet seemed to iron out the toxicity of copper to some extent. The activity of the enzyme reached near normal level and at high concentration of the vitamin (500 mg vit C/kg diet) the enzyme activity was almost stable giving an indication of effective removal of superoxide radicals from the cells. In general, it is reported that low concentrations of ascorbate are required for pro-oxidant conditions, while high concentrations are needed for antioxidant conditions (Buettner and Jurkiewicz, 1996). The exceptionally low (compared to both control and intoxicated groups) activity of superoxide dismutase in gills observed on 4^{th} day after supplementation with 250mg/kg diet of vitamin C (Fig. 5.3.1.6) needs special mention. This is supposed to be because at low concentrations, ascorbate shows pro oxidant activity (Scarpa *et al.*, 1983, Buettner and Jurkiewicz, 1996) capable of generating amounts of superoxide radicals. This being an addition to the copper-induced superoxide production might have inhibited the activity of the enzyme, which regained the same as noted after 8^{th} day of exposure. This restoration of activity might be due to the continued supplementation of vitamin C reaching to a level sufficient to attain the antioxidant property. Thus, the 'crossover' effect of vitamin C vividly explains the situation. The result was in agreement with Palace et al., (1998) who observed significant reduction in the early mortality syndrome in lake trout (*Salvelinus namaycush*) inhabiting contaminant laden waters due to enhanced activity of superoxide dismutase and high vitamin C content in tissues.

High concentration of vitamin C in the diet was found to restore the activity of superoxide dismutase in liver and kidney of the fishes. Adham et al (2000) reported a powerful antioxidant effect of vitamin C on

biological water-soluble compartments of *Clarias gariepinus* and found that it reacts directly with superoxide anions, hydroxyl radicals and various lipid hydroperoxides. The same mechanism may be operating in the tissues thereby helping in the restoration of normalcy in superoxide dismutase activity.

It was interesting to observe that the activity value of the two glutathione – dependent enzymes (glutathione peroxidase and glutathione reductase) in liver, and kidney exhibited significant ($P< 0.05$) increase in comparison with control group and significant ($P< 0.05$) decrease when compared to copper intoxicated group. This was accompanied by a corresponding increase in reduced glutathione (when compared to the intoxicated group) content and decrease in lipid peroxidation (Fig.5.3.1.7,8,13,14,16,17,19,20). Here, it is worth mentioning an exception that the hepatic tissue maintained high activity of glutathione peroxidase accompanied by decreased level of glutathione (reduced) when compared to that of control group and no significant change from that of intoxicated group. This condition observed was independent of the vitamin C concentration in the diet. It is assumed that glutathione peroxidase continues to scavenge hydrogen peroxide at the expense of glutathione. As the liver of the ascorbic – acid supplemented group of fishes exhibited reduction in necrosis and pycnosis (Fig.5.3.1.22 and 23), a restoration from damage is inferred. The kidney of the fish also exhibited features of recovery from damage like disappearance of casts, and desquamation (Fig. 5.3.1. 24 and 25). Ascorbate; the deprotonated form of vitamin C occurring under physiological pH in animal tissues functions as a metal chelator and oxygen scavenger. Furthermore, ascorbate is effective in trapping both singlet oxygen and superoxide anions (Elbarassi et al., 2004). This also may be a reason for the repair

effects observed in liver and kidney of the experimental fish. Ahmad et al., (2000) assessed the activities of glutathione peroxidase in various tissues of *Channa punctatus* in response to paper mill effluents and found a time – dependent increase in glutathione level and activities of glutathione peroxidase in liver. Temporal and concentration dependent downturn in glutathione peroxidase activity in gills approaching near normalcy indicates that the tissue is recovering from the oxidative damage. To justify further, the morphology of branchial apparatus recorded total recovery from epithelial lifting, squamous metaplasia, and ballooning degeneration with associated reduction in hyperplasia of respiratory epithelium, and lamellar fusion (Fig. 5.3.1.26 and 27). The decrease in the content of reduced glutathione in gills when compared to control group (Fig. 5.3.1.21), point to its utilization by glutathione peroxidase.

