BIOCHEMICAL EFFECTS OF THE PESTICIDE CHLORPYRIFOS ON THE FISH *OREOCHROMIS MOSSAMBICUS* (PETERS)

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Certificate

This is to certify that the thesis entitled "**Biochemical effects of the pesticide Chlorpyrifos on the fish O*reochromis mossambicus* (Peters)**" is an authentic record of the research work carried out by Ms.Aniladevi Kunjamma K P under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, in partial fulfilment of the requirement for the degree of Doctor of Philosophy in Biochemistry of Cochin University of Science and Technology, and that no part thereof has been presented for the award of any other degree, diploma or associateship in any university.

Cochin-16
April 2008

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Declaration

I hereby declare that the thesis entitled "**Biochemical effects of the pesticide Chlorpyrifos on the fish *Oreochromis mossambicus* (Peters)**" is a genuine record of research work done by me under the supervision and guidance of Prof. Dr. Babu Philip, Professor of Marine Biochemistry, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology. The work presented in this thesis has not been submitted for any other degree or diploma earlier.

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ABSTRACT OF THE THESIS

Man uses a variety of synthetic material for his comfortable materialistic life. Thus human interactions may become harmful for various terrestrial and aquatic lives. This is by contaminating their habitat and by becoming a threat to organisms itself. Thus the application and dispersal of several organic pollutants can lead to the development of several mutated forms of the species when exposed to sublethal concentrations of the pollutants. Otherwise, a decrease in number or extinction of these exposed species from earth's face may happen. Pesticides, we use for the benefit of crop yield, but its persistence may become havoc to non-target organism. Pesticides reaching a reservoir can subsequently enter the higher trophic levels. Organophosphorus compounds have replaced all other pesticides, *due to its acute toxicity and non-persistent nature.*

Hence the present study has concentrated on the toxicity of the largest market-selling and multipurpose pesticide, chlorpyrifos on the commonly edible aquatic organism, fish. The euryhaline cichlid *Oreochromis mossambicus* was selected as animal model. The study has concentrated on investigating biochemical parameters like tissue-specific enzymes, antioxidant and lipid-peroxidation parameters, haematological and histological observations and pesticide residue analysis.

Major findings of this work have indicated the possibility of aquatic toxicity to the fish on exposure to the insecticide chlorpyrifos. The insecticide was found as effective to induce structural alteration, depletion in protein content, decrease in different metabolic enzyme levels and to progress lipid peroxidation on a prolonged exposure of 21 days. The ion-transport mechanism was found to be adversely affected. Electrophoretic analysis revealed the disappearance of several protein bands after 21days of exposure to chlorpyrifos. Residue analysis by gas chromatography explored the levels of chlorpyrifos retaining on the edible tissue portions during exposure period of 21days and also on a recovery period of 10days.

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Abbreviations

β	- beta
µg	- microgram
<	- less than
>	-greater than
0	- degree
2, 4-DNPH	-2, 4- dinitrophenyl hydrazine
AChE	- Acetylcholinesterase
ACP	- acid phosphatase
ALP	- Alkaline phosphatase
ALT	- Alanine amino transferase (Alanine transaminase)
ANOVA	- Analysis of Variance
ATP	- Adenosine triphosphate
ATPase	- Adenosine triphosphatase
BCF	- Bioconcentration factor
C	- Celsius
C^{14}	- Carbon isotope
Ca^{2+}	- calcium ion
ChE	- cholinesterase
cm	- centimeter
Con	- concentrated
Cumm	- cubic millimeter
dl	- deciliter
DNA	- Deoxy ribonucleic acid
E C	- enzyme commission number
EDTA	– Ethylene diamine tetra aceticacid
EPA	- Environmental Protection Agency
ER	- Endoplasmic reticulum
GC	- gas chromatography
GPX	- glutathione peroxidase
GR	- glutathione reductase

GSH	- reduced glutathione
GSSG	- oxidized glutathione
GST	- glutathione-S-transferase
H_2SO_4	- sulfuric acid
ha	- hectre
Hb	- hemoglobin
HCl	- Hydrochloric acid
Hg	- Mercury
hr	- hour
IU	- International unit
IUPAC	- International Union of Pure and Applied Chemistry
K^+	- potassium ion
K_2HPO_4	- di potassium hydrogen phosphate
KCl	- potassium chloride
kg	- kilogram
KH_2PO_4	- potassium dihydrogen phosphate
L	- Litre
LC_{50}	- lethal concentration causing 50% mortality
LSD	- Least Significant Difference
M	- molar
Mg^+	- magnesium ion
$MgCl_2$	- magnesium chloride
mL	- milliliter
mm	- millimeter
Mmol	- millimol
N	- normal
Na^+	- sodium ion
NaCl	- sodium chloride
NaCl	- Sodium chloride
NaOH	- sodium hydroxide
nm	- nanometer
nm	- nanometer
OD	- optical density
OP	- organophosphorus/ organophosphate

P value	- probability value
PAGE	- Polyacrylamide gel electrophoresis
PCV	- packed cell volume
p-nitrophenol	- para-nitrophenol
ppb	- parts per billion
ppm	- parts per million
RBC	- red blood corpuscle
RNA	- Ribonucleic acid
rpm	- rotations per minute
RT	- room temperature
SDS	- sodium dodecyl sulphate
SDS	- Sodium dodecyl sulphate
SOD	- superoxide dismutase
TCA cycle	- tricarboxylic acid cycle
TCA	- tetra chloro acetic acid
TCP	- 3,5,6-trichloropyridinol
TEMED	- N,N,N',N'-tetramethyl ethylene diamine
WBC	- white blood corpuscle

Chapter

1

INTRODUCTION

Contents

1.1 Environmental pollution

Man-made toxic chemicals are released into the environment during production, transportation as well as utilization, and thus pose a threat to living biota. Therefore the assessment of environmental hazards due to toxic substances is an important challenge to toxicologists and ecotoxicologists (Braunbeck, 1994).

Pollution is the unfavourable alteration of our environment, largely because of human activities. Environmental pollution, especially water pollution, has been increasing at an alarming rate due to rapid industrialization, civilization and green revolution. The deviation from the natural composition of a part of the environment results in adverse effects on life.

Pollutants are substances, which cause pollution. They may be the substances that occur in nature or unnatural substances released into the environment by human handiwork- eg. pesticides, herbicides etc (Kurian, 1997).

1.2 Pesticides

In the last 50years, there has been a steady growth in the use of synthetic organic chemicals such as pesticides. Pesticides are used widely all over the world to control the harmful effects of pests and hence to increase the agricultural productions. Pesticides include many specific chemical substances that can be grouped according to the type of pest they are intended to control. They represent artificial man-made materials, which are largely or entirely foreign to environment. The environmental impact of pesticide use has been discussed much due to its widespread use in parallel with the modernization of agricultural operations and indiscriminate permeation of the ecosystem with these pesticides.

In modern agricultural operations, a variety of pesticides such as organophosphate, organochlorine, carbamate and pyrethroid groups of pesticides and different types of inorganic fertilizers such as ammonium chloride, diammonium phosphate and urea are used. These have considerable advantages over the natural products in that they are potent, selective and comparatively cheap (Connell, 1984).

The discovery that organochlorine pesticides such as DDT, are highly persistent, bioconcentrate in food chains and can severely affect whole populations or species of wildlife has led to bans and restrictions in their use (Stickel, 1975). The decreased use of organochlorine pesticides has further expanded the market for less-persistent but acute toxic pesticides including organophosphate compounds.

1.3 Organophosphorus pesticides

Although research on organophosphorus compounds began as early as the 19th century, the insecticidal activity of the chemicals was discovered only in 1937. Organophosphates were rapidly developed as insecticides in Germany during World war II and in the 50's the commercial use of organophosphates expanded markedly. Presently, organophosphorus pesticides are used as insecticides, herbicides, nematicides, acaricides, fungicides, rodenticides and bird repellents throughout the world.

Insecticides are very important input in rice agro-system in India and its application has been reported to cause significant increase in crop yield. Among different molecules, granules of carbofuran and emulsifiable concentrates of chlorpyrifos and monocrotophos are used widely and they have been found effective against most of the insect pests (Pathak et al., 1974). Some of the properties that have attracted investigators to develop organophosphate esters as pesticides are their relatively high acute toxicity, the limited residual nature of the molecule and the relatively low cost of manufacture (Ronald, 1981). Organophosphorus insecticides were developed, for their insecticidal activity. Many are very toxic to vertebrates, and they can cause hazards to wildlife (Stanley and Bunyan, 1979). There are several acetylcholinesterase inhibitors, which are used particularly as pesticides and nerve gases in chemical warfare as well (Timbrell, 1982).

Organophosphorus insecticides are widely extended and they are used not only in farming purposes, but also in households, several industries, medicine and even as chemical weapons. Most organophosphorus insecticides are regarded as being non-persistent, but some reports have indicated that residues of

organophosphates remain essentially unaltered for extended periods in organic soils and surrounding drainage systems (Harris and Miles, 1975). It has also been observed that more water soluble, less persistent insecticides such as diazinon and parathion were not strictly absorbed by sediment while the less soluble, more persistent insecticides such as chlorpyrifos tended to be strongly absorbed by soil and sediments (Sharom et al, 1980).

According to WHO, the incorrect use of organophosphorus insecticides is responsible for a great number of cases of acute poisoning, characterized by the development of cholinergic syndrome and multiple chronic complications, with neuropathy being one of the most presented symptom. These complications are very important because their frequency is progressively increasing and they may go unnoticed (Carod, 2002).

A bewildering variety of pesticides, bought easily and used carelessly by farmers, are contaminating foodstuffs and posing health hazards, according to several surveys. Indiscriminate use of pesticides resulted in 3000 human kills in Madras and 361 in Maharashtra during 1971 (Tinbergen et al, 1976).

1.4 Aquatic toxicology

Aquatic toxicology has been defined as the study of the effects of chemicals and other toxic agents on aquatic organisms with special emphasis on adverse or harmful effects. It is apparent that addition of chemicals by humans to the earth surface has introduced or increased environmental stress for aquatic organisms and fishes, in particular. Dispersal of the pollutants results in contamination of natural terrestrial areas while water-runoff transfers quantities to fresh water areas and ultimately the oceans. Toxicants like pesticide and other chemicals find their ways into the fresh water bodies and have produced unexpected consequences on aquatic fauna.

Among the pollutants, pesticides rank a very important position, since pesticides and technical organic chemicals comprise the most dangerous group of pollutants. It is realized that these substances are totally alien to aquatic organisms.

Today, the use of pesticide is widespread on agricultural crops, rangelands, forests and wetlands and this undoubtedly exposes many wildlife species to chemical hazards. Many pesticides need to be resistant to environmental degradation so that they persist in treated areas and thus their effectiveness is enhanced. This property also promotes long-term effects in natural ecosystems. The excess amounts of these pesticides and chemicals produce unwanted and unwarranted residues, which pose a great threat to aquatic organisms (Ramasamy et al, 2007). They are toxicants capable of affecting all taxonomic groups of biota, including non-target organisms, to varying degrees, dependent on physiological and ecological factors.

Pesticides in running water may contaminate the ground water also. Lindane, methoxy-chlor and dieldrin have been found from time to time in the polluted seawater near agricultural sites. Marine organisms such as sea urchins, fishes etc. are likely to encounter these compounds, the major sources are run-off from treated farmlands, industrial domestic sewage, spillage and direct application to waterways such as in herbicide treatments and aquatic crop treatments (e.g.: rice production).

Pesticides are poisons and would be expected to have adverse effects on any non-target organism having physiological functions common with those of the target that are attacked or inhibited by the pesticide. Avian, mammalian, amphibian, piscine and reptilian species coming in direct contact with the insecticide application may also be affected.

Marine ecosystems have no or only limited capacities for metabolizing and degrading the synthetic organic compounds and their derivatives. Hence pesticides and technical organic chemicals released into marine and fresh waters tend to accumulate and cause long-term effects. Organic pollutants in the aquatic environment comprise a vast and ever-increasing range of compounds, including polyaromatic hydrocarbons (PAHs), PCBs, dioxins, nitroaromatics, aromatic amines, organophosphate and organochlorine pesticides and phthalate ester plasticizers. Physical, chemical and biological processes affect the distribution and fate of these substances in the marine environment. Such foreign compounds (xenobiotics) are fat-soluble and are therefore readily taken up from the water, sediment and food sources

into the tissues of aquatic organisms (Walker and Livingstone, 1992). Lipophilic nature of water-insoluble pesticides enhances its ability to cross the plasma membrane, when the pesticides come in contact with the aquatic organism.

Factors influencing the uptake and distribution of pesticides in biological systems are related to:

Inherent physical and chemical properties of the pesticide (eg: volatility, solubility in water and fat and sorption characteristics)

(1) Physiological characteristics of various species (eg: feeding behaviour, routes of uptake and habitat) and

(2) Ecosystem specific properties (eg: types of flow systems, temperature, pH, organic matter, and food web structure etc. (Connell, 1984).

The aquatic medium is a very efficient solvent for many chemical compounds or components there of. Consequently the aquatic organisms are extremely vulnerable to toxic effects resulting from absorption or oral intake of these contaminants from the immediate environment (Meyers and Hendricks, 1982).

The methods used for pesticide application (spraying and dusting) enable them to enter, and to pose a risk to, the aquatic ecosystem (Johnson, 1973). Due to their widespread distribution and toxic nature, pesticides may have a serious impact on the aquatic environment and have been shown to exert adverse effects on the associated organisms (Singh and Reddy, 1990). Recently, more concern has been expressed about chemicals such as organophosphates and avermectins used directly in support of the control of fish parasites, and in particular the marine crustacean parasites (Roth et al., 1993). These concerns relate both to possible effects on the environment and also to potential problems due to residues remaining in the fish. Such chemicals create toxicity in fish as well as in human beings when it is consumed. For these reasons such usages are now closely controlled in most developed countries (Woodburnt, 1995).

Indirect effects can be observed when the insecticide is moved from the application site to another site. There it may be accumulated at several trophic levels

to become toxic at the top of a food chain or reach the secondary site in concentrations that are toxic to non-targets. The overall effects of pesticides on non-targets can be categorized as follows- (a) reduction of species numbers (b) alteration of habitat with species reduction (c) changes in behaviour (d) growth changes (e) altered reproduction (f) changes in food quality and quantity (g) resistance (h) disease susceptibility (i) biological magnification (Pimentel, 1971).

During initial exposure to a xenobiotic, the first component, the concentration gradient between the environment and the fish, will be equal to the aqueous dissolved concentration of the xenobiotic in the environment. This will persist for some time because, immediately after entering the blood, lipophilic compounds will dissolve in lipids and bind to proteins (Schmieder and Henry, 1988). Fish are known to have very inefficient mixed function oxidase systems to detoxify these insecticides which make them vulnerable to environmental contaminants (Matsumura, 1980).

Adverse effects at the organismal level include both short-term and long-term lethality (expressed as mortality or survival) and sublethal effects such as changes in behaviour, growth, development, reproduction, uptake and detoxification activity and tissue structure. Adverse effects at suborganismal level include induction or inhibition of enzymes and/or enzyme systems and their associated functions. A working knowledge of aquatic ecology, one or more biological sub disciplines such as physiology, biochemistry, histology and behaviour and environmental chemistry is required to understand the effects of toxic agents on aquatic organisms. Thus toxicity tests are used to evaluate biochemical changes and the duration of exposure required to produce the criterion effects (Gary and Rand, 1995).

Species differences in behaviour, feeding ecology, receptor sensitivity and pharmacokinetics result in greater than 1 million fold variation in sensitivity to chlorpyrifos among species (Barron and Woodburn, 1995; Marshall and Roberts, 1978). Specifically, individual and species susceptibility to chlorpyrifos is related to the binding affinity of chlorpyrifos oxon to AChE and to its subsequent rate of inactivation.

The present study was undertaken to evaluate the toxicity of the largest market-selling and multipurpose insecticide chlorpyrifos, on the commonly available and edible aquatic organism fish. The study was carried out with special emphasis on behavioural, morphological, biochemical, haematological and bioaccumualtion effects of the insecticide, chlorpyrifos in the fish *Oreochromis mossambicus* (Peters).

1.5 Objectives of the study

The present investigation aims at elucidating the aquatic toxicity of the selected organophosphorus pesticide chlorpyrifos on fish. The research programme involved the biochemical studies by comparing a control group with the test group of fishes exposed to pre-determined dose of the pesticide. The study of biochemical effects and chemistry of the pesticide would lead our attention to the extent of toxic impact of the pesticide to reduce its unnecessary use and improve the yield from agricultural field employing the natural alternative methods to control pests.

Thus the main objectives of the present study include

- To study the oxidative stress by pesticide on piscine tissue biochemical parameters.
- To elucidate the levels of antioxidant enzymes of the fish exposed to the pesticide
- To find out the effect of pesticide on membrane stability
- To find out the gross anatomical and histological changes induced by the pesticide
- To study the changes in haemotological parameters and electrophoretic pattern of the serum proteins
- To quantitate the amount of pesticide residue in the edible body parts of the pesticide treated fish.

Chapter

2

Review of Literature

Contents

The chapter, review of literature presents the investigations made in different parts of the world, on aquatic organisms after exposure to pesticides, especially organophosphorus compounds. The scientific papers are mainly be categorized under those relating to the solubility of the selected pesticide, biochemical aspects, haematological findings, histological observations and pesticide residue-studies.

2.1: Solubility of chlorpyrifos

The rapid dissipation of chlorpyrifos from aquatic ecosystems has important implications for aquatic risk assessment. Toxicity profiles observed during prolonged, constant concentration exposure in the laboratory may not accurately reflect toxicological responses to pulsed and rapidly declining concentrations in water under field conditions.

The major chlorpyrifos derivative, 3,5,6-trichloro pyridinol (TCP), does not cause cholinesterase inhibition and is of low to moderate toxicity to aquatic and terrestrial biota. Evaluation of LD_{50} values (mg/kg) indicated that aquatic insects (Siegfried, 1993) might be more sensitive than terrestrial insects. Chlorpyrifos is primarily used to kill mosquitoes in the immature larval stages of development. It is generally considered to be non-persistent in the environment (Sharom et al, 1980).

Many organic substances are much more soluble in lipids than in water. These compounds enter animals because they are lipid soluble and then accumulate in the body fat of the animal. This bioaccumulation of substances in animals due to the high fat solubility of compounds has long been recognized (Randall et al, 1996).

According to Mace and Woodburnt (1995) the predominant determinant of chlorpyrifos toxicity to fish appears to be the test species, but toxicity may be influenced by exposure conditions, formulation, source and size of fish and water quality. Water hardness and pH do not appear to influence toxicity in laboratory tests because chlorpyrifos is nonpolar and non-ionizable. However, pH and temperature can affect the dissipation rate in water, which may influence

environmental exposures (Racke, 1993). Size of fish has been reported to influence toxicity in static tests (eg., El-Refai et al, 1976), possibly because absorption by the fish decreases the exposure concentration (Barron et al, 1993).

Technical grade chlorpyrifos generally appears to be of similar or greater toxicity than controlled release or emulsifiable concentrate formulations with lower LC_{50} values (Jarvinen and Tanner, 1982).

In many of the pesticide toxicity studies, acetone has been used as the vehicle. Acetone was used by Bakthavathsalam and Reddy (1983) to prepare the test solution of lindane and Miny and Sastry (1989) in the preparation of Monocrotophos solution. David (2005) used analytical grade acetone to prepare fenvalerate test solution and Shivakumar and David (2004) to prepare the solution of endosulfan. Acetone was found to be non-toxic to fish (Pickering et al, 1962). When embarking upon a series of toxicity studies, whenever possible, the test article to be investigated should be "technical grade" material of similar composition to what humans are expected to be exposed to and the vehicle used in formulating the test article is also appropriate for use as the control (Keller and Banks, 2006). According to Mallinckrodt Chemicals, acetone is expected to readily biodegrade and quickly evaporates, when released into water. This material has a log octanol-water partition coefficient of less than 3.0. This material is not expected to significantly bioaccumulate.

2.2: Biochemical studies

A number of studies have been made on the toxicity of different pesticides on aquatic and terrestrial organisms. Most of the studies dealing with the effects of pesticides on fish primarily focus on the short-term investigations involving whole animal responses such as gross abnormalities, behavioral changes in growth rate and mortality. Recently, more research is being conducted on physiological and biochemical responses of the agricultural pesticides on fish. In general, the pesticides increase the activities of some enzymes and decrease the activities of others, while the activities of a few enzymes remain unchanged in various tissues of

fish. Signs of acute toxicity in fish include increased cough frequency and ventilation volume, and decreased ventilation frequency (Bradbury et al, 1991).

The chronic toxicity of chlorpyrifos to fish has been evaluated in early life stage studies (embryo or larval stage through juvenile life stages) and full life cycle studies. In general, growth was the most sensitive measure of toxicity in the majority of chronic toxicity tests with chlorpyrifos (Cripe et al, 1986; Goodman et al, 1985a; Jarvinen and Tanner, 1982). In addition to effects on growth and survival, reported sublethal effects of chlorpyrifos include behavioural avoidance, changes in temperature preference and biochemical alterations (Mace et al, 1995).

Alterations in the chemical composition of the natural aquatic environment usually affect behavioural and physiological systems of the inhabitants, particularly those of the fish (Radhaiah et al, 1987). The fish show restlessness, rapid body movement, convulsions, difficulty in respiration, excess in mucus secretion, changes in colour and loss of balance when exposed to pesticides. Similar changes in behaviour are also observed in fishes exposed to different pesticides (Haider and Inbaraj, 1986). Liver, kidney, brain and gills are the most vulnerable organs of a fish exposed to the medium containing any type of toxicant (Jana and Bandyopadhyaya, 1987).

Fish species are sensitive to enzymatic and hormone disruptors. Chronic exposure to low levels of pesticides may have a more significant effect on fish populations than acute poisoning. Doses of pesticides that are not high enough to kill fish are associated with subtle changes in behaviour and physiology that impair both survival and reproduction (Kegley et al, 1999). Experimental exposure of fish to pesticides has been shown to depress protein values in brain, gills, muscle, kidney and liver. In the kidney and liver, stress-induced significant decrease in the protein content was observed by Tilak et al, 1991.

A significant decline was observed in the globulin content in the blood of chlorpyrifos-treated mice compared to the control mice. A significant decrease in acetylcholinesterase was evident in chlorpyrifos treated mice. Hegazi (1989)

reported that sublethal concentrations of chlorpyrifos reduced brain Acetylcholinesterase (AChE) of the catfish (*Clarias lazera*). He also reported that sublethal concentrations of chlorpyrifos reduced muscle and liver glycogen and blood glucose levels of *C.lazera*. The inhibition of acetylcholinesterase by organophosphate compounds has become an index of organophosphate pollution in the aquatic environment (Williams and Sova, 1966). Organophosphates effectively poison the enzyme by phosphorylation and thus block the hydrolysis of acetylcholine. This group of pesticides interferes with the process of synaptic transmission by inhibiting the activity of acetylcholinesterase. This enzyme is important for the neurological functioning of the sensory, integrative and neuromuscular systems in fish. The inhibition of this enzyme alters respiration (Klaverkamp et al, 1977), swimming (Post and Leisure, 1974) and social interaction (Symons, 1973) in salmonides. Chlorpyrifos causes many damages to human and animal health. Its effects on nervous system are well known through the inhibition of the acetylcholinesterase enzyme, which plays an important role in neurotransmission at cholinergic synapses by rapid hydrolysis of neurotransmitter acetylcholine into choline and acetate (Garcia et al., 2005). Several reports suggest that various organophosphorus pesticides at concentrations close to their LC_{50} values can induce a decrease in the enzyme level to 60-20% of their normal physiological activity in fish. Similar changes were found and used by various authors to evaluate the effects of these pesticides on fish (Salte et al, 1987).

The exposure of fish to organophosphates inhibits the activities of several enzymes such as glucose-6-phosphatase, acid phosphatase, pyruvate dehydrogenase, succinate dehydrogenase and acetylcholinesterase in brain. Inhibition in Cytochrome oxidase activity in brain, kidney, gill, liver and muscle have reported by Sastry and Sharma, 1980; Natarajan, 1984. Sastry and Sharma showed that the activity of alkaline phosphatase, ATPase and lactate dehydrogenase remained unchanged in the brain of *Ophiocephalus punctatus* after 15 days of exposure to an organophosphate pesticide. The exposure of methyl parathion to the freshwater fish, *Tilapia mossambica*, for 48hr decreased the activity of succinate and lactate dehydrogenase in the gill, liver and muscle tissues (Rao and Rao, 1979).

Reddy et al (1986) reported an increase in the activity of acid phosphatase in the hepatopancreas of the crab *Oziotelphusa senex* after Methyl parathion exposure. From the study it is inferred that the exposure attributed the stimulation of acid phosphatase activity, alteration in osteoblasts which resulted in more production and liberation of the enzyme, proliferation of smooth endoplasmic reticulum in the parenchymatous cells, that leads to increased production and release of microsomal enzymes. The pesticide may also induce peroxidation of lysosomal membrane leading to breakdown or increased permeability. Both can be responsible for the liberation of acid phosphatase thereby resulting in degeneration and necrosis in tissues (Gopalakrishnan, 1990).

Subburaju and Selvarajan (1989), reported changes in free sugar and amino acids, protein metabolism and lipid content in various regions of the brain of *Tilapia mossambica* exposed for 4days to 0.7µg/L chlorpyrifos. Reported behavioral effects from acute exposures have included immobility and erratic swimming (Subburaju and Selvarajan, 1988; Thirugnanam and Forgash, 1977).

A marked inhibition of DNA, RNA and protein contents was observed in the liver of the fish *Brachydanio reieo* by malathion and carbaryl. The effect of these pesticides on the *in vitro* protein synthesis by liver of the freshwater teleost *Channa punctatus* was studied by Saxena et al. (1988). Exposure period dependent depletion in protein content in endosulfan treated fish *Oreochromis mossambicus* was reported by Ganesan et al. (1989). Dose-dependent depletion of protein content in *Barsillus bendelisis* under toxicity to thiodon was reported by Deoray and Wagh (1991).

The organochlorine compounds have been shown to behave as antithyroid materials in fish. They reduce the metabolic activity and oxygen consumption in various tissues (Brown, 1957). The adverse effects of endosulfan and its isomers on the tissue protein, glycogen and lipid content of the freshwater fish, *Channa punctatus*, has been reported (Murty and Devi, 1982). The organochlorine pesticides have been shown to inhibit the activity of Na^+K^+-ATPase in several tissues of fish (Davis et al, 1971; Desaiah and Koch, 1975a). Dalela et al. (1988) have reported

inhibition in the activity of ATPase in several tissues of a freshwater teleost, *Channa gachua.*

Tripathi and Shukla (1990) have shown that an exposure for 7days of an organophosphate pesticide, methyl parathion and an organochlorine pesticide, endosulfan caused a decline in the efficiency of TCA cycle and the anaerobic glycolytic pathway as reflected by the reduced activities of cytoplasmic malate dehydrogenase, mitochondrial malate dehydrogenase and lactate dehydrogenase in the liver and the skeletal muscle of the freshwater catfish, *Clarias batrachus.*

Since mature chloride cells are in permanent contact with the surrounding water they form an obvious target for aquatic pollutants (Mallatt, 1985; Evans, 1987).

2.3: Histological studies

The pesticides in aquatic ecosystems affect nontarget organisms such as fishes and prawns (Gupta, 2007). A number of pathological changes have been reported in fishes exposed to different organochlorine, organophosphate, carbamate and synthetic pyrethroid pesticides (Vijayalakshmi and Tilak, 1996; Tilak et al, 2001 a, b). Pesticide hazard on fish mortality, growth and tissue damage have been amply reported by Wildish et al. (1971) and Jackson (1976). Sudha and Mehrotra (1999) observed severe damage in the outermost serosa of muscle layers, necrosis in intestinal villi and increase in the number and size of muscle cells under sublethal exposure of carbaryl for a period of one month. Ramachandra (2000) observed that *Channa punctatus* on sublethal exposure of malathion caused significant reduction in the ovarian weight and diameter of developing oocytes and also degeneration of growing oocytes.

Braunbeck (1994) reports that the typical reaction of a rainbow trout hepatocyte exposed to organic toxicants is likely to include (1) disturbance of the highly cytoplasmic compartmentation; (2) augmentation of nucleoli and nuclear deformation in conjunction with a stimulation of karyokinesis, but not of cytokinesis; (3) reduction and gradual disintegration of rough endoplasmic reticulum; (4)

proliferation of smooth endoplasmic reticulum, peroxisomes and lysosomal elements; (5) increased heterogeneity of mitochondria and peroxisomes; (6) glycogen depletion; and (7) an immigration of macrophages. Other unique alterations include induction of peroxisomal cores after endosulfan exposure, granulocyte invasion with atrazine or condensation of tremendous amounts of glycogen in multinucleate hepatocytes of 4-chloroaniline-contaminated rainbow trout.

Mallatt (1985) extensively reviewed the morphological changes taking place in fish gills in the presence of environmental pollutants. The most common changes, applicable to a wide range of pollutants, are: lifting of the epithelium covering the secondary lamellae, increased number of lymphatic spaces, changed blood flow patterns and appearance of granulocytes in the epithelium. Furthermore, hypertrophy and hyperplasia of epithelial cells including mucous and chloride cells are often observed. Svobodova et al, 1994 reported that considerable circulatory disorders are the dominant histopathological changes on chronic exposure to copper in all the organs (particularly gills) of the rainbow trout fingerling. These disorders contribute to the respiratory epithelium and liver tubule epithelium vacuolisation and disintegration, in hepatocyte vacuolisation, and in damage to the brain nerve cells (wrinkling and hyperchromasia).

2.4: Haematological studies

Haematological characteristics are tools for screening pathological status. The haemotological parameters constitute a good indicator of physiological responses (Blaxhall, 1972). Many reports have been published on the toxic effects of pesticides on haematology of fishes (Koundinya and Ramamurthi, 1979; Sharma and Gupta, 1984; Thakur and Pandey, 1990). A significant decrease in red blood cell (RBC) count, Haemoglobin (Hb) content and packed cell volume (PCV) has been observed earlier in fishes exposed to different pesticides (Koundinya and Ramamurthy, 1979; Sambasiva Rao et al., 1955) and such decreasing effect has been primarily attributed to a condition of hypochromic microcytic anaemia (Bhai et al, 1971; Raja Rishi, 1986). Shankar (1975) has reported a significant increasing trend, observed in the number of white blood cell (WBC), in catfish exposed to sublethal concentration of

phosphamidon. Mean corpuscular volume (MCV) and Mean corpuscular haemoglobin (MCH) along with Mean corpuscular haemoglobin concentration (MCHC) showed appreciable decrease in exposed fishes. Similar observations were reported by Thomas et al. (2007).

But Subramanian and Ramalingam (2003) reported that Hb content showed an increase in fishes exposed to 0.001ppm of DDT, 0.95ppm of malathion and 0.09ppm of mercury chloride. RBC count showed an increase when exposed to DDT and mercury when compared to control. Packed cell volume also showed an increase in DDT and mercury. Erythrocyte sedimentation rate showed no significant change in both control and test groups.

According to the reports by Subburaju and Selvarajan (1988), reduction in acetylcholinesterase in plasma, red blood cell and brain in a variety of fish species acutely exposed to chlorpyrifos. Effects of exposure on the blood chemistry of fish include decreased arterial oxygen, CO_2, pH, haematocrit and haemoglobin levels (Bradbury et al., 1991).

Reports on variations of qualitative tissue proteins are very few and are much wanting, especially with reference to pesticide toxicity (Arockia et al, 2007). A comparative electrophoretic study on the tissue proteins of some catfishes was carried out by Hussain and Siddiqui (1974).

Svobodova et al (1994) repoted that significant increases in the erythrocyte count and haematocrit was found in carp after toxic exposure to organophosphorus pesticides. The changes like an increased volume of erythrocytes, mean corpuscular volume (MCV) and a decreased level of mean corpuscular haemoglobin concentration (MCHC) have also been reported after acute exposure to organophosphorus pesticide.

2.5: Studies on pesticide residues

Bioconcentration factors (BCF) for chlorpyrifos in invertebrates and fish range from 42 to 5100mL/g, depending on the species, exposure concentration, and

exposure conditions. Neely and Blau (1977) modelled the disposition of chlorpyrifos in a pond environment; the model estimated the BCF in fish to be 700mL/g and predicted the maximum concentration would occur in 37.5days under conditions of a declining water concentration (Mace, 1995). Environmental monitoring programs have been conducted to determine the occurrence of chlorpyrifos and other pesticides in surface waters as a result of agricultural inputs. In general, levels of chlorpyrifos in surface water have ranged from nondetectable to aqueous concentrations of <0.04-0.134μg/L (Natale et al, 1988) and 0.2-1.6μg/L (Braun and Frank, 1980). The effects of chlorpyrifos in aquatic ecosystems have been assessed in a variety of field studies by monitoring changes in population densities and functional parameters in estuarine and agricultural environments, following accidential introductions into aquatic systems, and in lotic and lentic systems. Mace et al (1995) reviewed the report by Ludwig et al (1968) that an application of 0.028kg/ha resulted in no apparent effects on caged shrimp, minnows, blue crabs or fish (multiple species). A second application of 0.056kg/ha resulted in reduction in brown shrimp abundance and mortality of small fish (species not specified); no mortality of larger fish or blue crabs was observed. Maximum chlorpyrifos concentrations measured in oysters were 0.042, 0.006 and less than 0.005mg/kg at 1, 2, and 3 days following application respectively. The ecological effects of chlorpyrifos on aquatic agricultural environments have been evaluated in rice fields. Linn (1968) made general observations of the toxicity of aerially applied chlorpyrifos on caged and released fish (green sunfish (*Lepomis cyanellus)*, black bullheads) in rice fields (California, U.S). Linn concluded that application rates of 0.028kg/ha had negligible effects on fish survival, whereas 0.056kg/ha appeared to cause mortality of sensitive fish species.

Forgash (1976) and Thirugnanam and Forgash (1977) evaluated the toxicity of four sequential applications of granular chlorpyrifos (0.028kg/ha) on a salt marsh environment (New Jersey, U.S) during a 10-week period. There were no significant effects on marsh grasses (growth of roots or shoots, total productivity), aquatic invertebrates (eg. Isopods, amphipods, snails, shrimp), terrestrial invertebrates or birds (eg. Seaside sparrow, sharp-tailed sparrow). Caged killifish (*Fundulus*

heteroclitus) exhibited abnormal behaviour within 24hr of the first application. Fish mortality was 18% following the first application and 36% following the second application; 59% of fish exhibited abnormal behaviour eg. Immobility, loss of equilibrium, reduced feeding . Growth of young fish was reduced for at least 2 weeks following the final chlorpyrifos application. Live fish exhibited a 96% depression of brain AChE activity. Average inhibition of brain AChE activity was 62% at 69 days after the final application of chlorpyrifos. A recent report indicated that 10.5% of farm-raised channel catfish (*Ictalurus punctatus*) sampled fillets contained detectable chlorpyrifos residues (Santerre et al, 1999). Data from the National Contaminate Biomonitoring Program (EPA, 1992) showed that chlorpyrifos was found, in nine out of sixteen wild channel catfish samples with concentrations above the detection limit (2.5ppb).

Currently chlorpyrifos is used for different purposes. A number of studies in various aspects are in running stage to investigate the toxicity of this insecticide on terrestrial and aquatic organisms.

Chapter

3

Effects of Chlorpyrifos on Behavioural and Biochemical Parameters in *Oreochromis Mossambicus*

Contents

3.1 Introduction

The first study to examine metabolism of chlorpyrifos in fish was conducted by Smith et al. (1966) using goldfish, *Carrassius auratus*. Fishes were placed in water containing 0.3mg/L chlorpyrifos and assayed after 48hr for residues. In addition to chlorpyrifos, several metabolites were tentatively identified, including a major one, 3, 5, 6-trichloropyridinol (TCP) and several minor ones (desethyl chlorpyrifos oxon and TCP phosphate).

Determination of the changes in enzyme activities is one of the used methods of monitoring environmental pollution. Enzyme changes in numerous aquatic organisms such as microsomal mixed function oxidase from marine species; cytochrome P-450 dependent monoxygenase in crab and superoxide dismutase activity in fishes have all been used as bioindicators of water pollution. Several researchers have advocated the use of fish and other invertebrate organisms as bioindicators of water quality because they produce evidence of relatively stable concentrations compared with chemical analysis of water itself (Achuba, 2002).

Sublethal effects cover the effect of all those concentrations, which are not lethal for individuals, even after prolonged exposures, but increase the population mortality, decrease its size, or changes in composition. Thus a group of effects that affect the growth rate, metabolism, reproductive potential, behaviour or which impair the defense mechanism of an organism are referred to as sub lethal effects. Sublethal exposures can affect organisms without obvious signs of injury. The most obvious effect of exposure to a pollutant is rapid death, and it is common practice to assess this type of toxicity by the LD_{50} or a similar type of measurement. These measures are used to assess the environmental impact of pollutants (Moriarty, 1983).

Organophosphorus and organochlorine compounds have been shown to interact with membrane events including nerve conductance, and plasma membrane and organelle enzyme activities (Binder et al. 1976; Rosenstock et al. 1990;

Schneider, 1975). The levels of enzymatic and non-enzymatic biological molecules are more prone to variation under different pathological conditions and toxicological environment. Thus their levels in tissues and body fluids are of diagnostic value since any change in the level may affect the homeostasis of the organism.

The present study was undertaken to determine the levels of total protein content. Protein constitutes the building block and the basic molecule for any biochemical reaction. Determining the acetylcholinesterase (AChE) level is necessary to monitor the organophosphorus toxicity. Hence the level of brain-specific acetylcholinesterase (AChE) was studied. Ready source of energy is glucose and its metabolism is of great importance for the normal existence of any living organism. Thus the anaerobic-glycolytic enzyme, lactate dehydrogenase (LDH) and aerobic glycolytic enzyme, succinate dehydrogenase (SDH) were considered for the study. Liver is the major organ of detoxification. Liver plays the role in activation and detoxification of chlorpyrifos, by desulfuration to chlorpyrifos oxon. Hence the liver-specific enzymes alanine aminotransaminase (ALT) and alkaline phosphatase (ALP) were included for the toxicological investigations of chlorpyrifos in fish.

Swimming behaviour of fish is frequently assessed as a tool in toxicity investigations because altered locomotor activity can be the indicator of the effects to the nervous system (Venkateswara Rao et al., 2005). Thus the investigations were made on biochemical parameters as well as behavioral patterns.

LDH interconverts lactate and pyruvate and has very important role in carbohydrate metabolism. LDH acts as a pivotal enzyme between glycolytic pathway and TCA cycle. It catalyses the conversion of pyruvate into lactate, under anaerobic conditions (Lehninger, 1993). A fish under stress prefentially meets its energy requirements through anaerobic oxidation (Wallice Luiz, 1998). LDH activity depends on its five isoenzymes and the activity changes under pathological conditions (Martin et al, 1983).

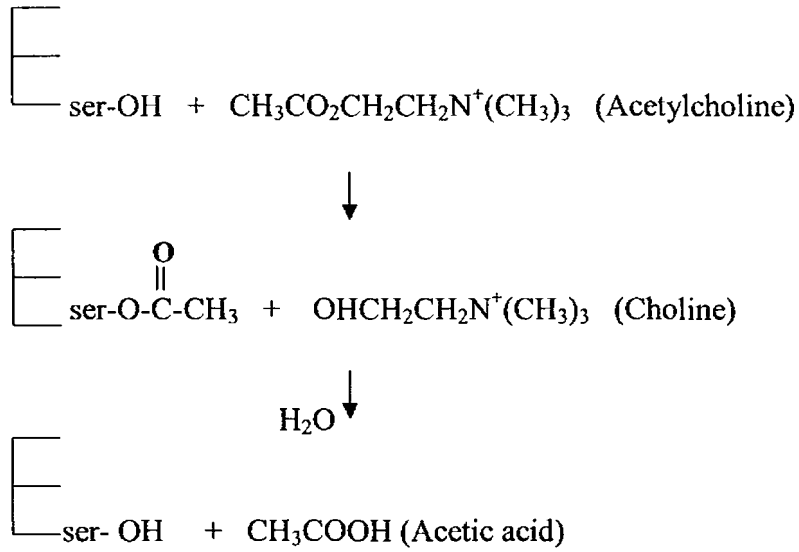
FAD-dependent succinate dehydrogenase facilitates interconversion of succinate and fumarate and it is mitochondrially localized. Reduction in SDH

activity is reported to be associated with a corresponding decrease in other oxidative enzymes like malate dehydrogenase, isocitrate dehydrogenase and cytochrome-c-oxidase under various stress conditions (Dhalla et al, 1971).

Five AChE fractions have been identified by electrophoresis in the brain homogenates of goldfish (*Carassius auratus*). Polyacrylamide gel electrophoresis (PAGE) indicates that one or more molecular forms can represent AChE of the fish brain. Fish brain and muscle primarily possess AChE (Abou-Donia and Menzel, 1967; Coppage, 1971; Habig et al, 1988). The muscle tissue of the majority of fish species, like blood serum, has lower activity for AChE than in the brain. Fish brain is essentially nerve tissue and is rich in AChE as compared to butarylcholinesterase (BChE) which is found in blood. (Whittaker, 1951). In fish living in natural waters, even a relatively low concentration of organic phosphoric acid esters is capable of causing considerable AChE inhibition (Williams and Sova, 1966). This has been attributed to the enhanced accumulation of chemical pollutants in fish. ChEs of aquatic animals appear to have varying sensitivities to organophosphates (Kozlovskaya, 1993). ChE activities of fish have been recognized a potential biochemical indicator for toxic effects of these insecticides (Gruber and Munn, 1998). With repeated inputs of anticholinesterase chemicals to the aquatic environments, fish may be exposed to acutely lethal to sublethal concentrations. The degradation of the chemical would allow the affected fish to recover from the poisoning (Chandrasekera, 2005).

The toxicity of organophosphorus and carbamate insecticides is mainly due to the inhibition of AChE, the enzyme which cleaves the neurotransmitter acetylcholine, thereby interfering with proper neurotransmission in cholinergic synapses and neuromuscular junctions. Although both types of insecticides have a common mode of action, organophosphorus insecticides are irreversible inhibitors of AChE whereas carbamates are often considered as reversible inhibitors of the enzyme as a relatively weak bond is formed between the carbamate and AChE (Ecobichon, 1992).

AChE breaks down ACh to acetic acid and choline. A serine residue in the active site of the enzyme participates in the breakdown of ACh as shown in Fig 3.1.



where ser-OH indicates serine residue in the active site of the enzyme

Fig.3.1: Mechanism of action of Acetylcholinesterase enzyme

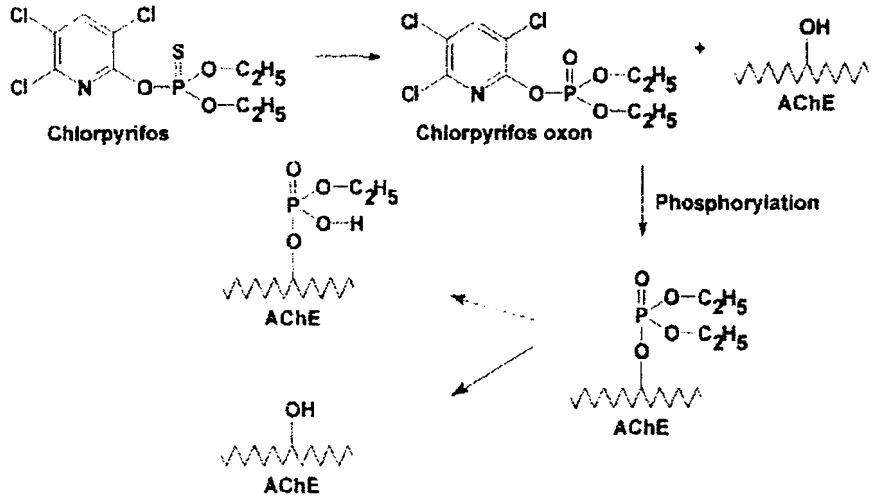


Fig.3.2: Diagram of activation of chlorpyrifos to the oxon form, phosphorylation and recovery of acetylcholinesterase (John Giesy, 1999).

The enzyme mediated hydrolysis of ACh depends on the attack by a hydroxyl group attached to a serine molecule in the enzyme (Fig.3.1). This hydroxyl group reacts with ACh to liberate choline. The acetylated enzyme then reacts with water to regenerate the enzyme and to release acetic acid. This hydroxyl group in AChE is also the site of action for organophosphorus insecticides. Organophosphorus molecule becomes attached to the serine group of the enzyme, in much the same way as does ACh, (Fig. 3.2). In contrast to the reaction with ACh, the phosphorylation of the enzyme with organophosphorus compound is irreversible. Hence, organophosphorus insecticides decrease the availability of AChE and ACh accumulates at nerve endings (Moriarty, 1983).

Organophosphorus compounds inhibit a whole range of esterase enzymes. Inhibition of acetylcholinesterase (AChE) enzyme produce symptoms of acute poisoning. Acetylcholine (ACh), the natural substrate for AChE, is one of the principal known transmitters of impulses across synapses between adjacent nerve endings, and across neuromuscular junctions. Nerve impulses stimulate the release of ACh, which transmits the stimulus across the gap to the adjacent nerve or muscle cell. The ACh is normally broken down rapidly by hydrolysis, catalyzed by the enzyme AChE. Inhibition of AChE means that ACh persists much longer. This leads to disturbance in normal nerve functions and a sufficiently severe disturbance ends in death (Eto, 1974).

Organophosphorus insecticides can produce in birds and mammals, a second lesion quite unrelated to the inhibition of AChE (Johnson, 1981; Baron, 1981). Symptoms do not appear until 1-2 weeks after exposure, when the hind limbs, and in severe cases the fore limbs too, become paralysed. Originally, this condition was referred to as demyelination, because the myelin sheath that surrounds most nerves degenerate. It is now considered that the primary damage is not to the myelin sheath but to the nerve axon itself and the condition is more correctly described as delayed neurotoxicity. The primary lesion occurs in an esterase, "neurotoxic esterase", which belongs to the same group of enzymes as does AChE. Symptoms of poisoning appear after a delay of 1-2 weeks, if more than about 80% of this enzyme is

phosphorylated and so inhibited by an organophosphorus insecticide (Moriarty, 1983).

Acid phosphatase and alkaline phosphatase are enzymes, which catalyze the hydrolysis of orthophosphoric acid esters at optimum pH levels below 7.0 and above 7.0 respectively. Phosphatase enzymes are considered as an important toxicological tool to study the pesticide effect that reflects the change in physiological and biochemical pathways (Ramano Rao et al, 1996).

Interestingly, the animals have the capacity to regulate and modulate the inherent diversions in their metabolism to meet the altered physiological or environmental conditions (Hoar, 1976). This is easily done to meet the energy demands under attenuated or imposed stress conditions to facilitate synthesis of extra energy to overcome such impeding situations.

In the present study an attempt was made to investigate the biochemical adaptability of the animal to overcome the chlorpyrifos-induced stress.

3.2 Materials and Methods

3.2.A Materials used for the study

a. Chlorpyrifos

Chlorpyrifos [CAS 2921-88-2] is an organophosphorothioate compound

Chemical name:	O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate
Synonyms:	The common name chlorpyrifos is in general use. Another nonproprietary name, trichlorpyriphos has been discontinued.
Trade name:	Dursban® and Lorsban®
Chemical characteristics:	Chlorpyrifos is a white granular crystalline solid with a strong odour
Melting point:	42^0 to 43.5^0C

Vapor pressure: $1.87X10^{-5}$ mm Hg at 25^0C and $8.87X10^{-5}$mm Hg at 35^0C.

Solubility: It is readily soluble in acetone, benzene, chloroform, methanol and isooctane at 50, 790, 630, 45 and 79 g/100g of solvent, respectively, but less soluble (0.4ppm) in water at 23^0C. Hydrolysis in water occurs least readily at about pH 6 and very readily above pH 8 (Brust, 1966). The partition coefficient between n-octanol and water is 66,600 (Kenaga, 1971).

It does not mix well with water, so it is usually mixed with oily liquids before it is applied to crops or animals. It may also be applied to crops in a micro encapsulated form (ATSDR, 1997).

Uses: It is an organophosphorus insecticide that has been widely used in the home and on the farm. It is the active component of a number of formulations used in crop and turf applications (Racke, 1993) as well as for control of household pests (Tomlin, 1994). Chlorpyrifos is a potential replacement for organochlorine insecticides (eg.

dieldrin and aldrin) which are now either discouraged or banned for control of termites (Wright et al, 1988).

For household applications, chlorpyrifos has been used as a 0.25 to 0.5 percent spray for the control of structural pests like cockroaches, ants and termites; it has also been an active ingredient in some pet flea and tick collars. On the farm, it is used to control ticks on cattle and as a spray to control crop pests.

Fig: 3.3 a&b Chlorpyrifos

Chlorpyrifos has been used for mosquito control at dosages of 0.0125 to 0.05 lb/A (Kenaga, 1971).

Commercially available Chlorpyrifos 20% EC (CLASSIC 20) insecticide has several uses (as listed in the direction list along with the commercially available pack).

Table 3.1: List of crop and common name of the pests, for which chlorpyrifos is applied.

A. CROP and COMMON NAME OF THE PESTS

Cereals:	Pulses:	Sugar cane	Fibre crops	Oil seeds	
PADDY	BEANS	GRAM	COTTON	GROUND NUT	MUSTARI
Hispa Leaf roller Stem borer	Aphid Pod borer	Cut worm Pod borer	Black bug Early shoot &stalk borer Pyrilla	Aphid Boll worm Gall midge White fly Cut worm	Aphid Root grub

Vegetables			Fruit trees			Miscellaneous
BRINJAL	CABBAGE	ONION	APPLE	BER	CITRUS	TOBACCO
Shoot borer	Daimond Back-moth	Root grub Aphid	Aphid	Leaf hopper	Black citrus	Ground bee

B. **Noncropped area**

Building (Pre & Post construction treatment)

Forestry

C. **Cropped area**

For seed treatment of wheat, Barley and Gram

For soil treatment of wheat and sugarcane

Environmental entry: Chlorpyrifos enters the environment through direct application to crops, lawns, domesticated animals as well as through household applications. Chlorpyrifos may also enter the environment through volatilization, spills and the disposal of chlorpyrifos waste.

Metabolism: After chlorpyrifos enters the body, it is converted by the liver into other forms of the compound that may or may not be less toxic than the original compound. The major nontoxic metabolite formed by the liver is 3,5,6-trichloropyridinol or TCP. TCP is primarily eliminated from the body through urine. In addition to chlorpyrifos, TCP is a metabolite of methyl chlorpyrifos and triclopyr.

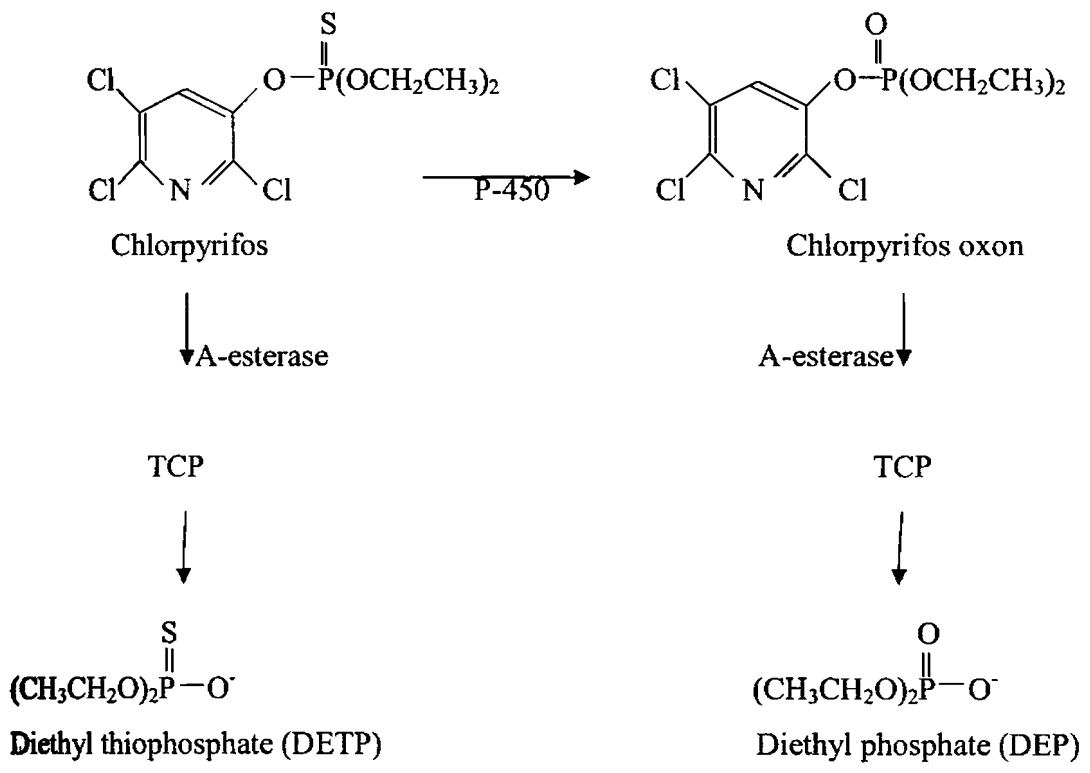


Fig.3.4: Diagramatic representation of metabolism of chlorpyrifos [Adapted from Drevenkar et al, 1993 (ATSDR, 1997)].