The activity pattern of glutathione S transferase in liver of *Oreochromis mossambicus* revealed a concentration and duration (of vitamin C in diet) independent turn back when compared to the intoxicated group of fishes. As the condition backed by constancy of glutathione level and decreasing lipid peroxidation existed, inferred a mending of the antioxidant mechanism. Contrary to this, the kidney and gills of the vitamin treated fishes showed an increase in lipid peroxidation (when compared to the control group), even though the rate was significantly ($P< 0.05$) lower than the intoxicated group. Curiously enough, the level of glutathione in these tissues never regained the control status. Even under these circumstances, the kidney and gills maintained the increased activity of glutathione S transferase. It is understood that the increased activity of glutathione reductase in kidney (Fig. 5.3.1.14) helped to provide the supply of glutathione for the activity of glutathione

S transferase. Contrary to this, the gills revealed very low activity of glutathione reductase (Fig. 5.3.1.15). In spite of this, the tissue maintained high level of reduced glutathione (Fig. 5.3.1.21). In this context, it is deduced that the gills derived the glutathione from sources other than the activity of glutathione reductase. This should be so, because, the gills recovered from injury as evidenced by the microphotograph (Fig. 5.3.1.26).

Remarkable reduction in lipid peroxidation together with maintenance of elevated antioxidant enzyme activities and partial recovery from histological lesions exhibited by liver, kidney and gills of the malachite green stressed fishes after supplementation with vitamin C are clear cut indications of healing effect of the latter.

The liver of the malachite green intoxicated but ascorbic acid supplemented group of fishes revealing a synchronized high activity of catalase and glutathione peroxidase (Fig. 5.3.2.1 and 7) signifies the efficient removal of hydrogen peroxide derived from the toxicity of the test dye. The vitamin concentration in the diet imparts no significant effect ($P > 0.05$) on the level of activity of these enzymes. The decrease observed in the activity of glutathione peroxidase on 12^{th} and 16^{th} day after ascorbic acid treatment, when compared to 4^{th} and 8^{th} days may be connected with the corresponding activity increase of catalase in these days of concern, i.e the latter is superseding the former on 12^{th} and 16^{th} days in the scavenging of hydrogen peroxide. As glutathione peroxidase is involved in the abolition of organic hydroperoxides also, this situation seems to be normal. A condition akin to this was reported by Lushchak and Bagnyukova (2006) The dietary load (of vitamin C) - independent raised activity of superoxide dismutase in hepatic tissue accompanied by low levels of malondialdehyde is a signpost of the tissue gaining recovery

from the risk of accumulation of superoxide radicals. Similarly, dietary concentration (of vitamin C)- independent stepwise decrease in the hepatic glutathione S transferase activity reaching to that of control batch of fishes indicates the restoration of normalcy to some extent due to the glutathione- conjugated excretion of the toxicant by the liver. The activity data of glutathione reductase in the liver of vitamin supplemented group with higher than the drastic decrease on 16^{th} day after exposure to malachite green treated group and control group but less than the peak activity observed on 4^{th} and 8^{th} day after exposure also is an indication of lessening of oxidative damage (Fig. 5.3.2.13). This is especially so because the hepatic tissue revealed significant declension in the level of lipid peroxidation (Fig. 5.3.2.16). Though less than the control group of animals, the recovered level (when compared to the intoxicated group) reduced glutathione in liver, implies that this secondary antioxidant regained its activity due to the incorporation of vitamin C in the diet. Since liver is the major site for detoxification of exogenous and endogenous electrophilic compounds, it is therefore, important that this tissue possesses adequate amounts of the detoxifying enzymes like glutathione S transferase and its substrate, reduced glutathione (Abdalla et al., 2003).

Kidney of vitamin C supplemented *Oreochromis mossambicus* displayed a raised level of lipid peroxidation even in the presence of high level of glutathione (reduced); an indication of sustenance of oxidative stress. The assumption is supported by the declension in activity of glutathione S transferase and glutathione reductase, the magnitude of which was less than that of the control group though it was high on the 4^{th} day after vitamin treatment. This trend was independent of the amount of ascorbic acid in the diet. It is assumed that the degree of lipid peroxidation

outweighed the activity of these enzymes in kidney leading to the gradual declension in activity with the progress of exposure period. Nevertheless, the coordinated functioning of catalase and glutathione peroxidase in renal tissue seemed to eradicate hydrogen peroxide from the tissue. So also, the elimination of superoxide radicals from the tissue reached the maximum on 16th day after complementation with ascorbic acid, as the activity value of superoxide dismutase reached the maximum on the day. Curiously enough, the histopathological changes (Fig. 5.3.2.24 and 25) were not favoring a complete recovery from malachite green-induced damage. Except for reduction in tubular constriction and eosinophilic infiltration, the kidney remained unaltered after vitamin supplementation. In this context, the liver seemed to combat the dye-induced toxicity better than the kidney, both in respect of enzymatic profile and histological improvement. In other words, the lowered activity of glutathione related enzymes in kidney suggest that the renal tissue is less effective in detoxification and excretion of the dye.

The branchial apparatus of the fish, on the other hand, maintained a more or less healthy condition when compared to the dye-stressed group, as concluded from the activity status of antioxidant enzymes and changes in morphology. Though not reached to the magnitude of control group, malondialdehyde levels were significantly lower ($P< 0.05$) when compared to the intoxicated lot of fishes, indicating some degree of recovery from oxidative injury (Fig.5.3.2.18). As indicated by fig. 5.3.2.21, the content of reduced glutathione remained at a significantly higher ($P< 0.05$) level when compared to the intoxicated group, retaining the control status after an intermittent declension on 8th and 12th days. This retention after a lag probably indicates adaptation to the situation. To underline this assumption, the maintenance of high activity of glutathione

dependent enzymes like glutathione peroxidase, glutathione S transferase and glutathione reductase was referred (Fig.5.3.2.9, 12 and 15). Though the gills managed to maintain high level of catalase activity, thereby eliminating hydrogen peroxide from the tissue, the dismutation of superoxide radicals was at a diminished rate as assumed from the activity of branchial superoxide dismutase (Fig. 5.3.2.6). Though showed some degree of improvement when compared to the intoxicated group of fishes, its activity never turned back to control levels indicating the latent state of the enzymic machinery. The gills of the fish regained normalcy as indicated by considerable lessening of secondary lamellar fusion and curling and disappearance of exfoliation (Fig. 5.3.2.26 and 27). However, the commendable increase in hyperplasic state of respiratory epithelia is assumed as a progressive change helping in the increase of diffusion distance between respiratory blood and water-borne xenobiotic (Banerjee, 2007., Bernet et al., 1999), although the extreme of it may lead to asphyxiation and death.

None of the assessed biochemical parameters of liver, kidney and gills was dependent on the concentration of vitamin C in diet, indicating the sufficiency of 250mg/kg of ascorbic acid to trigger the lessening of oxidative stress.

The activity profiles of the preventive antioxidant enzymes in liver like catalase, superoxide dismutase, and glutathione peroxidase and glutathione reductase justifies the potential of vitamin C to impart recovery from oxidative stress resulting from the exposure to Metacid 50. As can be expected, the liver was found to defend the pesticide toxicity via the elevated activity of the efficient xenobiotic-conjugating enzyme glutathione S transferase. As in the case of copper and malachite green, the concentration of vitamin C elicited no significant difference in any of