In rat and mouse, chlorpyrifos is bioactivated in the liver to chlorpyrifos oxon via cytochrome P-450 dependent desulfuration (Ma and Chambers, 1994; Sultatos

and Murphy, 1983). The oxon is rapidly hydrolysed to TCP, probably by A-esterase (Barron et al, 1993).

b. *Oreochromis mossambicus*

The selection of organisms for toxicity test is mainly based on certain criteria like its ecological status, position within the food chain, suitability for laboratory studies, genetically stability and uniform populations and adequate background data on the organism. The euryhaline teleost cichlid fish *Oreochromis mossambicus* (common name-tilapia) was selected for the present study due to its wide availability and suitability as model for toxicity testing (Ruparrelia et al., 1986) and also due to sustainability in laboratory conditions. The fish shows a well adaptive nature with the changing environment.

The **Systematic position** of the fish is

Kingdom : Animalia
Phylum : Chordata
Class : Teleostomi
Subclass : Actinopterygii
Order : Perciformes
Family : Cichlidae
Genus : *Oreochromis*
Species : *mossambicus*

Fig.3.5: *Oreochromis mossambicus*

3.2.B Experimental design

1. Collection and maintenance of experimental animals

The fish, *Oreochromis mossambicus* was collected from Kerala Agricultural University Research station, Puthuvype, Cochin, Kerala. Animals were transported to laboratory in large aerated fibre glass and maintained in aquarium tanks containing well aerated dechlorinated tap water (with physico-chemical characteristics: pH 6.5-7.5, temperature 25±3^{0}C, salinity 0ppt and dissolved oxygen content of 7-8ppm in different 100L plastic tanks, for 20 days. The fishes were fed on commercial pelleted diet once a day, throughout the tenure of the chronic experiment. The acclimated healthy fishes of weight 15±3gm and size 8.5±1.5cm from both sexes were selected randomly for the studies.

2. Experimental design for LC_{50} determination

A semi-static acute toxicity bioassay was carried out to determine the LC_{50} value. The test solution of technical grade chlorpyrifos was prepared as 5gm% stock solution using acetone and diluted further as required. Fishes were divided into groups of six fishes in each group. They were kept deprived of feed and without aeration during the acute toxicity study period of 96-hr, in 20L-water containing plastic tubs. The tubs were kept closed to avoid the effect from sunlight and volatilization of pesticide from the surface water. They (group 1) were exposed to different concentrations of chlorpyrifos, in the range 1000-25ppb. Acetone was used as the vehicle to dissolve Chlorpyrifos. Controls receiving acetone (group 2) in equal volumes as pesticide treated group were also kept simultaneously to consider the mortality rate, if any, due to the effect of vehicle. Group 1 and group 2 were observed for a period of 96hr, with a parallel control, without either acetone or pesticide. The obtained mortality was subjected to probit analysis (Finney, 1971) and LC_{50} (the concentration of chlorpyrifos at which 50% mortality was observed) was calculated.

3. Experimental design for studies on Behavioral pattern and Biochemical analysis

After determining the LC_{50} value, fishes were divided into five groups in duplicates, with six fishes in each group. Group1-control, groupII-Acetone contol and groups III, IV and V were exposed respectively to 1/10, 1/5 and 1/3 sub-lethal concentrations of 96hr-LC_{50} value of chlorpyrifos.

Tank water was renewed every 24hr and fresh solution of chlorpyrifos was added (USEPA, 1975) to maintain the dissolved oxygen concentration at optimum level and pesticide concentration constant. During the exposure for 21days, the behavioural changes were noticed.

The fishes were subjected to starvation 24hr prior to the tissue collection for biochemical analysis. Sampling was done after 7days and 21 days of exposure to the pesticide. After the stipulated exposure periods, 6 fishes were removed from each pesticide-treated group and the respective controls were removed and sacrificed for tissue sampling. Tissues like Gills, liver and the whole brain were removed and kept frozen until analysis was performed. It is recommended that either the whole brain or half of the brain separated along the midline be used for enzyme analysis (Zinkl et al, 1991). This experimental set up was followed throughout the study. Different biochemical parameters were analyzed in gill, liver and brain tissue samples.

4. Biochemical analysis

All the reagents used in the present study were of analytical grade and were used without further purification.

a. Estimation of Protein:-

Protein content in tissues was estimated by the method of Lowry et al (1951).

Reagents:- 0.1N NaOH- 0.4gm NaOH dissolved in 100mL distilled water

Reagent A- 2gm Na_2CO_3 dissolved in 100mL distilled water

Reagent B- 5gm/L $CuSO_4.5H_2O$ dissolved in 10gm/L sodium-potassium-tartrate

Reagent C- Prepare on day of use by mixing 50mL Reagent A with 1mL Reagent B
Folin-Ciocalteau reagent- Dilute the commercial reagent with two times the volume of water on the day of use. This is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acids.
Standard solution of Bovine albumin- 100mg bovine albumin dissolved in 100mL 0.1N NaOH

Procedure:-

5% homogenate prepared in isotonic ice-cold solution was centrifuged. To the fixed volume of the supernatant, 1ml TCA was added and centrifuged. The precipitate was dissolved in 1ml 0.1N NaOH, 0.5ml was pipetted out to another tube. Making the volume to 1ml with distilled water, 5ml alkaline copper reagent was added and kept at RT for 10minutes. 0.5ml 1:2 diluted Folin's reagent was added and the tubes were kept in dark for 30minutes. The absorbance was read at 660nm.

b. Assay of Acetylcholinesterase activity (Acetylcholine hydrolase, E C.3.1.1.7)
Acetylcholinesterase activity was assayed by the method of Ellman et al, 1961).

Reagents:
Sodium phosphate buffer (0.1mol/L): pH 8
DTNB (dithionitrobenzoic acid, 10mmol/L): freshly prepared. Dissolved 39.6mg in 10ml of sodium phosphate buffer (0.1mol/L) containing 15mg sodium bicarbonate
Acetylthiocholine iodide (freshly prepared): 158.5mmol/L in the sodium phosphate buffer

Procedure:

Added 50µL of the enzyme source (5% tissue homogenate prepared in buffer) to 3ml of sodium phosphate buffer and incubated at RT for 5min. Added 10µL of DTNB followed by 20µL of acetylthiocholine iodide to give a final concentration of 1mmol/L of the substrate. Recorded the increase in absorbance at

412nm on a double-beam spectrophotometer against a blank of the above mixture prepared at the same time. In blank 50µL of the enzyme was replaced with 50µL of the buffer solution. Extinction coefficient of chromophore is $1.36x10^4$. The obtained values give the µmoles of substrate hydrolysed / minute/ mg protein.

c. Assay of Lactate dehydrogenase activity (L-lactate:NAD Oxidoreductase; E.C.1.1.1.27)

Lactate dehydrogenase activity was measured by the method of McQueen et al (1972).

Reagents:

Sorensen phosphate buffer (pH 7.2)- 6.7mmol/L

53.3mmol/L sodium pyruvate- 5.78mg/mL

0.18mM NADH- 3.7mg/mL in water

Procedure:

The tissue was homogenized in 6.7mmol/L phosphate buffer (pH 7.2). The homogenate was centrifuged at 20,000g for 30minutes in a refrigerated centrifuge at 0^0C. The supernatant obtained was used as the enzyme source. To the reaction mixture containing 3mL buffer and o.1mL NADH, the tissue homogenate was added. Mixed and incubated at 37^0C for 15minutes. 150µL pyruvate solution was added and the change in absorbance was measured at 340nm in a spectrophotometer. The activity was determined from the rate of oxidation of NADH. A standard graph was prepared and the activity of the enzyme was expressed as mg of NADH oxidized per hour per gm protein in the sample. Protein content of the sample was estimated by following the procedure of Lowry et al. (1951).

d. Assay of Succinate dehydrogenase activity (Succinate:Acceptor Oxidoreductase, E C 1.3.99.1)

Succinate dehydrogenase activity was estimated by the method of Nachlas et al, 1960.

Reagents:

0.33M sucrose

40µM sodium succinate:- 1.08gm in 100mL water

100µM of potassium phosphate buffer (pH 7)

4µM INT (2-p-ido dophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride: 0.202gm in 100mL toluene

Procedure:

Tissue homogenate was prepared in ice-cold 0.33M sucrose solution and centrifuged at 1000g for 15minutes. The supernatant was used for the assay.

The reaction mixture of 2mL contained 0.5mL sodium succinate, 0.5mL potassium phosphate buffer, 0.5mL INT and 0.5mL supernatant. The contents were incubated for 30minutes at 37^0C and the reaction was arrested by adding 5mL glacial acetic acid. The iodoformazan formed was extracted overnight in 5mL of Toluene at 5^0C. The absorbance of the colour was measured spectrophotometrically at 495nm against a toluene blank. The enzyme activity was expressed as µmoles of formazan formed/mg protein/hr.

e. Assay of Alanine aminotransferase activity (ALT) (DL-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2) ALT was assayed by the method of Mohun and Cook (1957).

Reagents:

Buffered substrate- Dissolve 15g K_2HPO_4, 2g KH_2PO_4, 300mg 2-oxoglutarate in 700-800mL H2O and add 17.8g DL-alanine. Check the pH and adjust to 7.4 with NaOH and make upto 1L with deionised water

2,4-DNPH- 200mg/L in acidified water

NaOH- 16g/L in water

Standard Pyruvate solution- 22mg of sodium pyruvate in 100mL distilled water

Procedure:

5% tissue homogenate was prepared in 0.33M sucrose and centrifuged at 1000g for 15minutes. The supernatant was used for the assay. Pipetted out 1mL each of buffered substrate into two test tubes labelled as test and control. Added 0.2mL of the supernatant to the tube labelled as test and incubated the tubes at 37^0C for 30minutes. After incubation, 0.2mL of the enzyme source was added to the control tube. 1mL of 2,4-DNPH reagent was added to each tube and kept at RT for 20minutes. The reaction was stopped by the addition of 10mL of 0.4N NaOH, vortexed and kept at RT for 5minutes. The absorbance was measured at 520nm. The values obtained were expressed as units/minute/mg protein. Standards are also treated in the same manner with 2,4-DNPH.

f. Assay of Alkaline phosphatase activity (E C 3.1.3.1)

Alkaline phophatase activity was assayed by the method of King and Armstrong (1934).

Reagents:

1. Disodium phenyl phosphate-100mmol/L- Dissolve 2.18gm (2.541gm of the dihydrate) in water and make up to 1L. Brought quickly to boil and cooled.
2. Sodium carbonate- Sodium bicarbonate (100mmol/L)- Dissolve 6.36gm of anhydrous sodium carbonate and 3.36gm of sodium bicarbonate in water and made up to 1L
3. Buffered substrate- Prepared by mixing equal volume of 1 and 2 reagents with a pH of 10.
4. Phenol reagent- One part of phenol reagent was diluted with two parts of distilled water
5. Stock standard of phenol (1gm/L)- Dissolved 100mg pure crystalline phenol in distilled water and made up to 100mL
6. Working standard solution- 10ml stock solution was diluted to 100ml with distilled water

Procedure

Different tissues were homogenized in 0.33M sucrose. Pippetted out 4mL buffer substrate in a test tube and placed in a water bath at 37^0C for a few minutes. Added 0.2mL homogenate preferably without removing from the water bath. Mixed, stoppered and left in the water bath at 37^0C for 15minutes. Removed and immediately added 1.8mL phenol reagent. At the same time, set up a control with 4ml buffered substrate and 0.2mL homogenate to which added immediately 1.8mL diluted phenol reagent. Mixed well and centrifuged.

Transferred 4mL supernatant from each and added 2mL of Na_2CO_3. Put up a series of standards treated with 2mL Na_2CO_3 and phenol reagent. Placed all tubes at 37^0C for 15minutes and absorbance was measured against the blank at 700nm. Reagent blank was prepared with 3.2mL water, 0.8mL diluted phenol reagent and 2mL Na_2CO_3 solution.

The obtained value corresponds to the liberation of phenol and activity was expressed as mg of p-nitro phenol formed/ minute/ mg protein.

3.3 Statistical Analysis

Statistical analysis was carried out by Single-factor ANOVA using Excel (Microsoft office) and followed by further LSD (Least significant difference) analysis (Zar, 1996). Significant difference in pesticide treated group from control group is represented along with Mean±SD.

3.4 Results

BEHAVIOURAL RESPONSE: Behavioural responses were found changed on exposure to the insecticide chlorpyrifos. In control group, fishes showed a tight school covering the part of bottom of the tank. They were found in well-co-ordinated manner and were alert to the slightest disturbances. The fishes exposed to acetone (9µL, the volume correseponds to chlorpyrifos solution in the highest

sublethal concentration.) were observed in the same behavioral pattern as that of contol group. No mortality was observed in acetone control group.

When exposed to pesticides, the shoal was observed as disturbed. Fishes were initially surfaced, followed by vigorous and erratic swimming showing agitation. Quick opercular and fin movements were observed initially and gradually became feeble and often showed gulping of air. Excess secretion of mucus was a prominent observation. Opercular opening became wider and exhibited respiratory distress. As the period of exposure increased, fishes were found to settle down to bottom and towards the final phase of exposure, fishes showed barrel-rolling indicating loss of equilibrium. Swimming with belly upwards and gradually became lethargic. Excess mucous of brownish hue was produced during intoxication. During initial phase of exposure fishes responded vigorously to mechanical stimulation but later failed to respond.

LETHALTOXICITY: Acute toxicity of chlorpyrifos on *Oreochromis mossambicus* was carried out by the semi-static method and its LC_{50} value for 96 hr is presented in Table-1. It is evident from the results that the chlorpyrifos can be rated as highly toxic to fish with an LC_{50} value of 82µg/L (82ppb).

Table 3.2. Acute toxicity of chlorpyrifos on *Oreochromis mossambicus*

Compound	Regression Equation	Acute Toxicity Range		
	$Y = (Y - bx) + bX$	95% confidence limit		Median LC_{50}
		Upper limit	Lower limit	(µg/L)
Chlorpyrifos	Y= -78.116+40.8X	83.007	81.25	82.14±0.88

BIOCHEMICAL RESPONSE: The responses to different biochemical parameters are given:-

Table 3.3 Effect of chlorpyrifos on gill total protein content in *O.mossambicus* exposed for 7days and 21 days (Fig.3.6)

Single-factor ANOVA revealed that after 7days of exposure, there was a significant difference (P<0.001) between different group means. From control group 27.33ppb group is significantly different followed by 16.4ppb group. LSD analysis revealed (LSD= 3.169) also that there was no significant difference in acetone control and 8.2ppb group when compared to control. No significant difference was observed between 8.2ppb and 16.4ppb; and between 16.4ppb and 27.33ppb groups.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	20.41±1.98	19.14±2.74^{NS}	18.75±2.10^{NS}	15.98±2.95^{c}	13.07±3.61^{c}
21 days	19.93±1.54	20.74±2.38^{NS}	13.44±3.58^{c}	11.31±4.45^{c}	9.47±4.16^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are in mg/100gmwet weight of the tissue.

c-significant at 0.001level ; NS-not significant.

Single factor ANOVA revealed a significant difference (P<0.001) between different group means. The chlorpyrifos treated groups, after 21 days of exposure, showed a significant difference from control. Further analysis by LSD (LSD=3.93) showed that the difference in 27.33ppb was significantly higher followed by 16.4ppb and 8.2ppb when compared to control. 8.2ppb showed a significant difference from 27.33ppb but not from 16.4ppb. No significant difference was observed between control and acetone control and also between 16.4ppb and 27.33ppb groups.

Table 3.4 Effect of chlorpyrifos on liver total protein content in *O.mossambicus* exposed for 7days and 21 days (Fig.3.7)

Single factor ANOVA and further analysis by LSD (LSD= 4.98) revealed that after 7 days of exposure, no significant difference was observed between different, pesticide-treated groups like 8.2ppb, 16.4ppb and 27.33ppb group. But control showed significantly ($P<0.05$) higher values compared to 27.3ppb and 16.4ppb groups.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	30.14±5.9	32.15 ± 5.54^{NS}	27.97 ± 3.79^{NS}	24.49 ± 2.55^{a}	24.21 ± 2.53^{a}
21 days	29.36±5.68	31.46 ± 6.31^{NS}	14.6 ± 3.12^{c}	11.41 ± 3.54^{c}	10.89 ± 2.83^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are in mg/100gm wet weight of the tissue

a-significant at 0.05level; NS-not significant

One-way ANOVA revealed that after 21 days of exposure, there was a significant difference ($P<0.001$) between different pesticide treated groups and control group. LSD value obtained was 5.22. LSD analysis revealed that 27.33ppb group showed significantly highest value followed by 16.4ppb and 8.2ppb groups. But no significant difference was observed between control and acetone control and also between any of the pesticide-treated groups.

Table 3.5 Effect of chlorpyrifos on brain total protein content in *O.mossambicus* exposed for 7days and 21 days (Fig.3.8)

Single-factor ANOVA revealed that no significant difference was observed at 0.05 level, after 7 days of exposure.

Exposure period	Controls	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	16.18±2.1	16.6±2.82^{NS}	15.37±2.53^{NS}	14.23±1.29^{NS}	14.09±2.57^{NS}
21 days	16.55±3.04	16.72±2.72^{NS}	11.79±2.44^{c}	8.83±2.84^{c}	8.17±3.22^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are in mg/100gm wet weight of the tissue.

c- significant at 0.001level; NS-not significant

There was a significant difference (P<0.001) between different group means, after 21 days of exposure. LSD analysis (LSD=3.31) revealed that 27.33ppb group showed the highest significance followed by 16.4ppb and 8.2ppb groups when compared to control. No significant difference was observed between control and acetone control. And there was no significant difference between 8.2ppb and 16.4ppb groups and also between 16.4ppb and 27.33ppb; but 8.2ppb group showed a significant difference from 27.33ppb group.

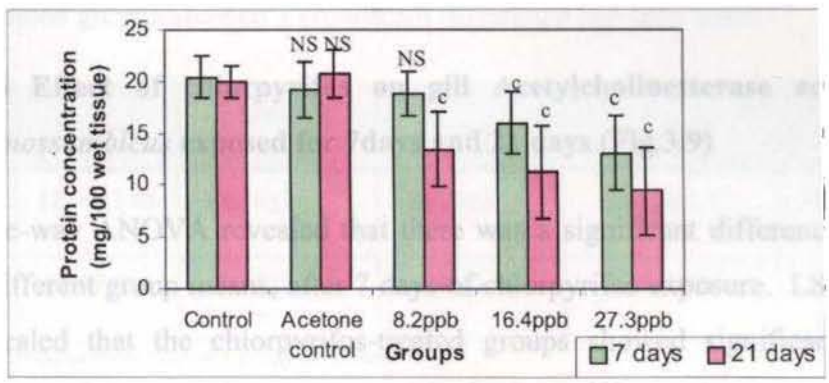


Fig.3.6: Effect of chlorpyrifos on gill total protein content in *O.mossambicus* exposed for 7 and 21days

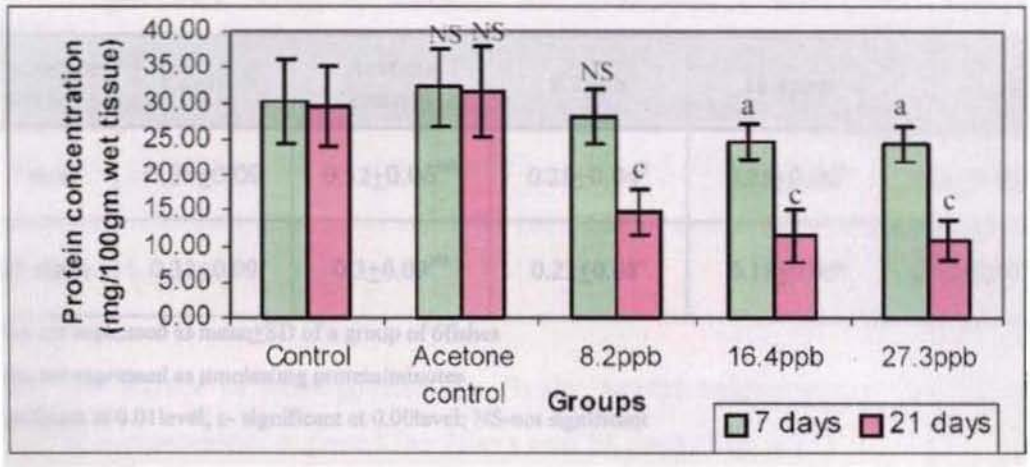


Fig.3.7: Effect of chlorpyrifos on liver total protein content in *O.mossambicus* exposed for 7 and 21days

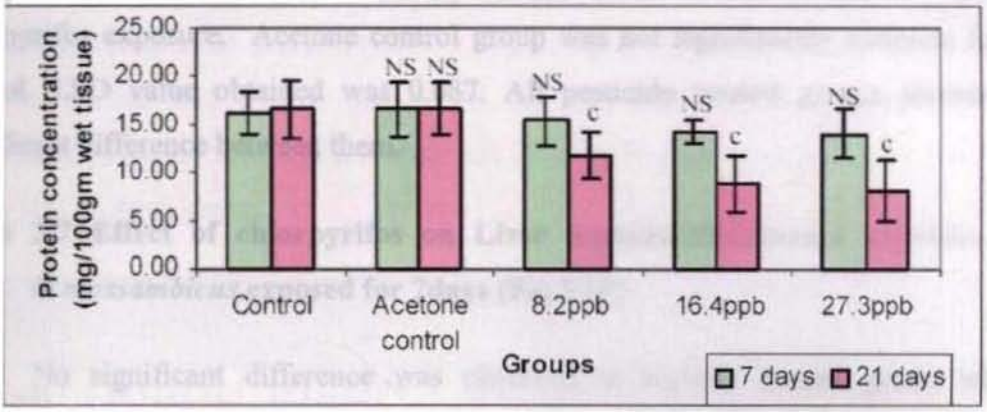


Fig.3.8: Effect of chlorpyrifos on brain total protein content in *O.mossambicus* exposed for 7 and 21days

Each histogram represents mean±SD of 6 values; superscript represents the group significantly different from control; a- P<0.05; b- P<0.01; c- P<0.001, NS-not significant

Table 3.6 Effect of chlorpyrifos on gill Acetylcholinesterase activities in *O.mossambicus* exposed for 7days and 21 days (Fig.3.9)

One-way ANOVA revealed that there was a significant difference (P<0.01) between different group means, after 7 days of chlorpyrifos exposure. LSD value of 0.067 revealed that the chlorpyrifos-treated groups showed significantly higher values compared to control. Acetone control group did not show any significant difference from control.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	0.35±0.09	0.32 ± 0.05^{NS}	0.28 ± 0.04^{b}	0.25 ± 0.06^{b}	0.21 ± 0.05^{b}
21 days	0.33±0.09	0.3 ± 0.09^{NS}	0.23 ± 0.08^{c}	0.18 ± 0.05^{c}	0.12 ± 0.04^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are expressed as µmoles/mg protein/minutes

b-significant at 0.01level; c- significant at 0.00level; NS-not significant

One-way ANOVA revealed that a significant difference ($P<0.001$) was observed in all pesticide-treated groups when compared to control, after 21 days of chlorpyrifos exposure. Acetone control group was not significantly different from control. LSD value obtained was 0.087. All pesticide treated groups showed a significant difference between them.

Table 3.7 Effect of chlorpyrifos on Liver Acetylcholinesterase activities in *O.mossambicus* exposed for 7days (Fig.3.10)

No significant difference was observed in acetone treated group when compared to control, after short-term exposure to chloerpyrifos. But single-factor ANOVA revealed that there was a significant difference ($P<0.001$) between control group and pesticide-treated groups. LSD analysis (LSD=3.526) revealed that all pesticide-treated groups showed a significant difference between them.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	12.04±3.01	12.38 ± 3.22^{NS}	18.56 ± 2.14^{c}	23.42 ± 2.9^{c}	28.08 ± 3.76^{c}
21 days	12.46±2.4	13.26 ± 1.26^{NS}	6.47 ± 1.89^{c}	2.6 ± 0.68^{c}	2.13 ± 0.94^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are expressed as µmoles/mg protein/minutes

c- significant at 0.001level; NS-Not significant

Single-factor ANOVA revealed that there was a significant difference ($P<0.001$) between chlorpyrifos-treated groups when compared to control, after 21 days of exposure. 27.33ppb group showed significantly the highest value followed by 16.4ppb group and 8.2ppb group. LSD analysis (LSD= 1.825) revealed that no significant difference was observed between control and acetone control and also between 16.4ppb and 27.3ppb.

Table 3.8 Effect of chlorpyrifos on Brain Acetylcholinesterase activities in *O.mossambicus* exposed for 7days and 21 days (Fig. 3.11)

After a short-term exposure, there was a significant difference ($P<0.05$) was observed in 27.3ppb group followed by 16.4ppb group when compared to control group. By further LSD analysis (LSD=3.58), no significant difference was observed between control and 8.2ppb group and between control and acetone control. Significantly higher values were observed in 8.2ppb group compared to 27.3ppb group and 16.4ppb group.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	14.92±2.71	16.15 ± 3.65^{NS}	14.16 ± 3.52^{NS}	10.61 ± 2.6^{a}	9.29 ± 2.27^{a}
21 days	13.98±3.93	14.68 ± 3.48^{NS}	5.87 ± 1.75^{c}	2.36 ± 0.74^{c}	2.26 ± 0.91^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are expressed as µmoles/mg protein/minutes

a-significant at 0.05level; c-significant at 0.001level; NS-nonsignificant

After 21 days of exposure, there was a significant difference ($P<0.001$) between control and different chlorpyrifos-treated groups. LSD value obtained is 2.75. But no significant difference was observed between control and acetone control and also between the higher sublethal concentration groups- 16.4ppb group and 27.3ppb group.

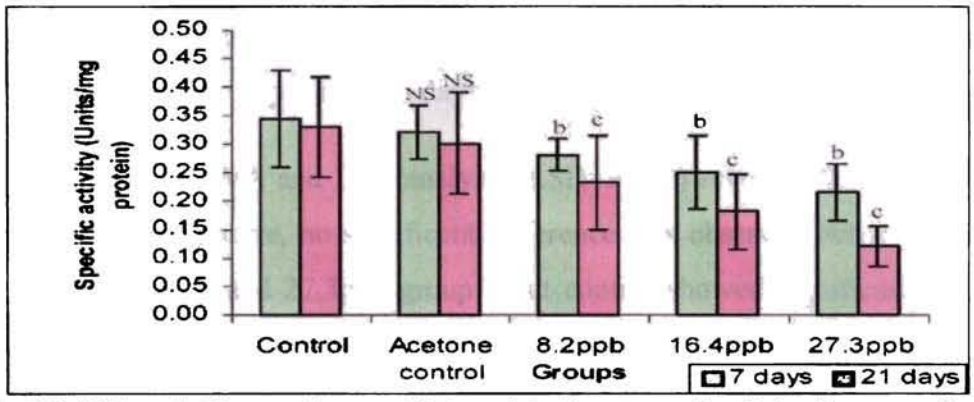


Fig.3.9: Effect of chlorpyrifos on gill Acetylcholinesterase levels in *O.mossambicus* exposed for 7 and 21days

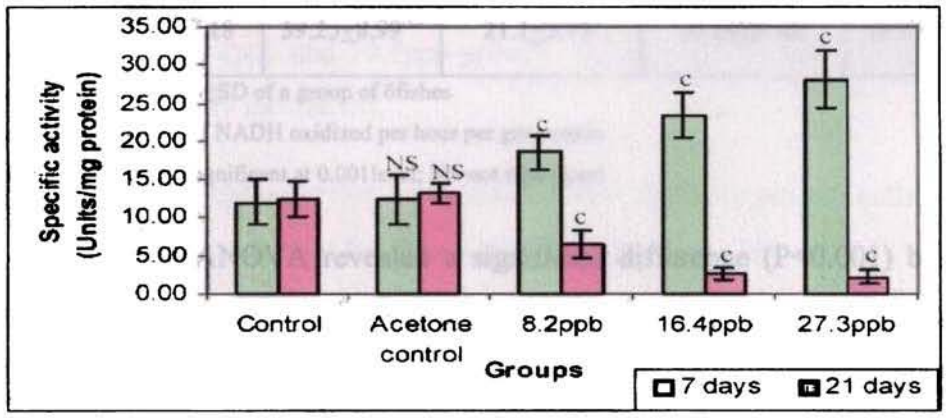


Fig.3.10: Effect of chlorpyrifos on liver Acetylcholinesterase activities in *O.mossambicus* exposed for 7 and 21days

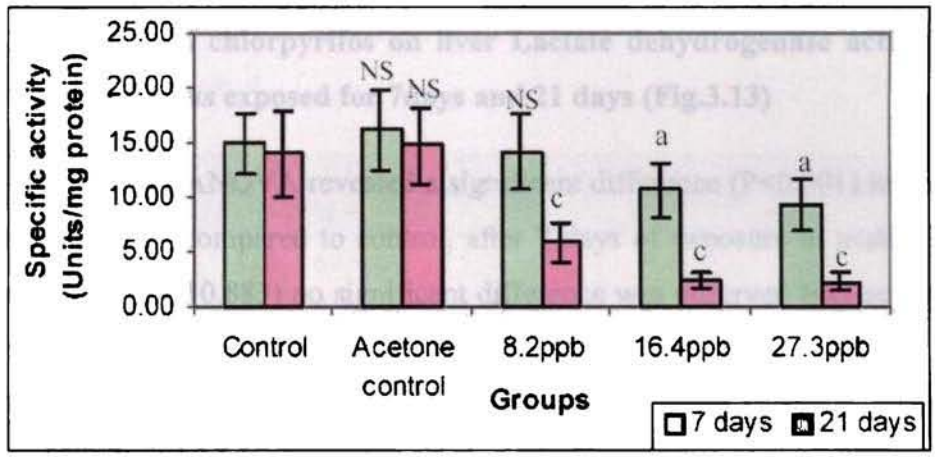


Fig.3.11: Effect of chlorpyrifos on brain Acetylcholinesterase activities in *O.mossambicus* exposed for 7 and 21days

One unit is µmole of acetyl thiocholine iodide hydrolysed/ minute
Each histogram represents mean±SD of 6 values; superscript represents the group significantly different from control; a- P<0.05; b- P<0.01; c- P<0.001, NS-not significant

Table 3.9 Effect of chlorpyrifos on gill Lactate dehydrogenase activities in *O.mossambicus* exposed for 7days (Fig.3.12)

One-way ANOVA and LSD analysis (LSD=9.652) revealed that after short-term chlorpyrifos exposure, no significant difference was observed between 8.2ppb group, 16.4ppb group and 27.3ppb group. But control showed significantly lower values ($P<0.05$) compared to the rest.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	33.92±7.79	35.19 ± 8.16^{NS}	44.77 ± 9.08^{a}	50.7 ± 9.87^{a}	49.6 ± 6.5^{a}
21 days	35.58±7.18	39.25 ± 8.99^{NS}	21.1 ± 3.77^{c}	20.88 ± 3.66^{c}	16.49 ± 4.16^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are expressed as mg of NADH oxidized per hour per gm protein

a-significant at 0.05level; c-significant at 0.001level; NS-not significant

Single-factor ANOVA revealed a significant difference ($P<0.001$) between control and different chlorpyrifos-treated groups, after a long-term exposure to chlorpyrifos. Further analysis by LSD (LSD=6.875), no significant difference was observed between control and acetone control and also between various pesticide-treated groups.

Table 3.10 Effect of chlorpyrifos on liver Lactate dehydrogenase activities in *O.mossambicus* exposed for 7days and 21 days (Fig.3.13)

Single-factor ANOVA revealed a significant difference ($P<0.001$) in 16.4ppb and 27.33ppb when compared to control, after 7 days of exposure to pesticide. By LSD analysis (LSD=10.883) no significant difference was observed between control and acetone control and also between control and 8.2ppb group. 8.2ppb group showed a significant difference from 16.4ppb and 27.33ppb groups. Difference between 16.4ppb and 27.3ppb was not significant.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	47.69±10.03	43.23±8.03^{NS}	50.18±11.7^{NS}	64.68±9.10^{c}	71.93±7.71^{c}
21 days	44.83±8.34	47.49±9.87^{NS}	38.28±6.77^{NS}	33.38±7.21^{c}	19.59±2.45^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are expressed as mg of NADH oxidized per hour per gm protein

c-significant at 0.001level; NS-notsignificant

A significant difference (P<0.001) was observed between control and 27.33ppb group followed by 16.4ppb group, after 21 days of exposure to pesticide. LSD value obtained was 8.497. It revealed that there is a significant difference in 27.3ppb group from 8.2ppb and 16.4ppb groups. 8.2ppb and 16.4ppb groups were observed as significantly indifferent.

Table 3.11 Effect of chlorpyrifos on brain Lactate dehydrogenase activities in *O.mossambicus* exposed for 7days and 21 days (Fig.3.14)

After 7 days of exposure, single-factor ANOVA revealed a significant difference (P<0.001) in 16.4ppb and 27.33ppb groups compared to control. By further analysis by LSD (LSD= 1.69) 8.2ppb was not significant different from control. But 8.2ppb was significantly different from 16.4ppb and 27.33ppb. No significant difference was observed between 16.4ppb and 27.33ppb and also between control and acetone control.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	4.55±1.33	5.02±1.67^{NS}	4.7±1.15^{NS}	7.30±1.36^{c}	7.84±1.72^{c}
21 days	4.55±1.33	4.48±1.27^{NS}	3.09±1.03^{c}	1.89±0.55^{c}	1.47+0.71^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are expressed as mg of NADH oxidized per hour per gm protein

c-significant at 0.001level NS-not significant

After 21 days of chlorpyrifos exposure, all the chlorpyrifos-treated groups showed a significant difference ($P<0.001$) from control when analyzed by One-way ANOVA. Further analysis by LSD (LSD=1.16) revealed that 27.33ppb group showed the highest significance followed by 16.4ppb and 8.2ppb. No significant difference was observed between control and acetone control and also between 16.4ppb and 27.33ppb groups. But a significant difference was observed in 8.2ppb from 16.4ppb and 27.33ppb.

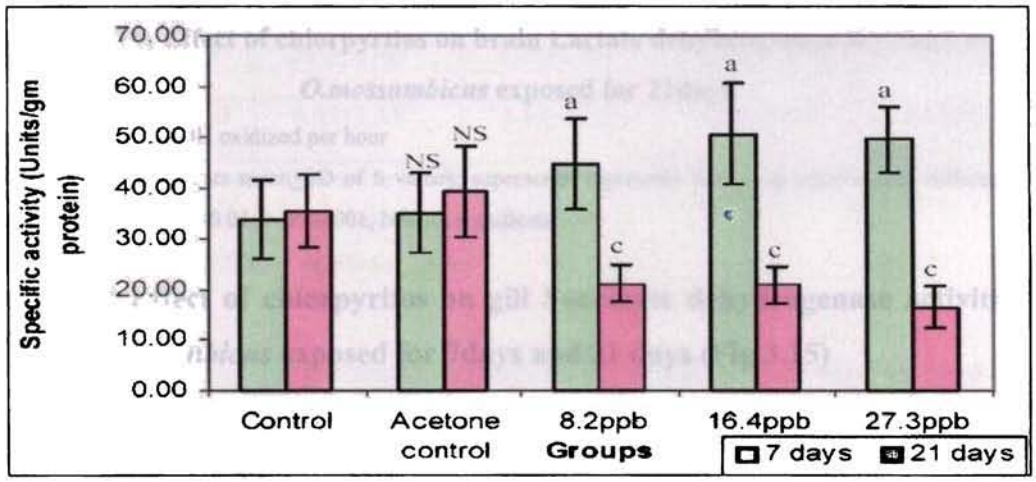


Fig.3.12: Effect of chlorpyrifos on gill Lactate dehydrogenase activities in *O.mossambicus* exposed for 7 and 21days

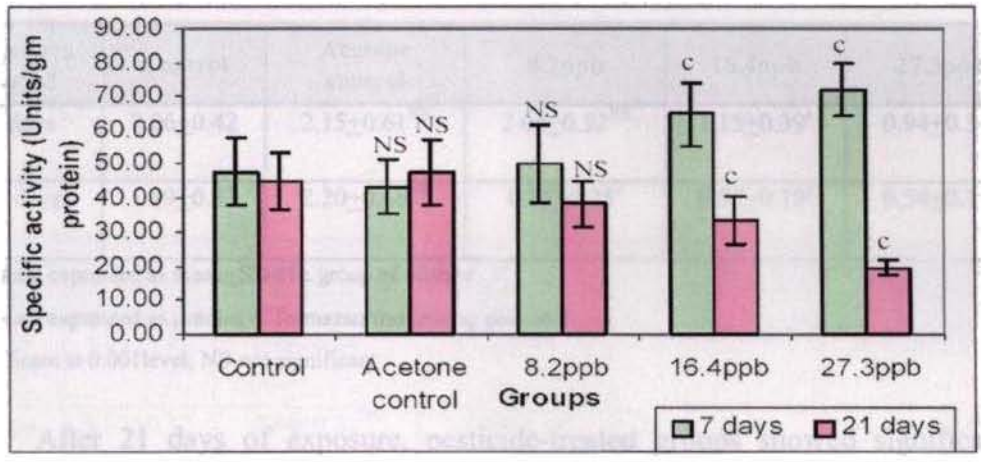


Fig.3.13: Effect of chlorpyrifos on liver Lactate dehydrogenase activities in *O.mossambicus* exposed for 7 and 21days

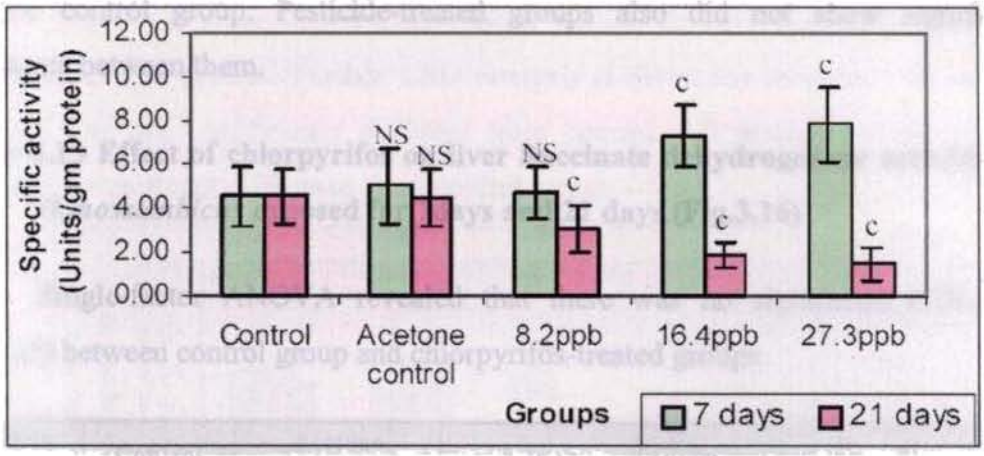


Fig.3.14: Effect of chlorpyrifos on brain Lactate dehydrogenase activities in *O.mossambicus* exposed for 21days

One unit is mg of NADH oxidized per hour

Each histogram represents mean±SD of 6 values; superscript represents the group significantly different from control; a- P<0.05; b- P<0.01; c- P<0.001, NS-not significant

Table 3.12 Effect of chlorpyrifos on gill Succinate dehydrogenase activities in *O.mossambicus* exposed for 7days and 21 days (Fig.3.15)

LSD value obtained was 0.54. One-way ANOVA revealed that chlorpyrifos-treated groups – 16.4ppb group and 27.3ppb group are significantly different (P<0.001) from control group. No significance was observed between control and acetone control group, after 7 days of chlorpyrifos exposure.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.3ppb
7 days	2.06±0.42	2.15 ± 0.61^{NS}	2.04 ± 0.52^{NS}	1.15 ± 0.39^{c}	0.94 ± 0.37^{c}
21 days	1.89±0.33	2.20 ± 0.66^{NS}	0.85 ± 0.25^{c}	0.55 ± 0.19^{c}	0.54 ± 0.19^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are expressed as µmoles of formazan formed/mg protein/hr

c-significant at 0.001level; NS-not significant

After 21 days of exposure, pesticide-treated groups showed significantly (P<0.001) lower values compared to control group. LSD analysis with LSD value, 0.425 showed that no significant difference was observed between control group and

acetone control group. Pesticide-treated groups also did not show significant difference between them.

Table 3.13 Effect of chlorpyrifos on liver Succinate dehydrogenase activities in *O.mossambicus* exposed for 7days and 21 days (Fig.3.16)

Single-factor ANOVA revealed that there was no significant difference (P>0.05) between control group and chlorpyrifos-treated groups.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.3ppb
7 days	5.86±1.37	5.97±1.794^{NS}	7.05±1.75^{NS}	6.96±1.59^{NS}	7.42±1.58^{NS}
21 days	5.86±1.37	6.47±1.53^{NS}	3.69±1.15^{c}	2.28±0.74^{c}	1.45±0.47^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are expressed as µmoles of formazan formed/mg protein/hr

c-significant at 0.001level; NS-not significant

There was a significant difference (P<0.001) between control and chlorpyrifos-treated groups. LSD value obtained was 1.297. Among pesticide treated groups, 27.3ppb group showed significantly higher value followed by 16.4ppb group and 8.2ppb group.

Table 3.14 Effect of chlorpyrifos on brain Succinate dehydrogenase activities in *O.mossambicus* exposed for 7days and 21 days (Fig.3.17)

There was no significant difference (P>0.05) between control and chlorpyrifos-treated groups.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.3ppb
7 days	0.84±0.20	0.86±0.24^{NS}	0.75±0.19^{NS}	0.89±0.25^{NS}	0.96±0.28^{NS}
21 days	0.82±0.13	0.84±0.21^{NS}	0.49±0.14^{c}	0.13±0.04^{c}	0.12±0.04^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are expressed as µmoles of formazan formed/mg protein/hr

c- significant at 0.001level; NS-not significant

There was a significant difference (P<0.001) between control and different pesticide-treated groups. Further LSD analysis (LSD=0.15) revealed that acetone control was not significantly different from control. All pesticide-treated groups showed a significant difference from control group.

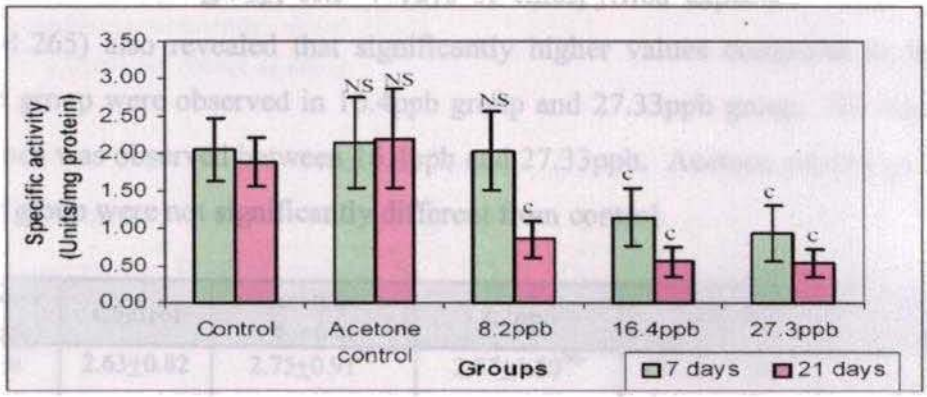


Fig.3.15: Effect of chlorpyrifos on gill Succinate dehydrogenase activities in *O.mossambicus* exposed for 7 and 21days

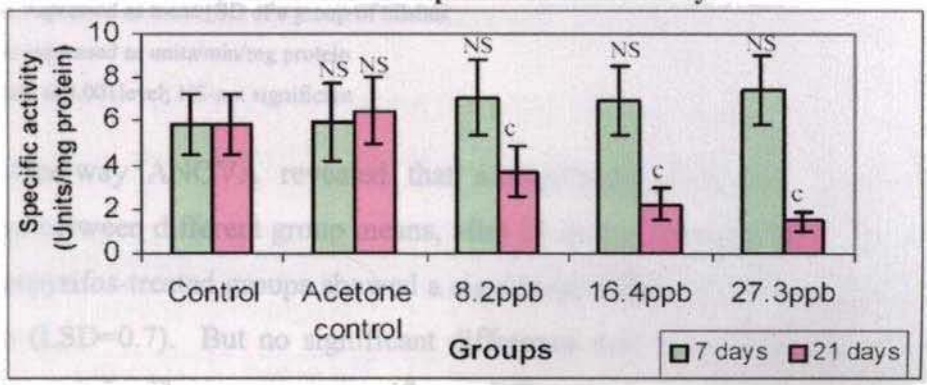


Fig.3.16: Effect of chlorpyrifos on liver Succinate dehydrogenase activities in *O.mossambicus* exposed for 7 and 21days

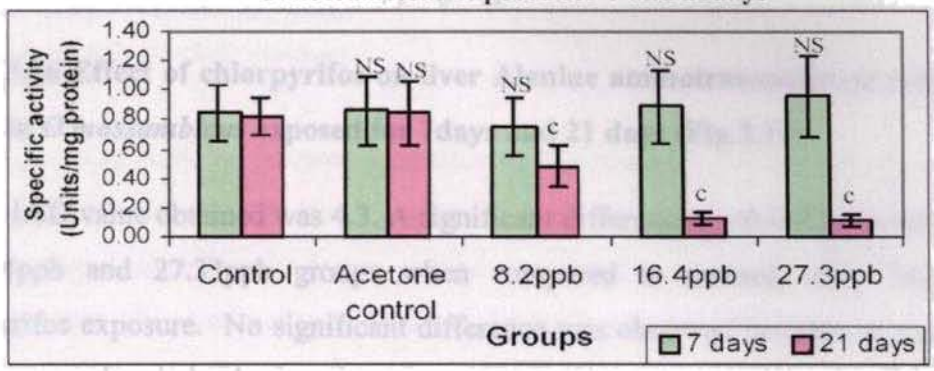


Fig.3.17: Effect of chlorpyrifos on brain Succinate dehydrogenase activities in *O.mossambicus* exposed for 7 and 21days

One unit is µmole of formazan formed/hr
Each histogram represents mean±SD of 6 values; superscript represents the group significantly different from control; a- P<0.05; b- P<0.01; c- P<0.001, NS-not significant

Table 3.15 Effect of chlorpyrifos on gill Alanine aminotransaminase activities in *O.mossambicus* exposed for 7days and 21 days (Fig.3.18)

There was a significant difference (P<0.001) in 16.4ppb and 27.33ppb groups compared to control group, after 7 days of chlorpyrifos exposure. LSD analysis (LSD=1.265) also revealed that significantly higher values compared to those of control group were observed in 16.4ppb group and 27.33ppb group. No significant difference was observed between 16.4ppb and 27.33ppb. Acetone control group and 8.2ppb group were not significantly different from control.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	2.63±0.82	2.75±0.91^{NS}	2.87±1.20^{NS}	5.19±1.36^{c}	5.6±1.10^{c}
21 days	2.68±1.13	2.52±0.62^{NS}	1.04±0.29^{c}	0.55±0.33^{c}	0.22±0.13^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are expressed as units/min/mg protein

c-significant at 0.001level; NS-not significant

One-way ANOVA revealed that a significant difference (P<0.001) was observed between different group means, after 21 days of chlorpyrifos exposure. All the chlorpyrifos-treated groups showed a significant difference form control by LSD analysis (LSD=0.7). But no significant difference was found between control and acetone control. There was no significant difference between 8.2ppb and 16.4ppb and also between 16.4ppb and 27.33ppb groups.

Table 3.16 Effect of chlorpyrifos on liver Alanine aminotransaminase activities in *O.mossambicus* exposed for 7days and 21 days (Fig.3.19)

LSD value obtained was 4.3. A significant difference (p<0.001) was observed in 16.4ppb and 27.33ppb groups when compared to control, after 7days of chlorpyrifos exposure. No significant difference was observed between control and acetone control and also between control and 8.2ppb group. All chlorpyrifos-treated groups showed a significant difference between them.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	12.91±2.38	13.15±2.28^{NS}	15.95±1.89^{NS}	22.35±3.77^{c}	30.27±6.39^{c}
21 days	12.65±1.68	11.74±1.67^{NS}	16.73±1.88^{c}	16.02±1.45^{c}	14.43±2.30^{NS}

Values are expressed as mean±SD of a group of 6fishes

Values are expressed as units/min/mg protein

c-significant at 0.001level; NS-not significant

There was a significant difference (P<0.001) between control and chlorpyrifos-treated groups- 16.4ppb group and 8.2ppb group, after 21 days of pesticide exposure. Further analysis by LSD (LSD=2.1) revealed that there was no significant difference between control group and 27.3ppb group.

Table 3.17 Effect of chlorpyrifos on brain Alanine aminotransaminase activities in *O.mossambicus* exposed for 7days and 21 days (Fig.3.20)

One-way ANOVA revealed that there was no significant difference (P<0.05) between control and chlorpyrifos treated groups, after 7 days of exposure.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	1.02±0.32	1.04±0.34^{NS}	1.15±0.37^{NS}	1.06±0.36^{NS}	1.25±0.41^{NS}
21 days	1.01±0.21	0.96±0.29^{NS}	0.73±0.21^{c}	0.67±0.23^{c}	0.25±0.09^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are expressed as units/min/mg protein

c- significant at 0.05level; NS-not significant

One-way ANOVA showed a significant difference (P<0.001) between control and all chlorpyrifos-treated groups, after 21 days of exposure. Pesticide treated groups showed significantly lower values compared to control. No significant difference was observed between between 16.4ppb group and 8.2ppb group. But 8.2ppb group showed significantly higher values from 16.4ppb and 27.3ppb groups. LSD value obtained was 0.249.

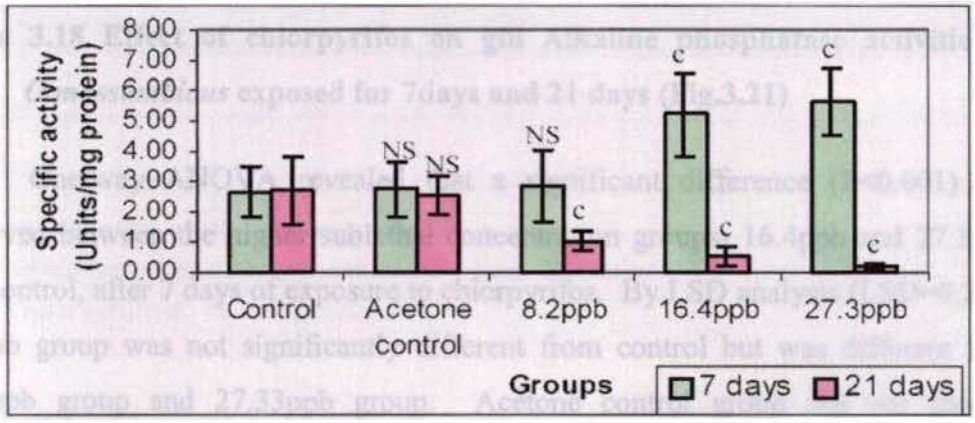


Fig.3.18: Effect of chlorpyrifos on gill Alanine aminotransaminase activities in *O.mossambicus* exposed for 7 and 21days

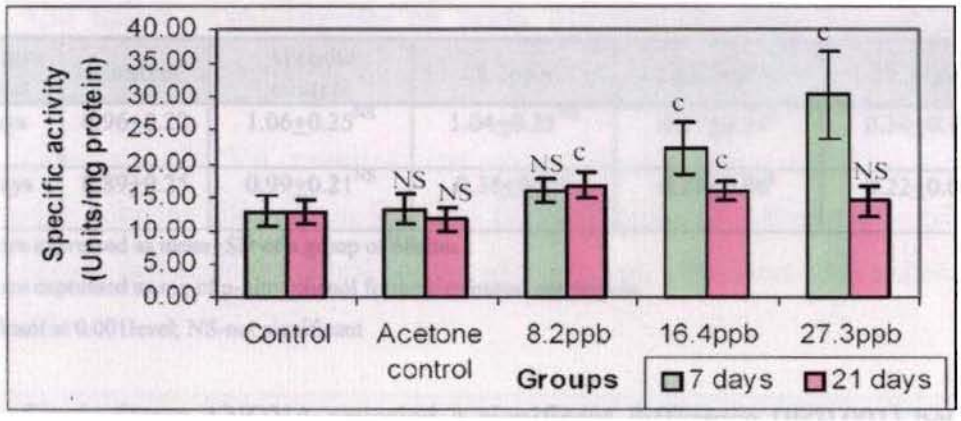


Fig.3.19: Effect of chlorpyrifos on liver Alanine aminotransaminase activities in *O.mossambicus* exposed for 7 and 21days

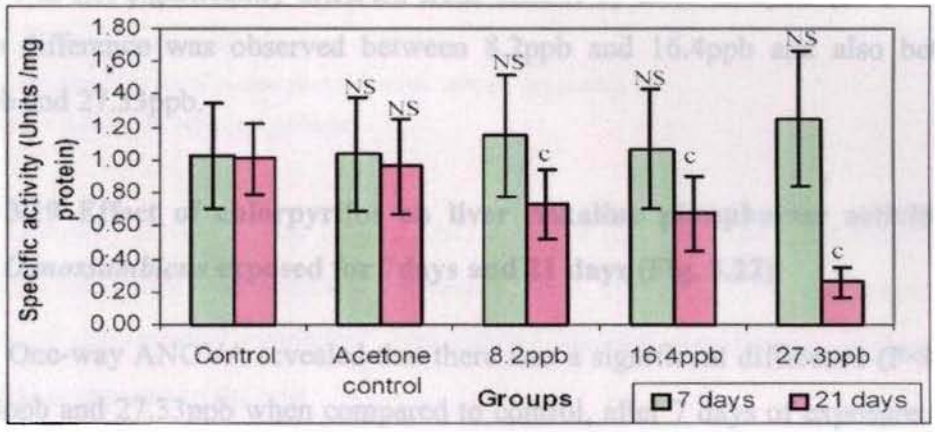


Fig.3.20: Effect of chlorpyrifos on brain Alanine aminotransaminase activities in *O.mossambicus* exposed for 21days

One unit is mmol of pyruvate formed/minute
Each histogram represents mean±SD of 6 values; superscript represents the group significantly different from control; a- P<0.05; b- P<0.01; c- P<0.001, NS-not significant

Table 3.18 Effect of chlorpyrifos on gill Alkaline phosphatase activities in *O.mossambicus* exposed for 7days and 21 days (Fig.3.21)

One-way ANOVA revealed that a significant difference (P<0.001) was observed between the higher sublethal concentration groups- 16.4ppb and 27.3ppb; and control, after 7 days of exposure to chlorpyrifos. By LSD analysis (LSD=0.238), 8.2ppb group was not significantly different from control but was different from 16.4ppb group and 27.33ppb group. Acetone control group did not show a significant difference from control. 16.4ppb and 27.33ppb did not show significant difference between them.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	0.96±0.22	1.06±0.25^{NS}	1.04±0.25^{NS}	0.57±0.19^{c}	0.34±0.10^{c}
21 days	0.89±0.25	0.99±0.21^{NS}	0.38±0.09^{c}	0.29±0.06^{c}	0.22±0.08^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are expressed as mg of p-nitro phenol formed/ minutes/ mg protein.

c-significant at 0.001level; NS-not significant

Single-factor ANOVA revealed a significant difference (P<0.001) between control and different chlorpyrifos-treated groups, after 21 days of exposure. Acetone control was not significantly different from control by LSD analysis (LSD=0.008). But no difference was observed between 8.2ppb and 16.4ppb and also between 16.4ppb and 27.33ppb.