the enzymatic activities. However, the lipid peroxidation status remained more or less elevated reluctant to return to the level equal to that of control group of fishes. Catalase activity in the liver of vitamin-supplemented group of fishes recorded significantly high activity when compared to control as well as intoxicated groups. Thus, the enzyme was assumed to shield the liver from the serious consequences of hydrogen peroxide (Fig. 5.3.3.1). Interestingly, the activity pattern of superoxide dismutase showed probably the maximum trying to keep away the harmful effects of superoxide accumulation. The hepatic glutathione peroxidase also behaved similarly with high activity at both the experimental concentrations of ascorbic acid and therefore the coordinated activity of catalase and glutathione peroxidase ensure a thorough defense against oxidative stress. As stated elsewhere, the hepatic tissue possesses high levels of organic toxicant metabolizing enzymes like glutathione S transferase. The progressively increasing glutathione levels accompanied by corresponding declension in its dependent enzyme activities is understood as these enzymes derive glutathione from the very beginning of the pesticide exposure which after the restoration of normal or near normal condition, retained the high levels. The overall picture of enzymatic activities, all of them maintaining high activity accompanied by reduction in lipid peroxidation suggests that vitamin C is capable of assisting the enzymatic machinery in the revival of oxidatively damaged tissue. In addition to this, there occurred some extent of revitalization of structural integrity of the hepatic tissue because of ascorbic acid supplementation (Fig.5.3.3.22 and 23). Drastic reduction of biliary hyperplasia, fatty degeneration and pycnosis need special mention. The findings are in conformity with the observations of Adham et al., (2000) who reported serious morphological changes in the vital organs of *Clarias gariepinus* resulting from vitamin C deficiency and associated oxidative

damage. Similarly, El Naggar and Lovell (1991) reported the efficacy of vitamin C in imparting structural integrity of liver in channel catfish (*Ictalurus punctatus*).

Kidney of *Oreochromis mossambicus* revealed high catalase activity throughout the experimental period (Fig. 5.3.3.2), an indication of continuation of oxidative defense when compared to the drastic declension on 16th day after exposure occurred in the intoxicated group. However, the significantly high ($P<0.05$) activity when compared to control is an indication of the persistence oxyradicals. On the other hand, the duration dependent increase in the activity of glutathione peroxidase accompanied by the duration dependent declension of reduced glutathione content (Fig.5.3.3.8 and 20), signifies the utilization of the latter by the former for maintaining the high activity thereby ensuring efficient scavenging of the pesticide-derived organic hydroperoxides and hydrogen peroxide. The pattern of high activity observed with superoxide dismutase, indicated revival of the superoxide removal as the activity of the enzyme declined significantly when compared to the control group of fishes (Fig.5.3.3.5). Significant declension in lipid peroxidation due to vitamin C complementation supports this assumption (Fig.5.3.3.17). The regeneration of glutathione (reduced) due to the activity of glutathione reductase (Fig. 5.3.3.14) seemed to assist the maintenance of glutathione resource required for the activity of glutathione S transferase and glutathione peroxidase. The efficacy of glutathione as a non-enzymatic antioxidant is clear from the dietary vitamin concentration-independent high activity of glutathione S transferase in renal tissue (Fig.5.3.3.11) which requires glutathione as the substrate for effective conjugation of the pesticide-derived metabolites. Contrary to the antioxidant enzyme profile, the histological damages suffered by the kidney seemed to improved less

due to vitamin C complementation (Fig. 5.3.3. 24 and 25) which need further studies for proper explanation.

The gills of the fish recorded commendable improvement of Metacid 50-induced toxicity due to the incorporation of vitamin C in the diet and lipid peroxidation; the index of tissue damage, was found to remain low and stable, independent of duration and concentration of ascorbic acid incorporation in the diet (Fig. 5.3.3.18). Similarly, the histological features of the gills indicated the revival from damage as there was considerable reduction in secondary lamellar fusion, hyperplasia, metaplasic state and disappearance of exfoliation of basal laminar cells and respiratory epithelia (Fig.5.3 3.26 and 27). The enhancement in lamellar curling was accompanied by decrease in lamellar fusion, as the recovery from the latter tends to maintain the bent nature of secondary lamellae. The vitamin supplementation facilitated the maintenance of high activity of catalase aiding in the removal of hydrogen peroxide. The drastic reduction in superoxide dismutase activity on 16^{th} day after exposure to Metacid 50, reaching the levels less than that of control group, was repaired after vitamin supplementation thereby facilitating the scavenging of superoxide radicals from the tissue (Fig. 5.3.3.6). The activity of glutathione peroxidase and glutathione S transferase recording high levels in the vitamin C supplemented group of fishes when compared to the pesticide-intoxicated group signifies the efficacy of these enzymes in countering the oxidative stress (Fig. 5.3.3.9 and 12). In addition to this, the activity of glutathione reductase decreased and reached a level equal to that of control group signifying the retention of its stability. The slight decrease in reduced glutathione levels on 12^{th} and 16^{th} after vitamin supplementation (Fig. 5.3.3.21) was accompanied by

corresponding high activity of glutathione peroxidase and glutathione S transferase denoting the utilization of the former by the latter.

To conclude, the supplementation of vitamin C in diet might help in rectification of oxidative damage in cardinal tissues of fishes.

Chapter -6

SUMMARY AND CONCLUSION

The present investigation mainly centres around the validity of analysing biochemical and histopathological biomarkers to assess toxic effects in fishes along with the exposition of ameliorative effects of vitamin C in repairing the damages caused by heavy metal, pesticide and drug toxicity. *Oreochromis mossambicus* is an interesting species to analyse such effects since the euryhaline capacity of the fish indicates the adaptability of the fish to changing salinity and differences of their habitat. The key enzymes concerned were assayed and histopathological evaluations were worked out and presented in the thesis.

The chapter on Introduction describes the aquatic ecosystems as the receptacle pollutants of anthropogenic origin, the concept and utility of biomarkers in environmental monitoring and the rationale behind the species selection for the present study. Harmful consequences of reactive oxygen species, cellular localization of antioxidant enzymes and the essentiality and role of vitamin C in piscine physiology are introduced based on literature survey.

Comprehensive information derived from investigations carried by different workers in the area of oxyradical research in general and in fishes in particular, are presented in the chapter on Review of Literature. The survey includes the studies concerned with the introduction of the concepts bioaccumulation, bioconcentration, biomagnifications and biomarkers, definition of oxidative stress, circumstances leading to

oxidative stress in aquatic organisms (including the presence of heavy metals, pesticides, and other xenobiotics), antioxidant machinery of fishes, relevance of vitamin C with reference to oxidative stress and bioassay techniques. An appraisal of malachite green as a therapeutic compound with reference to aquaculture and the significance of histopathological assessments in oxidative stress studies and pollution monitoring are also included in this part of the thesis.

Rapid lethal toxicity studies always gain attention as an important tool in bioassay methods. In this context, 96 hour LC_{50} assessment was carried out for copper and Metacid 50 in the test animal and the details are presented in the chapter on Lethal Toxicity Studies. The section introduces the test animal, brings out the methods of collection and maintenance in the laboratory, preparation of test solutions, procedures adopted, results and discussion. The 96 hour LC_{50} value determined for copper was 640 ppb and that for Metacid was 6.2 ppb. The behavioural responses of the fishes were more or less similar to both copper and the pesticide which included irritability to mechanical stimulation and strong phototrophic reactions. Rapid swimming, exhaustion and increased opercular rate were distinct behavioural responses.

The chapter entitled Sublethal Toxicity describes the biochemical and histopathological responses of the fish to the three test materials, *viz.* copper, Metacid 50 and malachite green. The section includes description about the major enzymatic and non enzymatic antioxidant defense mechanisms in fishes, the specific physiological roles played by the test organs (gills, liver and kidney) with special reference to xenobiotic absorption and/or biotransformation, localization of antioxidant enzymes in cells and their course of action, material and methods adopted for

sublethal toxicity assessment using antioxidative enzyme assays, histopathology and statistical analysis of data, results and discussion.