Table 3.19 Effect of chlorpyrifos on liver Alkaline phosphatase activities in *O.mossambicus* exposed for 7days and 21 days (Fig. 3.22)

One-way ANOVA revealed that there was a significant difference (P<0.001) in 16.4ppb and 27.33ppb when compared to control, after 7 days of exposure. Also LSD analysis (LSD=1.29) revealed that 8.2ppb is significantly different from the other two pesticide-treated groups. No significant difference was observed between control and acetone control and also between 16.4ppb group and 27.33ppb group.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	3.77±0.89	3.27±0.51^{NS}	3.61±0.72^{NS}	7.11±1.75^{c}	7.06±1.26^{c}
21 days	3.53±0.93	3.93±0.75^{NS}	4.11±1.33^{NS}	3.34±0.77^{NS}	3.23±0.56^{NS}

Values are expressed as mean±SD of a group of 6fishes

Values are expressed as mg of p-nitro phenol formed/ minutes/ mg protein.

c-significant at 0.001level; NS-not significant

After 21 days of chlorpyrifos exposure, no significant difference (P>0.05) was observed between control and different pesticide-treated groups.

Table 3.20 Effect of chlorpyrifos on brain Alkaline phosphatase activities in *O.mossambicus* exposed for 7days and 21 days (Fig.3.23)

One-way ANOVA revealed that no significant difference (P<0.05) was observed in Alkaline phosphatase levels in the brain tissue of different chlorpyrifos-treated groups and acetone control compared to control, after short-term exposure to chlorpyrifos.

Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
0.49±0.14	0.47±0.15^{NS}	0.41±0.11^{NS}	0.37±0.07^{NS}	0.38±0.09^{NS}
0.45±0.09	0.49±0.09^{NS}	0.19±0.04^{c}	0.08±0.02^{c}	0.08±0.02^{c}

Values are expressed as mean±SD of a group of 6fishes

Values are erxpressed as mg of p-nitro phenol formed/ minute/ mg protein.

c-significant at 0.001level; NS-not significant

One-way ANOVA and further LSD analysis (LSD= 0.069) revealed that no significant difference was observed between 8.2ppb, 16.4ppb, 27.3 ppb groups, after 21 days of chlorpyrifos exposure. But control showed significantly higher value (P<0.001) compared to the rest.

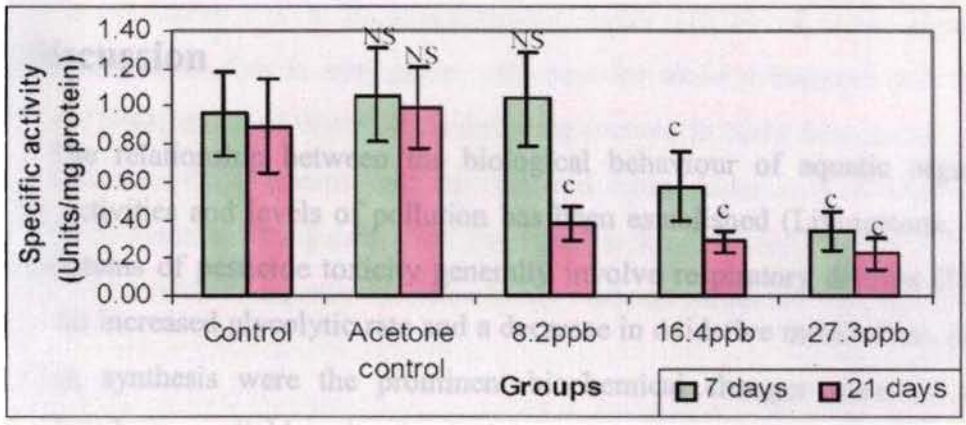


Fig.3.21: Effect of chlorpyrifos on gill Alkaline phosphatase activities in *O.mossambicus* exposed for 7 days and 21days

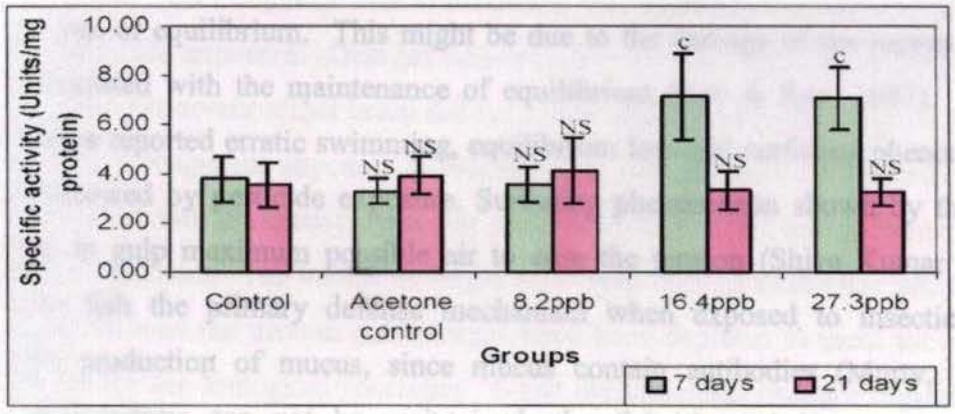


Fig.3.22: Effect of chlorpyrifos on liver Alkaline phosphatase activities in *O.mossambicus* exposed for 21days

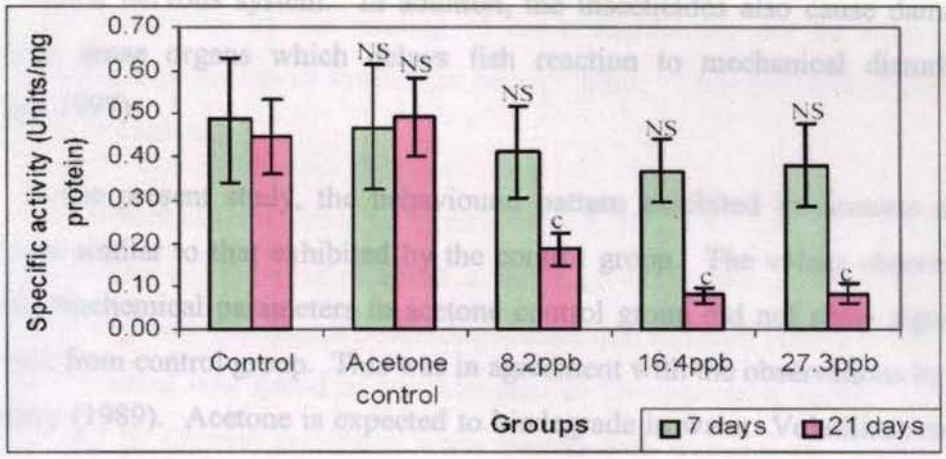


Fig.3.23: Effect of chlorpyrifos on brain Alkaline phosphatase activities in *O.mossambicus* exposed for 21days

One unit is mg p-nitro phenol formed /minute

Each histogram represents mean±SD of 6 values; superscript represents the group significantly different from control; a- $P<0.05$; b- $P<0.01$; c- $P<0.001$, NS-not significant

3.5 Discussion

The relationship between the biological behaviour of aquatic organisms, enzyme activities and levels of pollution has been established (Livingstone, 1991). The symptoms of pesticide toxicity generally involve respiratory distress (Holden, 1973). An increased glycolytic rate and a decrease in oxidative metabolism, protein, and RNA synthesis were the prominent biochemical changes observed during organophosphorus pesticide poisoning in fishes (Ghosh, 1987; Rao et al, 1986).

In the present study behavioural changes like irregular and erratic swimming indicate loss of equilibrium. This might be due to the damage of the region in the brain associated with the maintenance of equilibrium (Rao & Rao, 1987). Many studies have reported erratic swimming, equilibrium loss and surfacing phenomenon in fish followed by pesticide exposure. Surfacing phenomenon shown by the fish might be to gulp maximum possible air to ease the tension (Shiva Kumar et al., 2004). In fish the primary defense mechanism when exposed to insecticide is excessive production of mucus, since mucus contain antibodies (Murty, 1986). When homeostasis can not be maintained, the fish showed increased rate of respiration and nervous disorder due to damaged gill filaments and AChE inhibition in the central nervous system. In addition, the insecticides also cause damage to peripheral sense organs which delays fish reaction to mechanical disturbances (Fauziah, 1997).

In the present study, the behavioural pattern exhibited by acetone control group was similar to that exhibited by the control group. The values observed for different biochemical parameters in acetone control group did not show significant difference from control group. This was in agreement with the observations by Miny and Sastry (1989). Acetone is expected to biodegrade in water. Volatilization, with an estimated half-life of 20 hours, is expected to occur. Acetone does not significantly adsorb to sediment due to its high water solubility (ATSDR, 1994; Howard, 1990). Reported LC_{50} to *Lepomis macrochirus* (bluegill sunfish) is 8300

mg/L/96hr. Acetone has a Bioconcentration factor (BCF) of 0.69. Hence, its bioconcentration in fish is negligible. Agency for toxic substances and disease Regeistry (1994) reported that Fish do not store acetone in body from water. About half the acetone in a stream will be removed from water in less than a day. According to material safety data sheet from Mallinckrodt Chemicals, acetone is not expected to be toxic to aquatic life. The LC_{50}/ 96-hour values for fish are over 100 mg/L.

A significant difference was observed in the protein content of gill and liver tissues in *O.mossambicus* on short-term exposure to higher sublethal concentrations of chlorpyrifos. This might be due to moderate rate of protein depletion on a stress during insecticide exposure. Delayed neurotoxicity on toxic exposure might be the reason for the observation that brain did not show any significant change in protein content. On a long-term exposure to chlorpyrifos, a significantly high decrease in protein content in all the three tissues was observed. This might be due to metabolic utilization of proteins. The ketoacids might have been used to synthesize glucose by gluconeogenesis or the protein pools might have been depleted to meet the caloric requirement for the biological functions. Since the structural changes happened as evidenced from histological observations, the protein pools might have deteriorated and tried to involve in repair mechanisms. But the disrupted homeostasis and altered sterical conformations- by the binding of the pesticide-may not be allowing the protein synthetic apparatus to synthesize new proteins. Thus the protein showed a decrease in its content.

The present observation is in agreement with the investigations of Koundinya and Ramamurthy (1978) and revealed a decrease in protein content in *Tilapia mossambica* exposed to different pesticides. Sastry and Siddiqui (1984) reported that the protein content was found to be decreased in liver, muscle, kidney, intestine, brain and gill in *Channa punctatus* when treated with quinalphos. Yeragi et al. (2000) observed the decreased levels of proteins in gills, testis, ovaries and muscles of marine crab *Uca marionis* exposed to acute and chronic levels of malathion. Study by Aruna Khare et al. (2000) reported that the sublethal concentrations of

malathion showed a significant increase in the protein content which showed a gradual decrease on prolonged exposure to malathion. Also regarding brain, the observation from the present study is in consonance with the report by Praveena et al. (1994). According to the study, exposure to organophosphorus causes delayed neuropathy resulting in major changes in the concentration of protein and degeneration changes in the central nervous system, somatic and visceral sensitive system. A study by Svobodova et al (1994) reported that the level of total proteins in the blood plasma had a tendency to decrease in fish after a long-term exposure to pollutants.

On a short-term exposure to chlorpyrifos, Acetyl cholinesterase was found to be significantly decreased in gill tissue. A significant increase was observed in AChE activities in liver tissues. Hepatic tissues might have tried to keep the AChE levels in required amount for the biological reactions, by activating the enzyme synthetic process and thus to overcome the inhibition by the organophosphorus insecticide. The brain cells might have suffered from a delayed neurotoxicity. However, inhibition of enzyme might have adversely affected to bring the brain AChE activity to lower levels in 16.4ppb group and 27.3ppb groups. This might be due to the sufficient higher sublethal concentrations of chlorpyrifos capable to induce a toxic response.

On a long-term exposure to the insecticide, AChE levels are found to be significantly lowered in all the three tissues. This can be explained on the basis of the action of organophosphate insecticide on the enzyme and the impaired the synthesis of mew molecules of enzyme, by the damaged cells. Extensive studies in a variety of vertebrate and invertebrate species have conclusively shown that the acute toxic response noted with insecticidal organophosphate esters is due to the rapid inhibition of acetylcholinesterase, an essential component of nerve conduction in the central and peripheral nervous system.

Sahib and Rao (1980) reported that among the various tissues of Tilapia, the highest levels of AChE inhibition were noted in brain followed by muscle, gill and

liver. All organophosphate esters inducing a delayed neuropathy are either direct esterase inhibitors or are metabolically converted to inhibitors (Ronald Baron, 1981). Upon exposure to sublethal concentrations of organophosphorus insecticides, inhibition of brain and muscle AChE has been observed within 24hr, which indicates that bioactivation occurred rapidly after exposure (John Giesy et al, 1999). Brain plays a regulatory role in fish physiology and it is the most important organ in fish toxicology especially when pesticides are involved in their mode of action in the nervous system (Ware, 1983).

Chlorpyrifos was tested for its influence on the *in vitro* and *in vivo* brain AChE activity of *Fundulus heteroclitus* under laboratory as well as field conditions (Forgash, 1977). In *in vitro* studies, a 96-hr exposure of live fish to 1mg/L chlorpyrifos resulted in a maximum AChE inhibition of 24%. At a concentration of 2.1mg/L or higher, a 100% enzyme inhibition was observed after a 24-hr exposure period, followed by varying degrees of recovery, during the next 24-hr.

As repeated exposures appear to bring about increased depression of ChE activity in brain and liver tissues of aquatic organisms, intermittent anticholinesterase pesticide applications, especially organophosphorus insecticides, to the agricultural lands during cultivation seasons may manifest a threat to the fish populations inhabiting water bodies adjacent to these lands.

Lactate dehydrogenase (LDH) levels showed a significant increase in gill, liver and brain tissues of chlorpyrifos-treated fish groups after 7days of exposure to chlorpyrifos. This might be due to a shift from aerobic respiration to anaerobic respiration promoting the glycolytic rate and the conversion of pyruvic acid to lactic acid, under insecticide-induced stress. The tissues might have tried to compensate the incapability of oxygen consumption under stress condition. In most species of fish, the brain is readily accessible and easily removed. The brain is supplied with oxygenated blood that comes directly from the gills. The transfer, therefore, of toxic materials from the water across the gill membranes and to the reaction site is quick and direct. Succinate dehydrogenase (SDH) levels did not show significant changes

in liver and brain tissues. A significant decrease was observed in SDH level in gill tissue of fish of higher sublethal concentration groups (16.4ppb and 27.33ppb) on short-term exposure to chlorpyrifos. The enzyme might have been induced and synthesized at an increased rate to provide the required amount of ATP for biological functions.

In similar observations by Koundinya and Ramamurthi (1978) it is reported that depression in SDH and elevation in LDH activity in the fish *Tilapia mossambica* after exposure to sumithion favoured anaerobic metabolism. Subburaju and Selvarajan (1989) reported that at sublethal concentration, LDH activity in gills, brain, muscle, liver and ovary was elevated after the 7^{th} day onwards, while in the kidney it was found to be decreased. In contrast, after the 7^{th} day, SDH activity was decreased in gills, muscle, brain and liver except in the kidney where increase in SDH activity was observed. He interpreted this observation as a shift in the respiratory metabolism from aerobic to anaerobic in order to meet the enhanced energy demand under the toxic stress.

On long-term exposure to chlorpyrifos, the significant decrease in lactate dehydrogenase activities shown by gill and brain tissues might be due to tissue and cell destruction. Lower enzyme levels due to inhibition on synthetic process might have led the organism to a failure in meeting the energy demands even by anaerobic respiration. Liver tissues showed a decrease in enzyme activity, but not significant, in the 8.2ppb group. This might be due to the presence of enzyme in regenerating cells at certain regions of liver tissue, as observed in histopathological examination. It shows the potency of the hepatic cells to overcome the oxidative stress by meeting energy demands through anaerobic respiration even at prolonged exposure to chlorpyrifos at lower concentration. But 16.4ppb group and 27.33ppb group have shown significantly decreased enzyme activity. This is thought to be because of the aggressive damage happened to the tissue on exposure to chlorpyrifos. These findings can be correlated with morphological changes observed in histological observation in all the three tissues. The direct contact with the insecticide might have damaged the lipid and protein groups in gill tissue. This might have led to

mitochondrial disruption. Also the enzyme synthesis is expected to be adversely affected in the deteriorated gill tissues.

The results of the present study and a number of other reports reveal that the activity of mitochondrial enzyme SDH is decreased on exposure of fish to pesticides (Koundinya and Ramamurthi, 1978; Sastry and Siddiqui, 1984; Sahib et al, 1983).

Observations on the levels of LDH and SDH reveal that both anaerobic and aerobic metabolic pathways were adversely affected on long-term exposure of fish to the pesticide. The activity of SDH, the mitochondrial enzyme in all tissues was found to be decreased on long-term exposure to the organophosphate compound. Complete mitochondrial disruption releasing its contents might have led to the impaired aerobic respiration involving Kreb's cycle. These LDH and SDH changes might have subjected the organism to be lethargic until death occurs. Decreased SDH activity and an increased LDH activity has been reported in different tissues of liver, muscle, intestine, kidney, gill and brain of *Channa punctatus* when exposed to low and high concentration of phenyl mercuric acetate for short and long-term exposure (Karuppasamy, 2001). The LDH activity was reported as increased in gill, brain, muscles and liver tissues of *Channa punctatus* exposed to sublethal concentrations of metasystox (Natarajan, 1984) and phosphamidon in *Clarias batrachus* (Ghosh, 1987). Srinivas Reddy et al. (1993) reported that brain LDH decreased in *Channa punctatus* on exposure to Hexachlorocyclohexane for 15days.

Increase in ALT level was observed in gill and liver tissues on short-term chlorpyrifos exposure. This is assumed as an attempt by these tissues to overcome the xenobiotic toxicity. ALT is liver- specific cytoplasmic transaminase. The increased ALT activities in tissues suggest either increased operation of transamination or increased synthesis of aminoacids. This clearly indicates that stress brings about, the metabolic reorientation in the tissues by raising energy resources through transaminase systems. Similar studies have been reported by Tilak et al. (2004). An insignificant slight increase in ALT level was observed in brain tissue. Delayed neurotoxicity is expected to be the reason behind this observation. Certain

organophosphate esters produce a delayed neurotoxic response seven to fourteen days after acute poisoning (Baron, 1981).

Aspartate (AAT) and alanine (ALT) amino transferases are known to play strategic role in metabolizing l-amino acids for gluconeogenesis and also function as link between carbohydrate and protein metabolism under altered physiological, pathological and environmental stress conditions (Nicol and Rosen, 1963). A study on *O.mossambicus* reported that AAT and ALT enzyme activities were found to be increased in muscle, liver, gill and brain tissues of the fish after exposure to lindane in sublethal concentrations. The study inferred that the increased enzyme activity was due to increased utilization of aminoacids for energy synthesis, in fish suffering from toxic stress and energy crisis (Murthy et al, 1985).

But on long-term insecticide exposure, the structural alteration and the damage to the protein synthetic elements of the branchial cells might have led to a significantly decreased enzyme activity. Hepatic cells, the regenerating ones might have tried to overcome the stress conditions on long-term exposure so that the group means of chlorpyrifos treated groups showed a slight increase compared to group mean of control. The brain tissue on short-term exposure showed a slight elevation in enzyme activity, though not significant. But prolonged exposure was found to be effective to decrease the ALT levels significantly. This indicates that on exposure to higher sublethal concentrations, the degenerated and demyelinated neuronal cells are not capable to overcome the toxic stress induced by chlorpyrifos on long-term exposure.

On a short-term exposure, significant decrease was found in the gill ALP levels, which can be due to defective transphosphorylation reactions in the cells on direct contact with the insecticide. A dose-dependent response was observed in ALP activity in gill tissue. A significant increase in enzyme activities in liver tissue might be due to a stress-induced over activity of hepatobiliary cells, which have involved in detoxification mechanism. On long-term exposure, changes in ALP level were found to be insignificant. The only reason that can be expected is the presence of

regenerating hepatobiliary cells. But the enzyme level did not show any increase due to the presence of regenerating cells. Brain tissue, after 7 days of exposure to chlorpyrifos, did not show any significant change in ALP level. This is expected to be because of the delayed neurotoxicity on short-term insecticide exposure. Blood-Brain-Barrier might be protecting the brain from the immediate exposure to any foreign substance. But a significant decrease in brain ALP level was observed on long-term exposure. The decrease in enzyme activity after 21days of exposure to chlorpyrifos might have decreased the rate of transphosphorylation or uncoupling of oxidative phosphorylation. The present observation is in agreement with the investigations in liver tissue of *Oreochromis niloticus* exposed to malathion by Sarabadhikary and Sur (1982) and in *Clarias batrachus* exposed to mortal by Bhatnagar et al. (1996). A study by Mathivanan (2004) has reported toxicity of Quinalphos on alkaline phosphatase activity in *O.mossambicus*. When exposed to sublethal concentration for 15days alkaline phosphatase activity was found to be decreased in liver and instinal tissues. Shankar (1975) has stated that the decrease in alkaline phosphatase could be due to direct inhibition of the enzyme activity. Baby Shakila et al (1993) inferred that severe acidosis may be responsible for inhibition of alkaline phosphatase. This in turn could be adoptive for fish to meet the energy demand via anaerobic breakdown of glycogen. According to Mahendra and Agarwal (1983) inhibition of alkaline phosphatase acticvity by pesticide might be because, the enzyme serine residue at their active sites is inhibited by organophosphorus pesticide, quinalphos. It has been stated that organophosphorus compounds are general inhibitors of serine containing enzyme (Bell, 1970).

Acid and alkaline phosphatases are known as inducible enzymes and their activity goes up when there is a toxic impact and the enzymes begin to counteract the toxic effect. Murthy (1986) has reported that biochemical changes induced by pollutants disturb important enzymes, retard growth and reduce the fecundity and longevity. The observations from the present study also revealed changes in enzyme activities and protein content on exposure to the insecticide chlorpyrifos. In early stages of pesticide poisoning, fish usually showed increased activity but this subsequently decreased until death occurs (Holden, 1973). Responses to

organophosphorus insecticides by aquatic organisms are broad ranged depending on the compound, exposure time, water quality and species (Eisler, 1970; Fisher, 1991; Richmonds and Dutta, 1992). Subburaju and Selvarajan (1989) reported changes in free sugar and amino acids, protein metabolism and lipid content in various regions of the brain of *Tilapia mossambica* exposed for 4days to 0.7µg/L chlorpyrifos.

The effect of exposure to sublethal concentrations of the novel organophosphate insecticide, 2-butenoic acid-3-(diethoxyphosphinothioyl) methyl ester on biochemical parameters of *Oreochromis mossambicus* was studied by Venkateswara Rao (2006) for 30days and reported that acetylcholinesterase activity of brain, gill and muscle was inhibited by 67%, 77% and 73% respectively on day-30. The plasma and kidney alanine aminotransferase and aspartate aminotransferase activity increased, while decreases were observed in gill and liver. Increases in acid phosphatase and alkaline phosphatase activities were observed in plasma, gill and kidney. Lactate dehydrogenase activity decreased in liver and muscle, indicating tissue damage but a significant increase in LDH activity in gill and brain was observed.

A study by Sudheer Kumar (2006) reported that the AChE level was inhibited by chlorpyrifos and azadirachtin in all tissues of 7 and 15 days exposed fish. The inhibition was significant in all tissues except in brain and gill of 7days azadirachtin exposed fish. When the individual effects of chlorpyrifos and azadirachtin were compared on the inhibition of AChE, the effect of chlorpyrifos was greater than the azadirachtin in all tissues of pesticide exposed fish. Study by Haripriya et al (1995) in different regions of brain from albino rat exposed for 30days to monocrotophos, an organophosphate reported a decrease in acetylcholinesterase activity. In fish living in natural waters, even a relatively low concentration of organic phosphoric acid esters is capable of causing considerable AChE inhibition (Williams and Sova, 1966). This has been attributed to the enhanced accumulation of chemical pollutants in fish.

Chlorpyrifos is acutely toxic to some species of aquatic invertebrates and teleosts at nominal water concentrations ranging between 0.035 and 1.1μg/L. Sublethal effects were recorded in all species of organisms examined at concentrations below those causing mortality. These effects included bioconcentration from the medium by teleosts; cholinesterase activity reduction in brain and haematopoietic tissues; reduced growth; impaired reproduction, including sterility and developmental abnormalities; motor incoordination; convulsions and depressed population densities of aquatic invertebrates (Edward, 1988). Fish exposed to sublethal concentrations of many different types of pesticides exhibit changes in physiological actions, failures in reproduction and other effects (Holden, 1973; Brown, 1978).

Thus it is evident that chlorpyrifos is potent to change the enzyme levels and protein content on prolonged exposure. The changes in various biochemical molecules can lead to improper homeostasis and impaired metabolic processes.

Chapter

4

Effects of chlorpyrifos on different antioxidant enzyme activities in *Oreochromis mossambicus*

Contents

4.1 Introduction

Oxygen in its molecular state, O_2, is essential for many metabolic processes that are vital to aerobic life. This dependence on oxygen, forces aerobic life to withstand considerable toxicity (Ahmad, 1995). The oxygen paradox derives from the chemical nature of oxygen, which in its atomic form (O) is a free radical and in its molecular form (O_2) is a free bi-radical (Davies, 2000). The bi-radical nature of the oxygen molecule allows oxidation/ reduction reactions. However, monovalent reduction generates several reactive oxygen species (ROS) such as superoxide radical (O^{2-}), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-).