The analysis of data on the activity of primary antioxidant enzymes revealed interesting ways in which they behaved with enormous interplay to counter the oxidative stress induced by copper, Metacid 50 and malachite green; some of them submissive and some others defensive in the face of oxidative damage. The onset and progression of oxidative damage in the target tissues due to exposure to the experimental toxicants was indicated by the concentration and duration dependent increase in lipid peroxidation. The activity of the enzymes of first line defense category [i.e. preventive antioxidants concerned with the scavenging of superoxide radicals (superoxide dismutase), hydrogen peroxide (catalase), hydroperoxides (glutathione peroxidase) and regeneration of glutathione (glutathione reductase)] and the level of reduced glutathione, shifted significantly from that of control group of fishes. A concentration and duration dependent increase or decrease in the activity of different enzymes indicated oxidative damage. The high experimental concentration of copper, malachite green and Metacid 50 evoked different degree of activity in different tissues, the liver, the gill and the kidney of copper exposed fishes showing maximal activity of this enzyme capable of conjugated excretion of xenobiotics. However, activity of this enzyme decreased drastically in the gills at high concentration of the pesticide and its activity decreased with duration in the tissues under high concentration of malachite green. Thus the status of high glutathione S transferase activity as the specific biomarker of organophosphorous pesticides seems questionable as it applies more suitably to copper as indicated in the present study. Furthermore, it is understood from the current study that oxidative stress resulting from exposure to low concentration of toxicants

is defended more or less successfully by antioxidant enzymes and the mechanism often failed when the animal encountered high concentration of toxicants. Duration dependent increase in degenerative changes (pycnotic nuclei in hepatocytes and necrosis) occurs in hepatic tissue of the fish due to the toxicity of copper and malachite green. Non fatty vacuolation of hepatocytes and pancreatic tissue develops in liver due to the exposure to malachite green. Toxic exposure to Metacid 50 results in characteristic fatty degeneration in the liver of the fish. Kidney of the fishes exhibits inflammatory features due to exposure to the toxicants which includes swelling of glomeruli (glomerular shrinkage in the case of Metacid 50), thickening of Bowman's capsules, deposition of tubular casts and hyaline cytoplasm. The occurrence of intercapillary thickening of glomeruli, specifically known as 'wire loops' in histological terminology, was a special feature that resulted due to exposure to malachite green. The maintenance of more or less normal morphological nature of gills after exposure to malachite green indicates the free passage of the dye into the body of the fish. However, typical defensive (secondary lamellar hyperplasia and lamellar fusion) circulatory (hyperemia) and degenerative (necrosis and desquamation) changes manifest due to the toxicity of copper and Metacid 50. The significant increase in the intensity of damages in experimental tissues accompanying prolonged exposure, points to the risk of these fishes in chronically polluted environment. The study cautions against the liberal use of malachite green as an aquatic grade therapeutant. The present investigation recommends the utility of enzymatic and non enzymatic antioxidant responses as a biomarker to assess toxic effects.

The celebrated capability of vitamin C to repair oxidatively damaged tissues is tested, the details of which are illustrated in the fifth chapter.

The section covers the physiological role of the vitamin in fish followed by an account on material and methods. The results of antioxidant enzyme assays in the experimental tissues on supplementing the intoxicated fishes with vitamin C in diet, histopathology and discussion of the analyzed data are presented in the chapter.

It was found that biochemical and histological perturbations that resulted due to toxicity lead to certain range of restoration of the tissue morphology on supplying ascorbic acid through diet. The revival of antioxidant enzymatic activities that remained suppressed due to toxicity with associated reduction in lipid peroxidation and partial recovery from histopathological lesions are significant indicators to such repair. The maintenance of almost normal structural integrity of gills in spite of very low activity of superoxide dismutase under malachite green stress underlines the independent ameliorative effect of vitamin C which is capable of eliminating superoxide radicals from biological systems.

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APPENDIX

‡†ETHOLOGICAL ASPECTS OF CHROMATOPHORE DISTRIBUTION IN *Oreochromis mossambicus* (Peters)

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Introduction

The interaction between changes in colour pattern and activity in fishes is a debated subject. Opinions vary among scientists about the biological mechanisms of changes in pattern of body colour but many of the behavioural studies are linked with changes in colour pattern. The present work is made to identify the colour patterns in *Oreochromis mossambicus* (mossambique tilapia) which resulted during different stress situations like exposure to the therapeutic dye namely malachite green, manipulations in light regimes and/or changes in background shades and breeding activity under various laboratory conditions. The behavioural aspects of chromatophore distribution that imparts the different patterns of body colour are discussed.

Material and methods

The specimens of *Oreochromis mossambicus* with a size ranging from 3.80 cm to 12.00 cm in total length (7.50 g to 35.78 g in weight) were purchased from the State Fisheries Station ,Puthuvypeen,Govt. of Kerala, India and are acclimatized to dechlorinated tapwater held in 1000 L glass aquaria. A pH of 7.28± 0.02 and temperature of 23 ± 1°c were maintained in the experimental water and vigorous aeration was provided. They were fed twice daily with commercial pellet feeds @ 10% of the biomass.

Both the juvenile and adult tilapia were subjected to visual examination in stocking aquaria and another group was exposed to one

tenth and one twentieth of the therapeutic concentrations(0.05and 0.025ppm respectively) of the dye viz. malachite green (a triphenyl methane dye in its zinc free oxalate form). Observations were also made to study the behaviour and associated changes in colour pattern in relation to manipulations in light intensity. Combinations of all experimental situations were applied in addition to handling stress and changes in background shades from transparent to black, white and sandy. A uniform light intensity of 1400 lux was provided in rearing tanks and control tubs and that supplied in experimental containers include 4200 and 8500 lux.

Opercular beat rates were determined with the aid of a stopwatch and ten 1 minute observations were made on fish from each tank. The fishes were drawn at random and examined under a dissection microscope. Melanophore counts were made using an ocular graticule. Anaesthetics were not applied as *Oreochromis* will remain for periods upto 10 minute in a flat tray when their eyes are covered. Localised contraction of melanophores in the skin was induced by the application of 0.025 adrenalin. Photographs of the specimen were taken as and when required.

Results and discussion

Variations in chromatophore concentration were observed when the specimens were exposed to malachite green. No commendable differences in body colour pattern were noticed under the two experimental dye concentrations of 0.05 and 0.025 ppm.

The seven different patterns developed during various situations were summarized in Table. 1. and Plate.1 (refer p.7 for plates)

Table 1. Patterns of body colour shown by *Oreochromis mossambicus* under different situations in the laboratory.

Sl. No	Name of pattern	Appearance of the body colour
1	Neutral or inactive	Body as a whole is pale or yellowish white
2	Frightened juvenile	Barred with conspicuous 'tilapia mark'
3	Frightened adult	Darkening of the dorsal half of the body – common to both sexes.
4	Aroused adult	Darkening of caudal, anal and pelvic fins- common to both sexes.
5	Courting female	Whole body turns black, margins of pectoral, dorsal and caudal fins reddish.
6	Brooding female	Striped with a distinct mid lateral stripe, blackening of eyes, jaw and operculum.
7	Aggressive male	Intensified darkening of the body with whitish jaw and operculum, margins of dorsal and caudal reddish.

It has been found that the opercular beat frequency is 52 beats per minute for control individuals which shot up as high as 58 to 70 beats per minute for specimens under the stress of the dye and enhanced light intensity. (Refer Table 2.)

Table 2. Opercular beat rates (beats min^{-1}) in *Oreochromis mossambicus* under different concentrations of malachite green and light intensities.

Control	MG= 0.025 ppm	MG=0.05 ppm	Lux=4200	Lux=8500	MG=0.025 ppm Lux=4200	MG=0.05 ppm Lux=8500
55	58	69	57	59	68	72
56	57	60	60	59	69	74
45	60	59	56	61	72	75
50	67	61	58	58	72	76
51	66	64	60	57	69	74
48	63	62	59	60	70	73
54	61	61	55	58	74	70
57	65	57	56	61	70	75
58	59	63	58	60	68	74
53	64	63	61	59	71	76
X=52	X=62	X=62	X=58	X=59	X=70	X=73

MG- Malachite Green, Lux- intensity of light supplied

In addition to the increase in opercular beat rates, the frightened individuals exhibited spurting motion with flushing of water. Of the two light regimes selected, the higher intensity was found to be disturbing more for the individuals, particularly for those under the stress of malachite green. The results show that the presence of high intensity of light and malachite green caused a significant increase ($t > 2.35$, d.f=4, $P<0.05$) in opercular beat rates compared with those in control tanks.