Oxidative stress can be defined most simply as the imbalance between the production of free radicals capable of causing peroxidation of the lipid layer of cells and the body's antioxidant defense. Free radicals are defined as atoms or molecules that contain one or more unpaired electrons. Oxidative stress occurs when the ROS generation rate exceeds that of their removal (Sies, 1986). Free radicals and other reactive oxygen species are derived either from normal essential metabolism in the human body or from external sources, such as exposure to rays, ozone, cigarette smoking, certain drugs, pesticides, air pollutants and industrial chemicals. Free radical formation occurs continuously in cells as a consequence of both enzymatic and non-enzymatic reactions. Its deleterious effects include oxidation of proteins, DNA and steroid components as well as peroxidation of unsaturated lipids in cell membranes. This produces unstable lipid hydroperoxides, the products of which, on decomposing, are highly reactive, threatening the cell integrity; in addition, these products can break down into free radicals that can perpetuate the destructive cycle of lipid-peroxidation chain reactions (Rosa, 2005). Enhanced lipid peroxidation, alterations in antioxidant activity, or imbalance in the cellular redox system may be used as early 'markers' of oxidative stress (Stripp and Trombetta, 1994).

When an extra electron is added to oxygen, free radical superoxide O^{2-} results. Some of these are formed accidentally in tissues, during the electron transfer process in mitochondria. Active phagocytes produce superoxide to kill bacteria.

Superoxide is also involved in the activity of the myeloperoxidase, produced by neutrophils which oxidizes chloride ions to the antibacterial hypochlorous acid, HOCl.

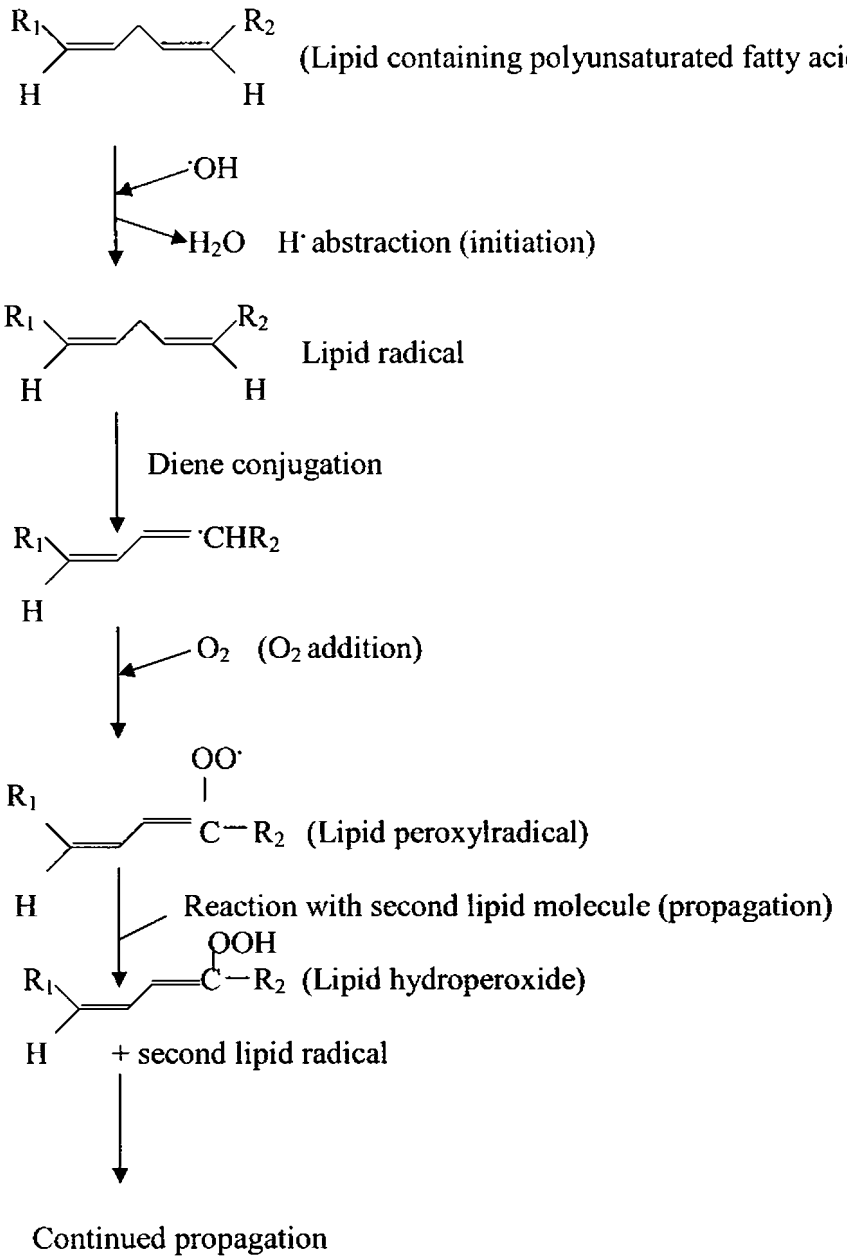


Fig 4.1: Hydroxyl radical (·OH)- mediated lipid peroxidation, a free radical chain reaction

Capillary endothelium releases a vasodilator in response to vasoactive compounds like acetylcholine and bradykinin. The endothelium also produces small amounts of superoxide. Superoxide levels in the body are kept within limits by the enzyme superoxide dismutase (SOD), which converts the superoxide to hydrogen peroxide. The latter itself is cytotoxic, however, H_2O_2 releases Ca^{++} from the membranes to cells, by damaging cell membranes. Proteolytic enzymes are activated by excess calcium, causing cell auto-destruction. Hydrogen peroxide also reacts with superoxide, separating another free radical, the $OH^{\cdot}$ This in turn causes its havoc on DNA and membranes. The enzyme glutathione peroxidase removes H_2O_2 by oxidizing reduced glutathione (Halliwell, 1989).

The first line of defense to oxidative stress is the use of antioxidant substances such as vitamin C, vitamin E, uric acid, glutathione and carotenoids. Also, diverse antioxidant enzymes prevent the cascade of oxidant reactions, intercepting and inactivating with the reactive intermediates of oxygen, closing the lipid-peroxidation cycle. Antioxidant enzymes are crucial in the effort to counteract oxygen toxicity when the supply of other antioxidant compounds is scarce or depleted (Ahmad, 1995). The antioxidant substances together with the enzymes constitute what are called "primary antioxidants" (Cadenas, 1995).

Water pollution and other forms of environmental stress can have a number of harmful effects (Salvato, 1976). Such effects include lipid peroxidation (Dianzani, 1987), which has a number of detrimental effects on biological membranes (Robons and Cotran, 1994). These effects are counteracted by the presence of antioxidant enzymes (Harris, 1992). The probable most important enzyme, is however, superoxide dismutase (Fridovich, 1986).

Contaminated water may contain a wide range of organic and metallic pollutants, including polynuclear aromatic hydrocarbons, organochlorine pesticides (DDT, dieldrin), polychlorinated dibenzo-p-dioxins, dibenzofurans and other heterocyclic, organophosphate fertilizers, estrogenic compounds and many metals. Most of these are powerful oxidants (Avci et al, 2005). Aerobic organisms have both

chemical and enzymatic defence systems against the toxicity caused by reactive oxygen species (Asada, 1978).

Like all aerobic organisms, fish is susceptible to the attack of reactive oxygen species and have developed antioxidant defenses demonstrated by research primarily dating to the 1980s. Specially adapted enzymes, such as catalase (CAT), superoxide dismutase (SOD), and enzymes dependent on glutathione (glutathione peroxidase (GPX) and glutathione reductase (GR) have been detected in most fish species investigated to date (Rudneva, 1997). High levels of non-enzymatic antioxidants have been detected in marine invertebrates and fish (Mezes, 1986; Kossmann, 1988). Thus high glutathione levels have been found in red blood cells of fish (Dafre and Reischl, 1990) even when compared with mammals. The glutathione of the erythrocytes protect the hemoglobin from spontaneous oxidation to methemoglobin and compared with other vertebrate species, fish hemoglobin has a higher tendency to oxidation (Rosa, 2005)

Most of the research on oxidative stress in fish focuses on toxicological aspects, such as the effects of different xenobiotics on antioxidant-enzyme activities and on the intensity of lipid-peroxidation (Di Giulio et al, 1989; Bainy et al, 1996; Zikic et al, 1996; Hai et al, 1997). These parameters have been proposed as biomarkers for contaminants. However, reviewing the available studies, Rosa reported that a clear trend was not found since in most cases the response is reportedly dependent on species, tissue, antioxidant parameter and/or time (2005).

Glutathione-S-transferase (GST) is a group of multifunctional isoenzymes, which play an important role in detoxification of toxic electrophiles by catalyzing the conjugation of these electrophiles with glutathione. GST is considered as first line of defense against oxidative injury along with other antioxidant enzymes, decomposing O_2 and H_2O_2 before interacting to form the more reactive $OH^{\cdot}$ (Anandakumar, 2007).

Glutathione reductase plays an important role in maintaining intracellular GSH concentration by converting oxidized glutathione back to GSH utilizing

NADPH as cofactor. In cytosolic and mitochondrial compartments, the oxidized glutathione (GSSG) is reduced at the expense of NADPH by the ubiquitous flavin containing enzyme GR (Schirmer and Siegel, 1988).

Malondialdehyde (MDA) is a marker of membrane lipid peroxidation (LP) resulting from the interaction of ROS and the cellular membrane (Aslan et al., 1997). The final membrane damage can lead to a loss of cellular homeostasis by changing the membrane characteristics (Swann et al., 1991). ROS are produced by the univalent reduction of dioxygen to superoxide anion (O_2^-), which in turn disproportionates to H_2O_2 and O_2 spontaneously or through a reaction catalyzed by superoxide dismutase (SOD). Endogenous H_2O_2 may be converted to H_2O either by catalase (CAT) or glutathione peroxidase (GPX). Otherwise, it may generate a highly reactive free hydroxyl radical (OH.) via a Fenton reaction, which is strongly believed to be responsible for oxidative damage. GPX converts H_2O_2 or other lipid peroxides to water or hydroxy lipids, and during this process glutathione (GSH) is converted to oxidized glutathione (GSSG). To recycle GSSG, the cell utilizes the enzyme NADPH-dependent GSH-reductase, the NADPH being supplied to the reaction by glucose-6-phosphate dehydrogenase (Bachowski et al, 1997).

The present study was performed to investigate the efficiency of antioxidant defensive system in *O.mossambicus* exposed to sublethal concentrations of LC_{50} value. Various antioxidant enzymes like catalase, GR, GST, GPX, and SOD were included under study. Since lipid peroxidation products are the substrates of these enzymes, the lipid peroxidation products like conjugated diene and malondialdehyde are also included under investigation.

4.2 Materials and Methods

Fishes were collected and maintained as described under section 3.2.B. The fishes exposed to acetone and also chlorpyrifos at different sublethal concentrations of LC_{50} value for a short-term period of 7 days and a long-term period of 21days.

a. Assay of Catalase activity (E C 1.11.1.6)

Assay of catalase was performed using the method of Maehly and Chance, 1954.

Reagents:

0.01M phosphate buffer (pH 7.0)

30mM H_2O_2

Procedure

20µL tissue homogenate prepared in phosphate buffer was added prior to analysis to a reagent mixture containing 3mL 0.01M phosphate buffer and 30mM H_2O_2. Decrease in absorbance was measured spectrophotometrically. Specific activity was expressed as IU/mg protein.

b. Assay of Glutathione reductase activity (E C 1.6.4.2)

Glutathione reductase was estimated by the method of David et al (1983).

Reagents:

Phosphate buffer- 0.12mol/L (pH 7.2)

15mM EDTA

9.6mM NADPH

65.3mM GSSG (Oxidised glutathione)

Procedure

Fixed volume of tissue homogenate in phosphate buffer was mixed with a reaction volume containing 2.6mL phosphate buffer, 0.1mL EDTA and 0.1mL GSSG. 0.05mL of NADPH was added prior to assay. Decrease in absorbance at 340nm was measured spectrophotometrically. Enzyme activity was expressed as Units/ mg protein.

c. Assay of Glutathione-S-transferase activity (E C 2.5.1.18)

Glutathione-S-transferase was determined by the method of Beutler et al (1986).

Reagents:

0.5M phosphate buffer (pH 6.5)

1-chloro-2,4-di nitro benzene : 25mM in 95% ethanol

Glutathione (GSH) : 20mM

Procedure:

Different tissues were homogenized in 0.5M phosphate buffer. The reaction mixture containing 200µL phosphate buffer, 20µL CDNB and 730µL distilled water were taken in the tube taken as control. Test tube marked as test contained 200µL phosphate buffer, 20µL CDNB and 680µL distilled water. Then the tubes were incubated at 37^0C for 10minutes. After incubation, added 50µL of GSH in both set of tubes. After mixing well, added 50µL of tissue extract in test sample tube. Increase in absorbance was noted at 340nm for 5minutes spectophotometrically. Values are expressed in nmoles of CDNB complexed/minute/mg protein. The extinction coefficient between CDNB-GSH conjugate and CDNB is 9.6mM/cm

d. Assay of Glutathione peroxidase activity (E C 1.11.1.9)

Glutathione peroxidase in different tissues was estimated by the method of Rotruck, 1973.

Reagents:

Tris buffer	: 0.4M, pH 7.0
Sodium azide solution	: 10mM
Tricarboxylic acid	: 10%
EDTA	: 0.4mM
H_2O_2	: 0.2mM
Glutathione (GSH)	: 2mM

Procedure:

Weighed sample of different tissues was homogenized in a known volume of Tris buffer. To 0.2mL of Tris buffer, 0.2mL EDTA, 0.1mL sodium azide and 0.5mL

homogenate were added and mixed well. To this mixture 0.2mL of GSH followed by 0.1mL H_2O_2 solution were added. The contents were mixed and incubated at 37^0C for 10minutes along with a control containing all reagents except tissue homogenate. After 10minutes, the reaction was arrested by the addition of 0.5mL of 10%TCA. Tubes were centrifuged and the supernatant was assayed for GSH. The values are expressed as µg of GSH/minute/mg protein.

e. Assay of Superoxide dismutase activity (E C 1.15.1.1)

Superoxide dismutase in different tissues was determined using the method of Kakkar et al., (1984).

Reagents:

0.33M Sucrose

n-butanol

0.052M sodium pyro-phosphate buffer (pH 8.3)

0.0025M Tris-HCl buffer (pH 7.4)

186µM phenazine methosulphate (PMS)

300µM Nitroblue tetrazolium (NBT)

780µM NADH

Glacial acetic acid

Procedure:

Weighed samples of tissues were homogenized in 0.33M sucrose and subjected to differential centrifugation under cold conditions to obtain the cytosol fraction. Before estimating the activity, precipitating the protein from the supernatant with 90% ammonium sulphate did an initial purification and this fraction was then dialysed against 0.0025M Tris-HCl buffer (pH 7.4). The supernatant was used as the enzyme source. Assay mixture contained 1.2mL of sodium pyrophosphate buffer, 0.1mL PMS, 0.3mL NBT, 1.3mL distilled water and 0.1mL of tissue sample.The tubes were kept at 30^0C for 90sec and the reaction was stopped by the addition of 0.1mL of glacial acetic acid. Reaction mixture was shaken vigorously with 4mL of n-

butanol. The mixture was allowed to stand for 10minutes and centrifuged. The upper butanol layer was removed. Absorbance of chromogen in butanol was measured at 560nm against n-butanol blank. A system devoid of enzyme was taken as control. One unit of enzyme activity is defined as the enzyme concentration required to inhibit chromogen production by 50% in one minute under the assay conditions and specific activity is expressed as units/mg protein.

f. Estimation of Malondialdehyde

The amount of malondialdehyde was estimated by the method of Niehaus and Samuelson (1968).

Reagents:

TCA-TBA-HCl reagent: 15% (w/v) trichloro aceticacid and 0.375% (w/v) thiobarbituric acid in 0.25N HCl.
0.1M Tris-HCl buffer : pH 7.5

Procedure:

The tissue homogenate prepared in Tris-buffer was mixed thoroughly with 2mL TCA-TBA-HCl reagent.1ml distilled water was added, mixed and heated for 15minutes in a boiling water bath. It was then cooled and centrifuged for 10minutes at 600g. The absorbance of the coloured solution was read spectrophotometrically at 535nm against a reagent blank, free of tissue extract. Extinction coefficient of malondialdehyde is $1.56x10^5M^{-1}cm^{-1}$. The obtained values are expressed as millimoles/100gm wet weight of the tissue.

g. Estimation of conjugated diene

Amount of conjugated dienes was estimated by the method of Retnagal and Ghoshal (1966).

Procedure:

1mL of tissue homogenate of different tissues was mixed thoroughly with 5mL of chloroform:methanol (2:1) followed by centrifugation at 1000g for 5minutes to separate the phases. 3mL of the lower chloroform layer was recovered using micropipetter and placed in a test tube and dried in a 450C water bath under a stream of nitrogen. Obtained residue was dissolved in 1.5mL cyclohexane. 0.3mL NBT was added, mixed and absorbance was read at 233nm against blank of cyclohexane. Molar extinction coefficient of conjugated diene is $2.52x10^4$ $M^{-1}cm^{-1}$.

4.3 Statistical analysis

Statistical analysis was carried out by Single-factor ANOVA and followed by further LSD (Least significant difference) analysis (Zar, 1996). Significant difference in pesticide treated group from control group is represented along with Mean±SD.

4.4 Results

Responses of various antioxidant enzymes and lipid peroxidation parameters are recorded.

Table 4.1: Effect of chlorpyrifos on Gill Catalase activities in *O.mossambicus* exposed for 7days and 21days (Fig. 4.2)

Single-factor ANOVA revealed that after 7 days of chlorpyrifos exposure, there was a significant difference ($P<0.001$) between control and different chlorpyrifos-treated groups. Further analysis by LSD (4.964) revealed that acetone control was not significantly different from control. 27.33ppb was highly significant followed by 16.4ppb and 8.2ppb. 8.2ppb showed a significant difference from 16.4ppb and 27.33ppb groups. No significant difference was observed between 16.4ppb and 27.33ppb groups.

Exposure period	Control	Acetone Control	8.2ppb	16.4ppb	27.33ppb
7 days	20.25±2.71	23.96±2.74^{NS}	29.94±3.86^{c}	39.85±5.61^{c}	43.22±5.58^{c}
21 days	21±2.76	22.63±3.18^{NS}	28.10±3.48^{c}	15.63±2.67^{c}	13.90±3.16^{c}

Values are expressed as mean±SD of six fishes.

Values are expressed as IU/mg protein

c-significant at 0.001level; NS-not significant

Single-factor ANOVA and further analysis by LSD (LSD=3.538) revealed that after 21 days of exposure, chlorpyrifos-treated groups are significantly different ($P<0.001$) from control group. But no significant difference was observed between control and acetone control. 8.2ppb showed significantly higher values from 16.4ppb and 27.33ppb groups. No significant difference was observed between 16.4ppb and 27.33ppb groups.

Table 4.2: Effect of chlorpyrifos on Liver Catalase activities in *O.mossambicus* exposed for 7days and 21 days (Fig.4.3)

Single-factor ANOVA and further analysis by LSD (LSD=8.983) revealed that after 7 days of exposure, chlorpyrifos-treated groups are significantly different ($P<0.001$) from control group. The 27.33ppb group showed the highest significance followed by 16.4ppb and 8.2ppb. No significant difference was observed between control and acetone control. There was a significant difference between insecticide-treated groups.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	30.33±4.69	28.73±3.5^{NS}	41.91±5.28^{c}	52.81±6.97^{c}	67.41±13.85^{c}
21 days	32.52±4.66	31.72±4.78^{NS}	46.99±3.97^{c}	19.99±3.21^{c}	18.35±3.38^{c}

Values are expressed as mean±SD of six fishes.

Values are expressed as IU/mg protein

c-significant at 0.001level; NS-not significant

One-way ANOVA revealed that a significant difference (P<0.001) was observed between control and different insecticide-treated groups, after 21 days of exposure. By further LSD analysis (LSD=4.36) there was no significant difference observed between control and acetone control. Though 8.2ppb showed a significant difference from 16.4ppb and 27.33ppb, no significant difference was observed between 16.4ppb and 27.33ppb groups.

Table 4.3: Effect of chlorpyrifos on Brain Catalase activities in *O.mossambicus* exposed for 7days and 21 days (Fig.4.4)

One-way ANOVA revealed no significant difference (P>0.05) in brain catalase activities on a short-term exposure.

Exposure period	Control	Acetone Control	8.2ppb	16.4ppb	27.33ppb
7 days	13.34±2.41	13.14±2.94^{NS}	14.42±2.70^{NS}	15.52±1.29^{NS}	15.58±2.55^{NS}
21 days	11.81±1.51	12.75±3.27^{NS}	16.93±1.71^{c}	22.64±1.77^{c}	21.77±4.13^{c}

Values are expressed as mean±SD of six fishes.

Values are expressed as IU/mg protein

c-significant at 0.001level; NS-not significant

The LSD value obtained was 3.102 after the statistical analysis by single-factor ANOVA. After 21 days of chlorpyrifos exposure, there was a significant difference (P<0.001) between control and insecticide-treated groups and also between pesticide-treated groups. But no significant difference was observed between control and acetone control.

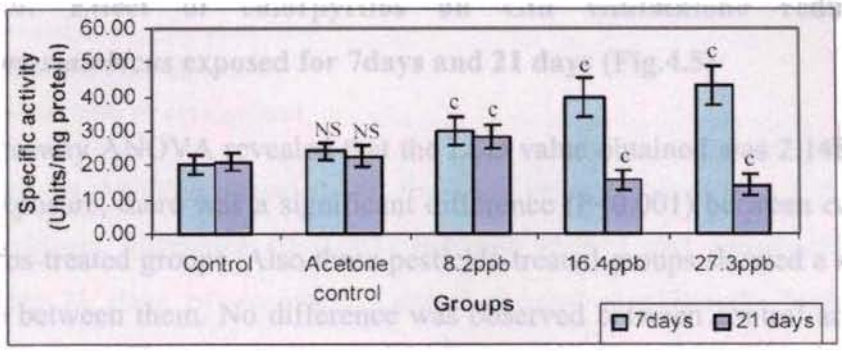


Fig.4.2: Effect of chlorpyrifos on Gill Catalase activities in*O.mossambicus* exposed for 7 and 21days

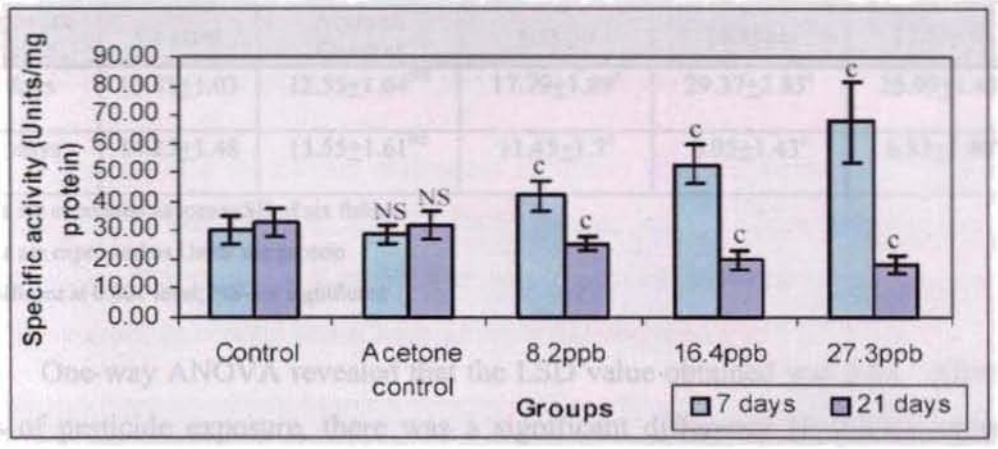


Fig.4.3: Effect of chlorpyrifos on liver Catalase activities in *O.mossambicus* exposed for 7 and 21days

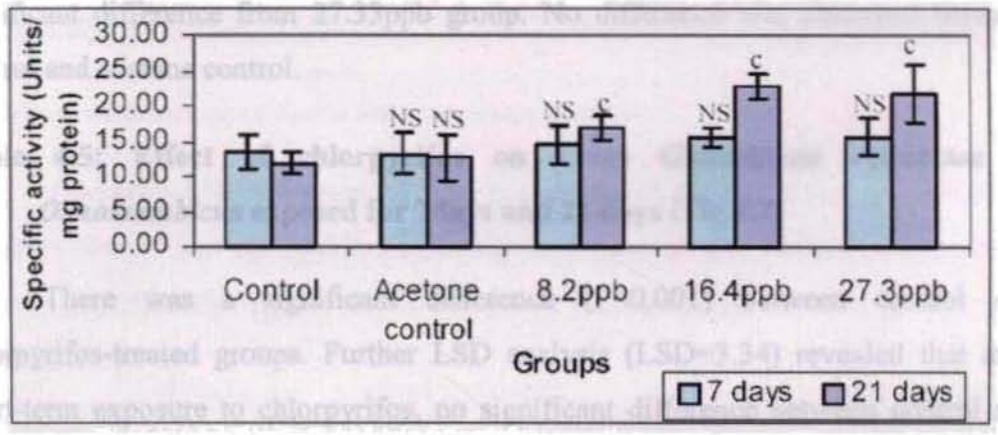


Fig.4.4: Effect of chlorpyrifos on Brain Catalase activities in *O.mossambicus* exposed for 7 and 21days

One unit is one micromole of H_2O_2 decomposed/ min
Each histogram represents mean±SD of 6 values; superscript represents the group significantly different from control; a- P<0.05; b- P<0.01; c- P<0.001, NS-not significant

Table 4.4: Effect of chlorpyrifos on Gill Glutathione reductase in *O.mossambicus* exposed for 7days and 21 days (Fig.4.5)

One-way ANOVA revealed that the LSD value obtained was 2.148. After 7 days of exposure, there was a significant difference (P<0.001) between control and chlorpyrifos-treated groups. Also these pesticide-treated groups showed a significant difference between them. No difference was observed between control and acetone control.

Exposure period	Control	Acetone Control	8.2ppb	16.4ppb	27.33ppb
7 days	13.81±1.03	12.55±1.64^{NS}	17.79±1.89^{c}	29.37±2.83^{c}	25.99±1.48^{c}
21 days	14.25±1.48	13.55±1.61^{NS}	11.45±1.7^{c}	6.95±1.43^{c}	6.83±1.80^{c}

Values are expressed as mean±SD of six fishes.

Values are expressed as Units/ mg protein

c-significant at 0.001 level; NS-not significant

One-way ANOVA revealed that the LSD value obtained was 2.04. After 21 days of pesticide exposure, there was a significant difference (P<0.001) between control and chlorpyrifos-treated groups. The pesticide-treated groups were significantly different from one another. But 16.4ppb group did not show a significant difference from 27.33ppb group. No difference was observed between control and acetone control.

Table 4.5: Effect of chlorpyrifos on Liver Glutathione reductase in *O.mossambicus* exposed for 7days and 21 days (Fig.4.7)

There was a significant difference (P<0.001) between control and chlorpyrifos-treated groups. Further LSD analysis (LSD=3.34) revealed that after short-term exposure to chlorpyrifos, no significant difference between control and acetone control. 8.2ppb was not significantly different from control.

Exposure period	Control	Acetone Control	8.2ppb	16.4ppb	27.33ppb
7 days	20.88±2.64	17.97±4.43^{NS}	24.03±1.82^{NS}	30.41±3.31^{c}	44.09±0.97^{c}
21 days	19.31±4.08	21.05±2.92^{NS}	13.03±1.92^{c}	7.74±1.51^{c}	4.09±0.97^{c}

Values are expressed as mean±SD of six fishes

Values are expressed as Units/ mg protein

c-significant at 0.001level; Ns-not significant

One-way ANOVA revealed that the LSD value obtained was 2.924. There was a significant difference (P<0.001) between control and chlorpyrifos-treated groups, after 21 days of exposure. Control showed significantly higher values compared to pesticide-treated groups. No difference between control and acetone control.

Table 4.6: Effect of chlorpyrifos on Brain Glutathione reductase in *O.mossambicus* exposed for 7days and 21days (Fig.4.8)

There was a significant difference (P<0.01) between control and pesticide-treated groups like 16.4ppb and 27.3ppb groups, after a short-term exposure to chlorpyrifos. No significant difference was observed between either control and acetone control or control and 8.2ppb group. LSD obtained was 5.25.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	24.97±4.62	24.61±3.50^{NS}	27.19±3.49^{NS}	30.47±2.95^{b}	35.47±6.99^{b}
21 days	23.96±4.52	23.61±3.44^{NS}	14.27±2.31^{c}	9.24±2.17^{c}	8.90±2.18^{c}

Values are expressed as mean±SD of six fishes

Values are expressed as Units/ mg protein

b-significant at 0.01level; c-significant at 0.001level; NS-not significant

One-way ANOVA revealed that there was a significant difference (P<0.001) between control and chlorpyrifos-treated groups after 21 days of exposure. Control showed significantly higher values compared to chlorpyrifos-treated groups. LSD value obtained was 3.578.

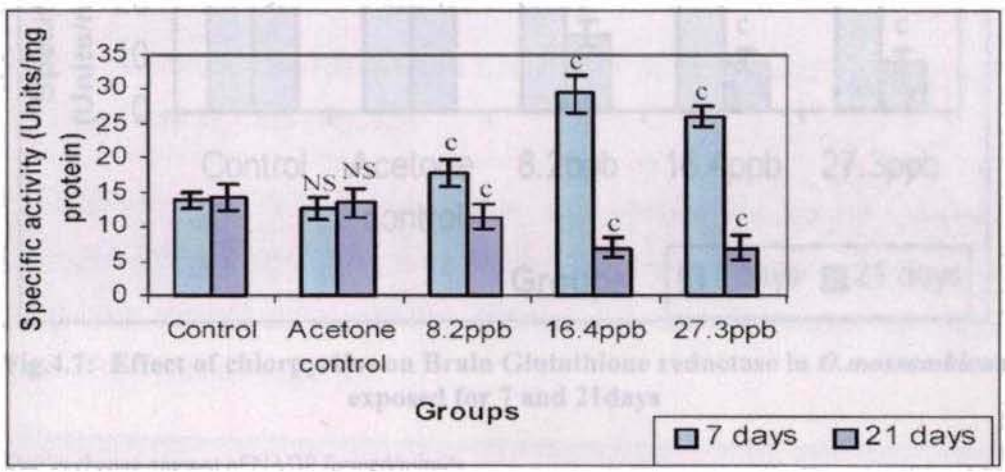


Fig.4.5: Effect of chlorpyrifos on Gill Glutathione reductase in *O.mossambicus* exposed for 7 and 21days

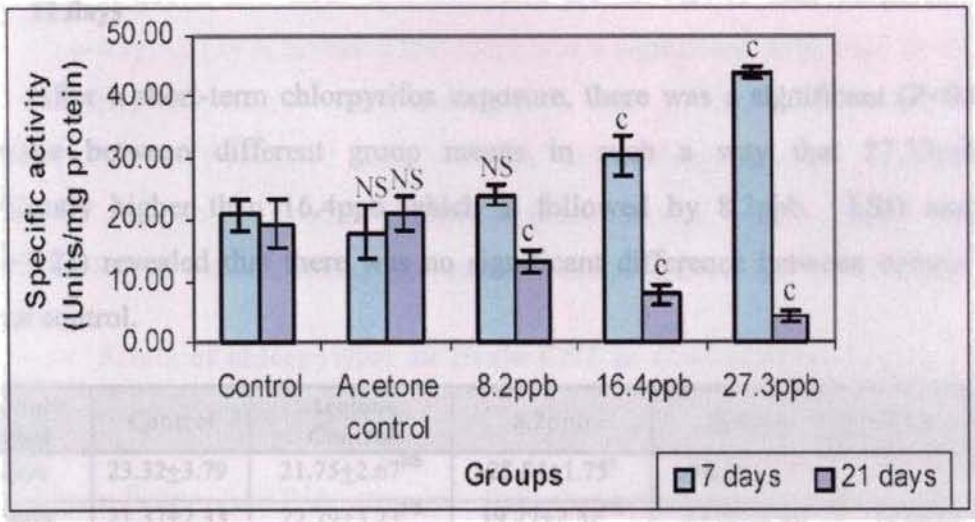


Fig.4.6: Effect of chlorpyrifos on Liver Glutathione reductase in *O.mossambicus* exposed for 7 and 21days

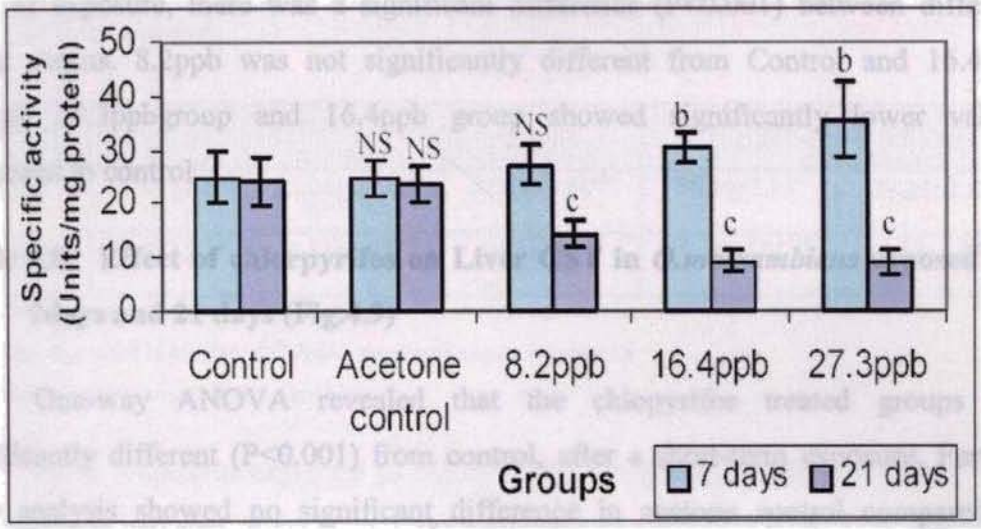


Fig.4.7: Effect of chlorpyrifos on Brain Glutathione reductase in *O.mossambicus* exposed for 7 and 21days

One Unit is change amount of NADP formed/minute
Each histogram represents mean±SD of 6 values; superscript represents the group significantly different from control; a- P<0.05; b- P<0.01; c- P<0.001, NS-not significant

Table 4.7: Effect of chlorpyrifos on Gill GST in *O.mossambicus* exposed for 7days and 21 days

After a short-term chlorpyrifos exposure, there was a significant (P<0.001) difference between different group means in such a way that 27.33ppb is significantly higher than 16.4ppb which is followed by 8.2ppb. LSD analysis (LSD=3.22) revealed that there was no significant difference between control and acetone control.

Exposure period	Control	Acetone Control	8.2ppb	16.4ppb	27.33ppb
7 days	23.32±3.79	21.75±2.67^{NS}	28.84±1.75^{c}	36.26±2.5^{c}	45.9±2.6^{c}
21 days	21.37±2.33	22.79±3.23^{NS}	19.27±2.32^{NS}	17.27±2.32^{c}	10.99±2.21^{c}

Values are expressed as mean±SD of six fishes
Values are expressed in nmoles of CDNB complexed/minute/mg protein.
c-significant at 0.001level; NS-not significant

One-way ANOVA revealed that the LSD value obtained was 3.282. After 21 days of exposure, there was a significant difference (P<0.001) between different group means. 8.2ppb was not significantly different from Control and 16.4ppb groups. 27.3ppbgroup and 16.4ppb group showed significantly lower values compared to control.

Table 4.8: Effect of chlorpyrifos on Liver GST in *O.mossambicus* exposed for 7days and 21 days (Fig.4.9)

One-way ANOVA revealed that the chlopyrifos treated groups are significantly different (P<0.001) from control, after a short-term exposure. Further LSD analysis showed no significant difference in acetone control compared to control. LSD value obtained was 5.75.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	31.89±4.26	30.1±4.12^{NS}	42.46±5.42^{c}	52.36±4.63^{c}	60±6.16^{c}
21 days	33.23±3.49	31.26±3.46^{NS}	26.63±6.06^{c}	11.12±2.98^{c}	9.31±3.33^{c}

Values are expressed as mean±SD of six fishes
Values are expressed in nmoles of CDNB complexed/minute/mg protein
c-significant at 0.001level; NS-not significant

One-way ANOVA revealed that there was a significant difference (P<0.001) between control and chlorpyrifos-treated groups, after 21 days of exposure. LSD analysis (LSD=4.57) revealed that 27.33ppb showed significantly higher values followed by 16.4ppb and then by 8.2ppb. Pesticide treated groups, except 16.4ppb group and 27.3ppb between them, showed a significant difference.

Table 4.9: Effect of chlorpyrifos on Brain GST in *O.mossambicus* exposed for 7days and 21 days (Fig.4.10)

Single-factor ANOVA revealed that there was a significant difference (P<0.001) between different group means, after a short-term exposure to chlorpyrifos. By LSD analysis (LSD=2.32), 8.2ppb did not show significant difference from control. Various Chlorpyrifos-treated groups showed significant difference between their group means. But acetone control was not significantly different from control.

Exposure period	Control	Acetone Control	8.2ppb	16.4ppb	27.33ppb
7 days	26.01±1.83	25.01±1.68^{NS}	27.44±1.66^{NS}	34.59±1.91^{c}	37.24±2.52^{c}
21 days	27.49±5.54	28.51±3.81^{NS}	19.35±2.43^{c}	15.26±3.98^{NS}	12.43±2.88^{NS}

Values are expressed as mean±SD of six fishes

Values are expressed in nmoles of CDNB complexed/minute/mg protein

c-significant at 0.001level; NS-not significant

After long-term exposure to chlorpyrifos, a significant difference (P<0.001) was observed between control and 8.2ppb chlorpyrifos-treated group, after long-term exposure to chlorpyrifos. Further analysis by LSD (LSD=4.68) revealed that control was not significantly different from acetone control. But 16.4ppb and 27.3ppb group did not show significant difference from control.

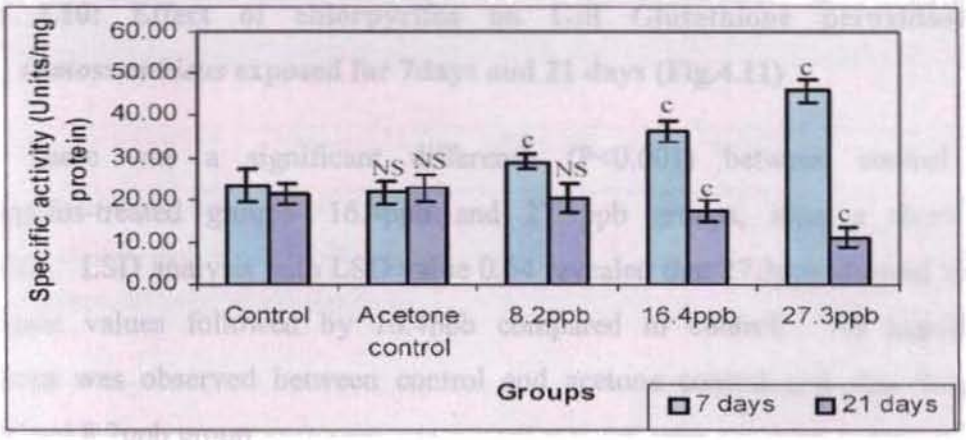


Fig.4.8: Effect of chlorpyrifos on Gill GST in *O.mossambicus* exposed for 7 and 21days

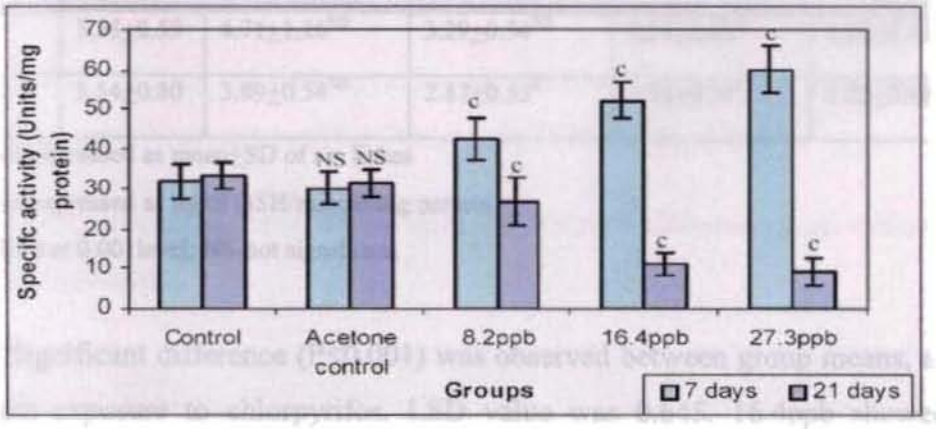


Fig.4.9: Effect of chlorpyrifos on Liver GST in *O.mossambicus* exposed for 7 and 21days

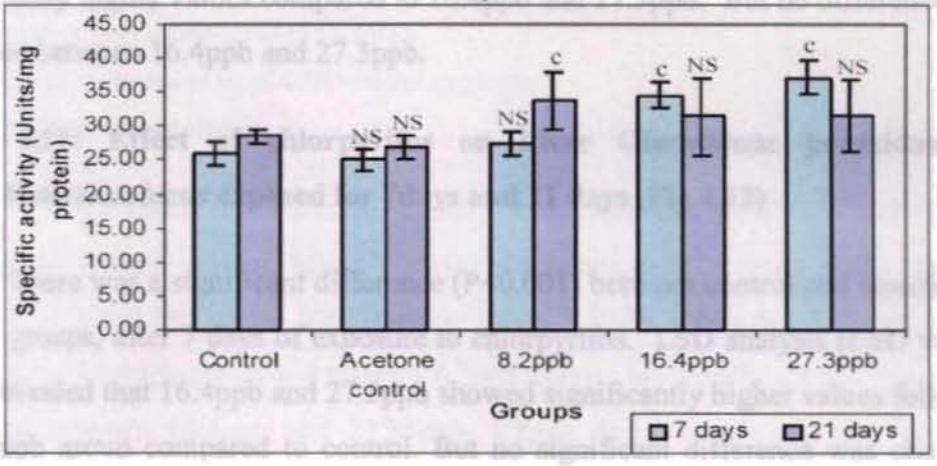


Fig.4.10: Effect of chlorpyrifos on Brain GST in *O.mossambicus* exposed for 7 and 21days

One Unit is nmoles of CDNB complexed/minute

Each histogram represents mean±SD of 6 values; superscript represents the group significantly different from control; a- P<0.05; b- P<0.01; c- P<0.001, NS-not significant

Table 4.10: Effect of chlorpyrifos on Gill Glutathione peroxidase in *O.mossambicus* exposed for 7days and 21 days (Fig.4.11)

There was a significant difference (P<0.001) between control and chlorpyrifos-treated groups- 16.4ppb and 27.3ppb groups, after a short-term exposure. LSD analysis with LSD value 0.64 revealed that 27.3ppb showed highly significant values followed by 16.4ppb compared to control. No significant difference was observed between control and acetone control and also between control and 8.2ppb group.

Exposure period	Control	Acetone Control	8.2ppb	16.4ppb	27.33ppb
7 days	3.38±0.59	4.71 ± 1.26^{NS}	3.29 ± 0.54^{NS}	4.24 ± 0.61^{c}	4.88 ± 0.53^{c}
21 days	3.54±0.80	3.89 ± 0.54^{NS}	2.87 ± 0.53^{c}	1.58 ± 0.31^{c}	2.02 ± 0.49^{c}

Values are expressed as mean±SD of six fishes

Values are expressed as μg of GSH/minute/mg protein.

c-significant at 0.001level; NS-not significant

Significant difference (P<0.001) was observed between group means, after a long-term exposure to chlorpyrifos. LSD value was 0.645. 16.4ppb showed the highest significant difference followed by 27.3ppb and 8.2ppb. 8.2ppb showed significantly higher values compared to 16.4ppb and 27.3ppb. But no difference was observed between 16.4ppb and 27.3ppb.

Table 4.11: Effect of chlorpyrifos on Liver Glutathione peroxidase in *O.mossambicus* exposed for 7days and 21 days (Fig.4.12)

There was a significant difference (P<0.001) between control and insecticide-treated groups, after 7 days of exposure to chlorpyrifos. LSD analysis (LSD value= 0.94) revealed that 16.4ppb and 27.3ppb showed significantly higher values followed by 8.2ppb group compared to control. But no significant difference was observed between control and acetone control. 8.2ppb group showed significant difference from 16.4ppb and 27.3ppb groups and no difference was observed between 16.4ppb and 27.3ppb groups.

Exposure period	Control	Acetone Control	8.2ppb	16.4ppb	27.33ppb
7 days	5.93±0.62	5.77±0.77^{NS}	7.09±0.87^{c}	9.52±0.93^{c}	9.42±0.85^{c}
21 days	5.31±0.72	5.46±0.86^{NS}	2.92±0.69^{c}	2.18±0.93^{c}	2.26±0.92^{c}

Values are expressed as mean±SD of six fishes

The values are expressed as µg of GSH/minute/mg protein.

c-significant at 0.001level; NS-not significant

One-way ANOVA revealed that there was a significant difference (P<0.001) between control and different pesticide-treated groups, after 21 days of chlorpyrifos exposure. From LSD analysis (LSD=0.917) no significant difference was seen between control and acetone control and also between chlorpyrifos-treated groups.

Table 4.12: Effect of chlorpyrifos on Brain Glutathione peroxidase in *O.mossambicus* exposed for 7days and 21 days (Fig.4.13)

No significant difference was observed between different group means, after 7 days of exposure.

Exposure period	Control	Acetone Control	8.2ppb	16.4ppb	27.33ppb
7 days	2±0.77	2.05±0.82^{NS}	2.9±0.58^{NS}	2.53±0.76^{NS}	2.84±0.79^{NS}
21 days	2.66±0.52	2.38±0.64^{NS}	1.47±0.35^{c}	1.16±0.65^{c}	0.67±0.33^{c}

Values are expressed as mean±SD of six fishes

The values are expressed as µg of GSH/minute/mg protein

c-not significant; NS-not significant

A significant difference (P<0.001) was observed between control and chlorpyrifos-treated groups, after 21 days of insecticide exposure. LSD analysis revealed (LSD=0.595) that significantly lower values given by 27.3ppb group followed by 16.4ppb group and then by 8.2ppb group. No significant difference was observed between 16.4ppb and 27.33ppb, 8.2ppb and 16.4ppb and also between control and acetone control.

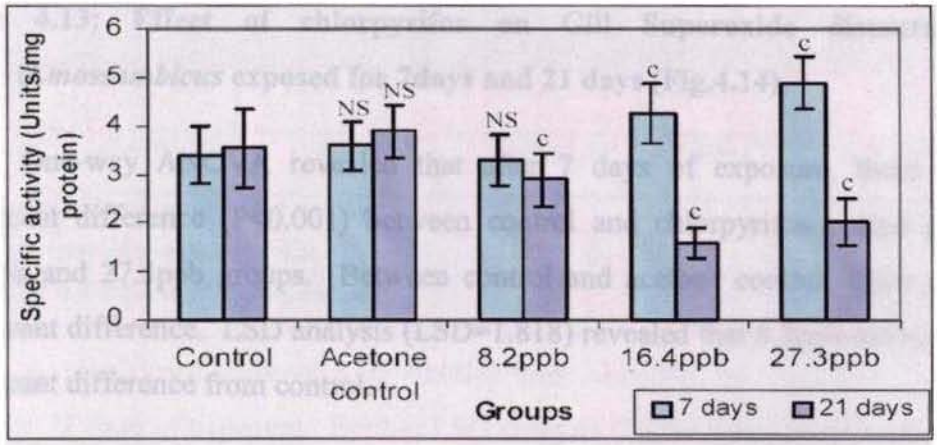


Fig.4.11: Effect of chlorpyrifos on Gill Glutathione peroxidase in *O.mossambicus* exposed for 7 and 21days

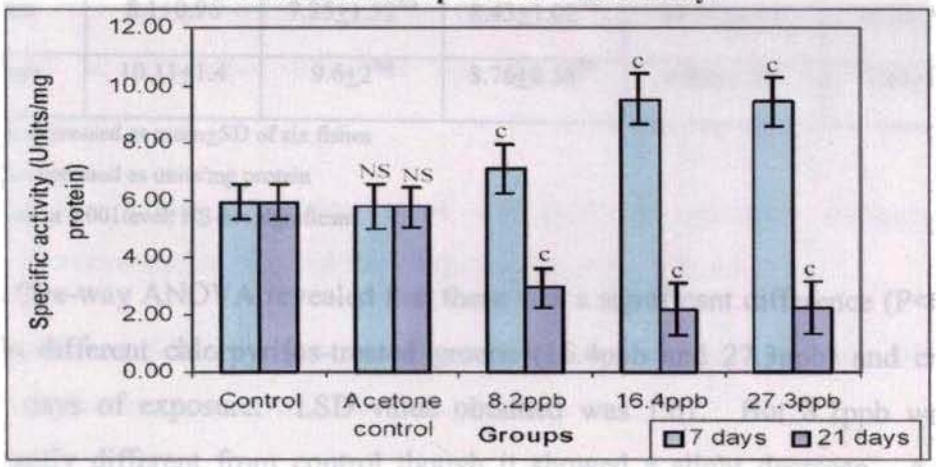


Fig.4.12: Effect of chlorpyrifos on Liver Glutathione peroxidase in *O.mossambicus* exposed for 7 and 21days

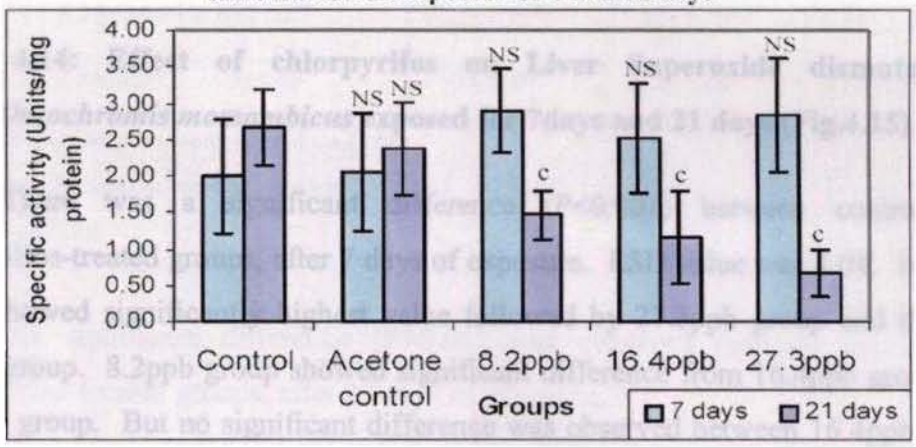


Fig.4.13: Effect of chlorpyrifos on Brain Glutathione peroxidase in *O.mossambicus* exposed for 7 and 21days

One Unit is one µg of GSH oxidized/minute; Each histogram represents mean±SD of 6 values; superscript represents the group significantly different from control; a- P<0.05; b- P<0.01; c- P<0.001, NS-not significant

Table 4.13: Effect of chlorpyrifos on Gill Superoxide dismutase in *O.mossambicus* exposed for 7days and 21 days (Fig.4.14)

One-way ANOVA revealed that after 7 days of exposure, there was a significant difference (P<0.001) between control and chlorpyrifos-treated groups, 16.4ppb and 27.3ppb groups. Between control and acetone control, there was no significant difference. LSD analysis (LSD=1.818) revealed that 8.2ppb did not show significant difference from control.

Exposure period	Control	Acetone Control	8.2ppb	16.4ppb	27.33ppb
7 days	8.1±0.96	7.25 ± 1.52^{NS}	8.43 ± 1.02^{NS}	14.31 ± 2.46^{c}	14.99 ± 1.45^{c}
21 days	10.11±1.4	9.6 ± 2^{NS}	8.76 ± 0.58^{NS}	6.98 ± 1.31^{c}	5.49 ± 1.46^{c}

Values are expressed as mean±SD of six fishes

Values are expressed as units/mg protein

c-significant at 0.001level; NS-not significant

One-way ANOVA revealed that there was a significant difference (P<0.001) between different chlorpyrifos-treated groups (16.4ppb and 27.3ppb) and control, after21 days of exposure. LSD value obtained was 1.61. But 8.2ppb was not significantly different from control though it showed a slight decrease. Acetone control was not significantly different from control.

Table 4.14: Effect of chlorpyrifos on Liver Superoxide dismutase in *Oreochromis mossambicus* exposed for 7days and 21 days (Fig.4.15)

There was a significant difference (P<0.001) between control and chlorpyrifos-treated groups, after 7 days of exposure. LSD value was 4.04. 16.4ppb group showed significantly highest value followed by 27.3ppb group and then by 8.2ppb group. 8.2ppb group showed significant difference from 16.4ppb group and 27.3ppb group. But no significant difference was observed between 16.4ppb group and 27.3ppb group.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	17.97±2.50	15.88±1.83^{NS}	26.12±3.21^{c}	38.04±3.37^{c}	36.99±5.46^{c}
21 days	18.47±1.94	17.8±3.08^{NS}	12.98±2.15^{c}	6.93±1.73^{c}	6.16±1.22^{c}

Values are expressed as mean±SD of six fishes

Values are expressed as units/mg protein

c-significant at 0.001level; NS-not significant

A significant difference (P<0.001) was obtained by One-way ANOVA analysis, 21 days of exposure. Further LSD analysis (LSD= 2.44) revealed that there was a significant difference between control and different chlorpyrifos-treated groups. 8.2ppb group showed significant difference from 16.4ppb group and 27.3ppb group and no significant difference was observed between 16.4ppb group and 27.3ppb group.