The average melanophore index of a bar and of an area adjacent to that specific bar are compared in Table. 3.

Table 3. Average chromatophore index (Hogben and Slome, 1931) of a bar and of an area near to that bar in *Oreochromis mossambicus*.

No of fish	Total length of fish (cm.)	Chromatophore index within a bar	Chromatophore index in the area adjacent to the bar
1	4.20	2.10	1.00
2	4.80	3.50	2.50
3	5.50	3.50	2.30
4	6.20	3.00	1.80
5	7.90	3.50	2.00
6	8.20	2.50	1.00
7	9.00	3.50	2.40
8	10.40	3.00	1.50
9	11.60	2.00	0.80
10	12.00	2.50	1.20

The neutral ‘inactive’ pattern, the colouration of frightened juveniles and the pattern exhibited by frightened adults are inferred as behavioural changes elicited according to the differences in situation. Those

individuals which are left undisturbed in a tank receiving a normal uniform light intensity of 1400 lux and a white or transparent background were found to be pale in colour.

Apart from changes in shade and light intensity, mere handling stress and dragging of a net in the tank were found to influence the body colour patterns. Presence of the dye and manipulation of photoperiod were the augmenting factors.

The various colour patterns can be explained with the help of informations about the development and localization of chromatophores in *Oreochromis mossambicus* . It is obvious that these colour patterns are liable to change during the life of the fish and with its mood or motivation. The juvenile patterns are basically variations of bars or stripes produced as a result of regularly spaced areas of high melanophore intensity. Melanophores distributed on distal part of the scale and those lying over their proximal portion are responsible for the formation of such markings. The chromatophore aggregation is so intense that the bars are get concealed in spite of the high melanophore concentration. Therefore it can be concluded that the presence of a nerve network that selectively influence the bar and stripe melanophore pattern cannot be overlooked. The appearance of bars and stripes is probably associated with the state of arousal leading to anxiety or fright. The ‘tilapia mark’ in juveniles was more intensely discernible in frightened state. The mark was always co existent with the barred pattern.

The tendency of the individuals to imitate the colour of the background may be presumably due to the optical impulse from the eye reaching the brain from where a motor impulse is transmitted to the muscle fibre connecting to the chromatophore. The same is applicable to

the responses to differences in light intensity. But the changes in colouration and markings of mature adults are evidently related to breeding behaviour as is the case with many of the cichlid species. But these adult patterns were retrievable to neutral pattern upon perturbations due to handling, variations in light intensity, and presence of the dye. Interestingly the adult patterns were more or less indifferent to such interferences in course of time. In this context, the possibility of a 'learning' process cannot be ruled out.

The maximum dispersion of different chromatophore systems suppressing the chromatophores of body musculature (containing melanophores, melanoirridophores and guanophores) is supposed to be the mechanism behind the pattern formation in aggressive individuals particularly in the aggressive males. The reddish hues developing on various fins are always attributed to the expansion of erythrophores.

Background matching as a means of predation avoidance (Endler,1978) could be an explanation for the association between colour change in tune with the changes in background colour. Communication among individuals under experimental situations is supposed to be another causative factor for the colour variations during stress. The interplay of melanocyte stimulating hormone(MSH) and melanocyte concentrating hormone(MCH) secreted by the pituitary and nerve connections to the muscle fibres underlying the scales is necessary to bring about the changes in patterns of body colour in fishes.

The role of manipulations in environmental factors and the influence of sex hormones in effecting the alterations in body colour patterns need further investigations.

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PLATE. 1. Situational colour patterns in *Oreochromis mossambicus*.

Brooding female

'Inactive'

Courting female

Frightened juvenile

Activated adult

Aroused adult

Aggressive male