Table 4.15: Effect of chlorpyrifos on Brain Superoxide dismutase in *O.mossambicus* exposed for 7days and 21 days (Fig.4.16)

One-way ANOVA analysis revealed that there was no significant difference between different group means, after 7 days of exposure.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	6.75±1.96	6.6±2.03^{NS}	6.22±1.35^{NS}	7.89±1.75^{NS}	8.82±2.10^{NS}
21 days	7.16±1.51	7.03±1.59^{NS}	5.72±1.68^{NS}	5.02±1.66^{NS}	4.35±1.54^{NS}

Values are expressed as mean±SD of six fishes

Values are expressed as units/mg protein

NS-not significant

No significant difference (P>0.05) was observed between control and chlorpyrifos–treated groups, after 21 days of exposure.

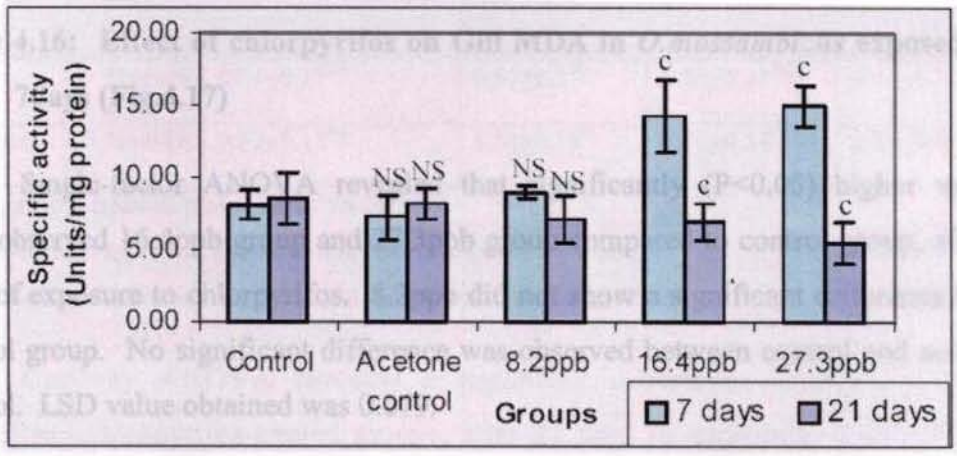


Fig.4.14: Effect of chlorpyrifos on Gill Superoxide dismutase in *O.mossambicus* exposed for 7 and 21days

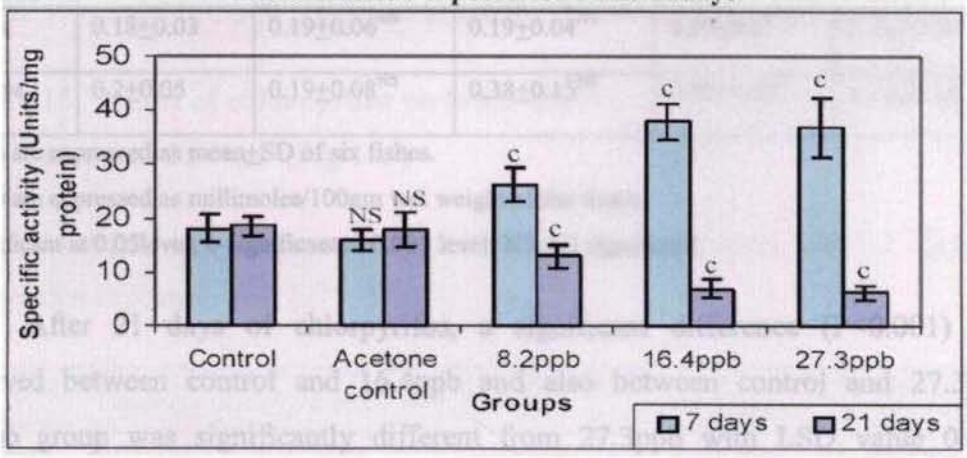


Fig.4.15: Effect of chlorpyrifos on Liver Superoxide dismutase in *O.mossambicus* exposed for 7 and 21days

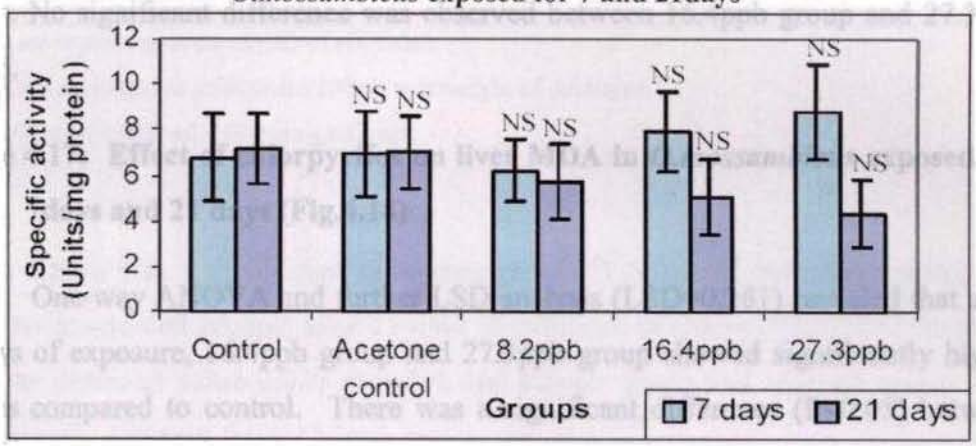


Fig.4.16: Effect of chlorpyrifos on Brain Superoxide dismutase in *O.mossambicus* exposed for 7 and 21days

One unit is the amount of enzyme that gives 50% inhibition of formazan formation/minute
Each histogram represents mean±SD of 6 values; superscript represents the group significantly different from control; a- P<0.05; b- P<0.01; c- P<0.001, NS-not significant

Table 4.16: Effect of chlorpyrifos on Gill MDA in *O.mossambicus* exposed for 7days (Fig.4.17)

Single-factor ANOVA revealed that significantly (P<0.05) higher values were observed 16.4ppb group and 27.3ppb group compared to control group, after 7 days of exposure to chlorpyrifos. 8.2ppb did not show a significant difference from control group. No significant difference was observed between control and acetone control. LSD value obtained was 0.079.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	0.18±0.03	0.19 ± 0.06^{NS}	0.19 ± 0.04^{NS}	0.30 ± 0.1^{a}	0.22 ± 0.09^{NS}
21 days	0.2±0.05	0.19 ± 0.08^{NS}	0.38 ± 0.15^{NS}	0.86 ± 0.58^{c}	1.12 ± 0.56^{c}

Values are expressed as mean±SD of six fishes.

Values are expressed as millimoles/100gm wet weight of the tissue.

a-significant at 0.05level; c-significant at 0.001 level; NS-not significant

After 21 days of chlorpyrifos, a significant difference (P<0.001) was observed between control and 16.4ppb and also between control and 27.3ppb. 8.2ppb group was significantly different from 27.3ppb with LSD value 0.425. 8.2ppb group showed a significant difference from 16.4ppb group and 27.3ppb group. No significant difference was observed between 16.4ppb group and 27.3ppb group.

Table 4.17: Effect of chlorpyrifos on liver MDA in *O.mossambicus* exposed for 7days and 21 days (Fig.4.18)

One-way ANOVA and further LSD analysis (LSD=0.261) revealed that after 7 days of exposure, 16.4ppb group and 27.3ppb group showed significantly higher values compared to control. There was a significant difference (P<0.05) between 8.2ppb group and 27.3ppb group.

Exposure period	Control	Acetone Control	8.2ppb	16.4ppb	27.33ppb
7 days	0.51±0.23	0.53 ± 0.17^{NS}	0.65 ± 0.3^{NS}	0.80 ± 0.21^{NS}	0.93 ± 0.21^{a}
21 days	0.75±0.16	0.62 ± 0.15^{NS}	1.51 ± 0.18^{c}	2.14 ± 0.34^{c}	2.25 ± 0.58^{c}

Values are expressed as mean±SD of six fishes

Values are expressed as millimoles/100gm wet weight of the tissue.

a-significant at 0.05level; c-significant at 0.001level; NS-not significant

One-way ANOVA revealed a significant difference ($P<0.001$) between control and chlorpyrifos-treated groups, after 21 days of exposure. LSD analysis with its value 0.376 revealed that no significant difference was observed between 16.4ppb group and 27.3ppb group and also between control and acetone control.

Table 4.18: Effect of chlorpyrifos on brain MDA in *O.mossambicus* exposed for 7days and 21 days (Fig.4.19)

No significant difference was observed at 0.05 level after 7 days of chlorpyrifos exposure.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	0.07±0.02	0.07 ± 0.03^{NS}	0.07 ± 0.02^{NS}	0.06 ± 0.02^{NS}	0.08 ± 0.02^{NS}
21 days	0.06±0.02	0.07 ± 0.03^{NS}	0.11 ± 0.04^{a}	0.14 ± 0.08^{a}	0.13 ± 0.04^{a}

Values are expressed as mean±SD of six fishes

Values are expressed as millimoles/100gm wet weight of the tissue.

a-significant at 0.05level; NS-not significant

There was a significant difference ($P<0.05$) observed between control and chlorpyrifos-treated groups, after 21 days of exposure to chlorpyrifos. LSD analysis with the obtained value 0.029 revealed that 8.2ppb group and 16.4ppb group were significantly different from 27.3ppb group.

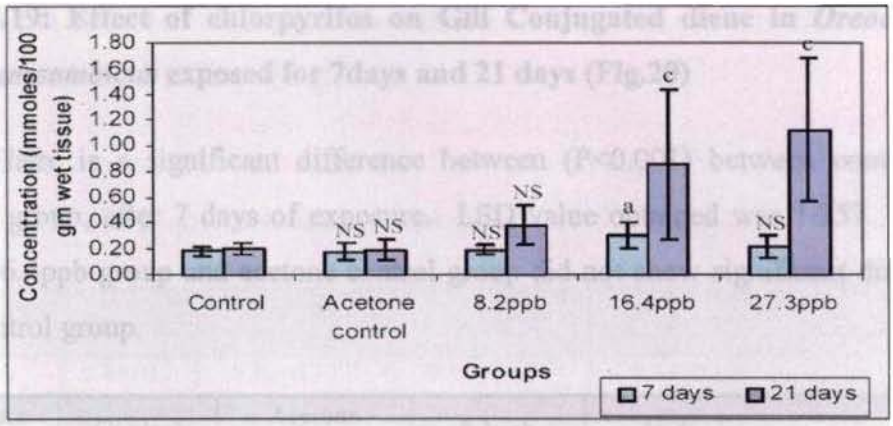


Fig.4.17: Effect of chlorpyrifos on Gill MDA in *O.mossambicus* exposed for 7 and 21days

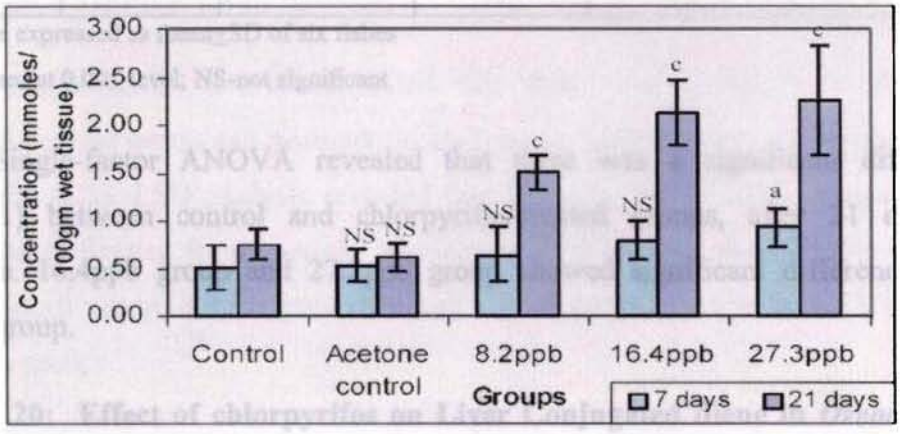


Fig.4.18: Effect of chlorpyrifos on liver MDA in *O.mossambicus* exposed for 7 and 21days

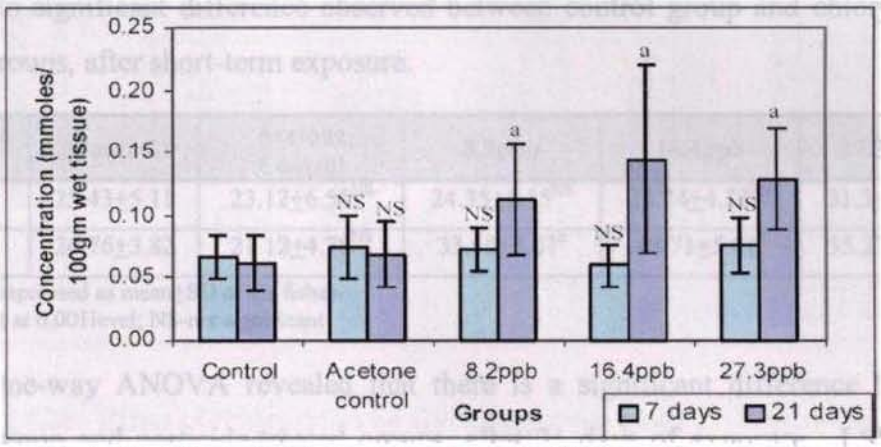


Fig.4.19: Effect of chlorpyrifos on brain MDA in *O.mossambicus* exposed for 7 and 21days

Each histogram represents mean±SD of 6 values; superscript represents the group significantly different from control; a- P<0.05; b- P<0.01; c- P<0.001, NS-not significant

Table 4.19: Effect of chlorpyrifos on Gill Conjugated diene in *Oreochromis mossambicus* exposed for 7days and 21 days (Fig.20)

There is a significant difference between (P<0.001) between control and 27.3ppb group, after 7 days of exposure. LSD value obtained was 1.257. 8.2ppb group, 16.4ppb group and acetone control group did not show significant difference from control group.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	4.13±0.86	4.17 ± 1.01^{NS}	4.68 ± 0.97^{NS}	5.03 ± 1.17^{NS}	7.17 ± 1.35^{c}
21 days	4.58±0.82	4.39 ± 1.21^{NS}	9.15 ± 0.95^{c}	11.54 ± 1.11^{c}	11.01 ± 1.24^{c}

Values are expressed as mean±SD of six fishes

c-significant at 0.001 level; NS-not significant

Single-factor ANOVA revealed that there was a significant difference (P<0.001) between control and chlorpyrifos-treated groups, after 21 days of exposure. 16.4ppb group and 27.3ppb group showed significant difference from 8.2ppb group.

Table 4.20: Effect of chlorpyrifos on Liver Conjugated diene in *Oreochromis mossambicus* exposed for 7days (Fig.4.21)

No significant difference observed between control group and chlorpyrifos-treated groups, after short-term exposure.

Exposure period	Control	Acetone Control	8.2ppb	16.4ppb	27.33ppb
7 days	25.43±5.11	23.12 ± 6.55^{NS}	24.35 ± 5.15^{NS}	28.74 ± 4.32^{NS}	31.3 ± 4.02^{NS}
21 days	24.76±3.82	21.12 ± 4.78^{NS}	33.69 ± 3.07^{c}	48.71 ± 5.81^{c}	55.23 ± 7.49^{c}

Values are expressed as mean±SD of six fishes.
c-significant at 0.001level; NS-not significant

One-way ANOVA revealed that there is a significant difference between control group and pesticide-treated groups, after 21 days of exposure. LSD value was 6.04. LSD analysis revealed that pesticide-treated groups showed a significant difference between them.

Table 4.21: Effect of chlorpyrifos on Brain Conjugated diene in *O.mossambicus* exposed for 21 days (Fig.4.22)

One-way ANOVA revealed that there was no significant difference between control group and pesticide-treated groups, after 7 days of exposure.

Exposure period	Control	Acetone Control	8.2ppb	16.4ppb	27.33ppb
7 days	0.46±0.2	0.50 ± 0.10^{NS}	0.55 ± 0.17^{NS}	0.64 ± 0.28^{NS}	0.67 ± 0.18^{NS}
21 days	0.42±0.12	0.49 ± 0.12^{NS}	0.65 ± 0.17^{NS}	1.21 ± 0.40^{c}	1.006 ± 0.2^{c}

Values are expressed as mean±SD of six fishes

c-significant at 0.001level; NS-not significant

Single-factor ANOVA revealed that there was a significant difference between control group and pesticide treated group, after 21 days of exposure. LSD value was 0.26. LSD analysis revealed that 16.4ppb and 27.3ppb groups showed ignificant difference from control. Acetone control was not significantly different from control group. 8.2ppb group showed significant difference from 16.4ppb and 27.3ppb groups.

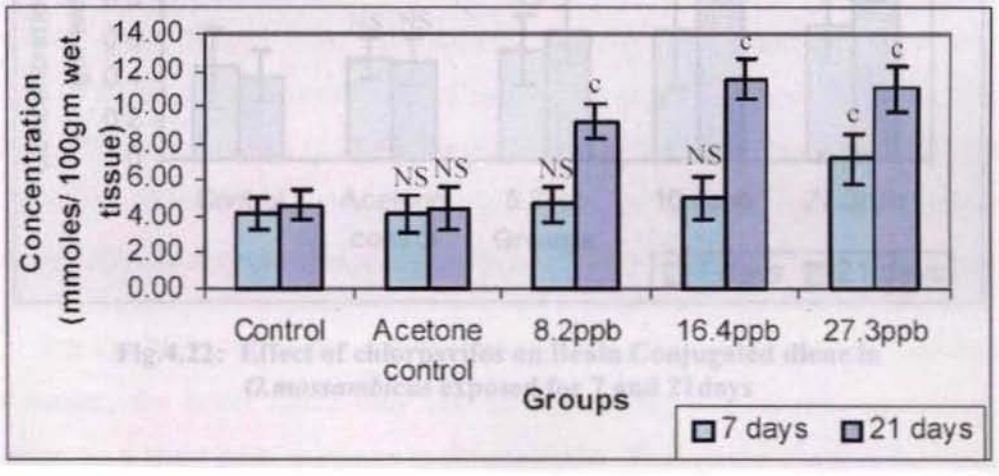


Fig.4.20: Effect of chlorpyrifos on Gill Conjugated diene in *O.mossambicus* exposed for 7 and 21days

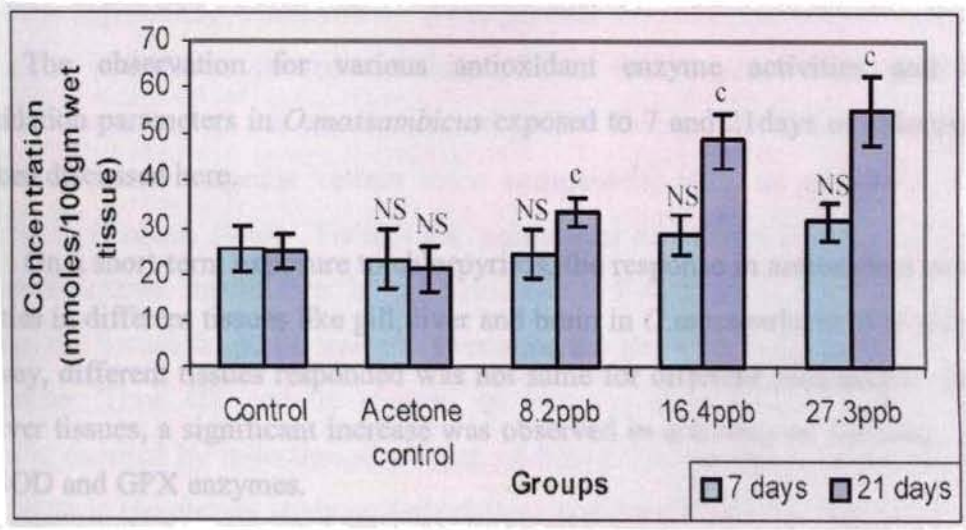


Fig.4.21: Effect of chlorpyrifos on Liver Conjugated diene in *O.mossambicus* exposed for 7 and 21days

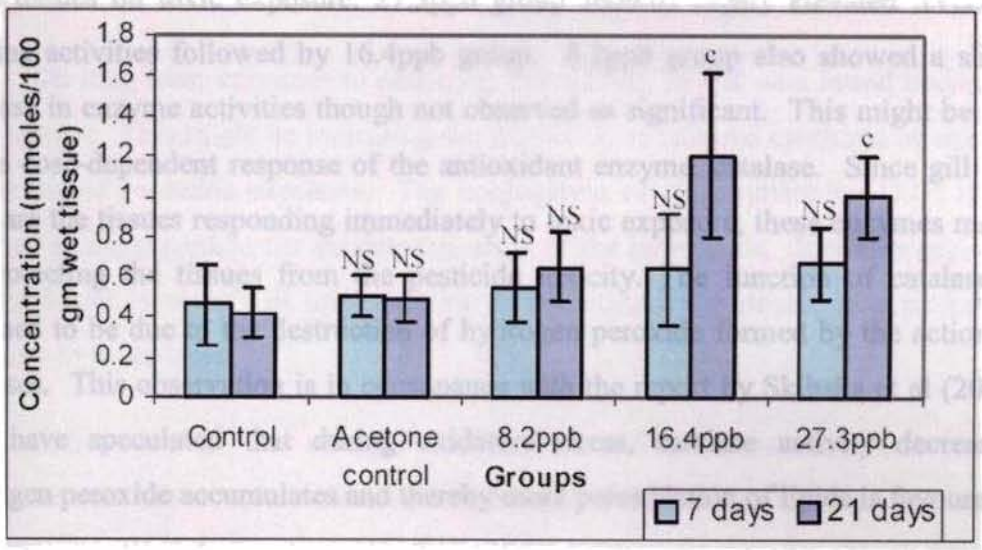


Fig.4.22: Effect of chlorpyrifos on Brain Conjugated diene in *O.mossambicus* exposed for 7 and 21days

Each histogram represents mean±SD of 6 values; superscript represents the group significantly different from control; a- P<0.05; b- P<0.01; c- P<0.001, NS-not significant

4.5 Discussion

The observation for various antioxidant enzyme activities and lipid peroxidation parameters in *O.mossambicus* exposed to 7 and 21days of chlorpyrifos has been discussed here.

On a short-term exposure to chlorpyrifos, the response in antioxidant enzyme activities in different tissues like gill, liver and brain in *O.mossambicus* was assayed. The way, different tissues responded was not same for different parameters. In gill and liver tissues, a significant increase was observed in activities of Catalase, GST, GR, SOD and GPX enzymes.

An increase was found in catalase activity in gill and liver tissues on short-term exposure to chlorpyrifos. This is expected to be due to induction of enzyme in these tissues on toxic exposure. 27.3ppb group showed highly elevated levels of enzyme activities followed by 16.4ppb group. 8.2ppb group also showed a slight increase in enzyme activities though not observed as significant. This might be due to the dose-dependent response of the antioxidant enzyme, catalase. Since gill and liver are the tissues responding immediately to toxic exposure, these enzymes might be protecting the tissues from the pesticide toxicity. The function of catalase is assumed to be due to the destruction of hydrogen peroxide formed by the action of oxidases. This observation is in consonance with the report by Skibska et al (2006) who have speculated that during oxidative stress, catalase activity decreases, hydrogen peroxide accumulates and thereby more peroxidation of lipids is favoured.

But the brain tissue has a protective covering of epithelial cells and blood-brain barrier, the brain tissue may not be responding immediately against toxic substance, on a short-term exposure to chlorpyrifos. Though the chances for delayed neurotoxicity can not be neglected, the lipophilic chlorpyrifos might be showing more affinity towards brain lipids. On long-term exposure the enzyme catalase was found increased in undamaged brain cells, providing a prolonged protection to the tissue.

Glutathione reductase (GR) was found significantly increased in all tissues on short-term exposure to chlorpyrifos. Since glutathione reductase converts oxidized glutathione (GSSG) to its reduced form (GSH), the enzyme level might have increased according to the requirement of GSH. Because GSH is an important defense mechanism against certain toxic compounds, such as some drugs and carcinogens (Robert, 1990). Though the metabolism of chlorpyrifos is in liver and the detoxification mechanism is by cytochrome P-450 monoxygenase and A-esterase, the tissues might be trying to overcome the stress by every kind of defense mechanism. Thus GR activity showed an increase after 7days of exposure to pesticide, resulted by induction as a mean of immediate response. Thus Increased GR activity in the present study on a short-term exposure to chlorpyrifos might be an adaptive mechanism to maintain intracellular GSH concentration. The decreased level of GPX activity under chlorpyrifos stress might be due to decreased availability of the cofactor GSH.

On long-term exposure to pesticide, the activity of GR was found decreased in all tissues. This might be indicating the inhibition or reduced synthesis of enzyme on prolonged pesticide exposure. The conjugation of chlorpyrifos by GSH is not reported as a mechanism for the detoxification of the insecticide. It can be expected that the lower levels of GR might be due to inhibition on synthesis on a prolonged exposure to insecticide.

Differences in the strength of the oxon-AChE association can play a significant role in the widely variable OP potencies exhibited by different species. It has been suggested that variations in conjugation of OP compounds with glutathione may also help explain species-specific OP susceptibility; glutathione conjugation with xenobiotics is generally considered protective in that it facilitates xenobiotic excretion (John Giesy et al, 1999).

GST in gill, liver and brain tissues was found as elevated on short-term exposure to chlorpyrifos. This might be because of the induction of this enzyme on

toxic exposure. But a dose-dependent relationship can be observed in brain tissue of 8.2ppb group that did not show a significant increase.

A number of synthetic chemicals or endogeneously generated electrophilic intermediates have been shown to induce GST (Chasseaud, 1979). The induction of GST aimed at the detoxification of the various xenobiotics by inactivating the potentially toxic electrophilic center of the substrate molecule and at the same time, form a hydrophilic conjugate for excretion (Kozak and Tate, 1982).

On long-term exposure, the GST activity was found significantly decreased in gill and liver tissues. Extensive deterioration of the tissues and the loss of normal metabolic processes for further synthesis of enzyme might have led to low levels of enzyme in these tissues. In brain tissue GST showed a tendency to decrease, though the decrease was not significant. It is expected that elevated levels of catalase and GST can provide an effective antioxidant protection against xenobiotic toxicity. Continuous synthesis of the enzyme in the undamaged brain cells might be the reason for GST and catalase that they did not show a significant decrease even on long-term exposure to chlorpyrifos.

Glutathione peroxidase level was found increased in gill and liver tissues on short-term exposure and decreased on long-term exposure. The increase might be because of the induction, as in the case of any other defensive anti-oxidant enzyme. The change was insignificant in brain tissue, though a slight increase was observed. This can be explained either on the basis of insufficient amount of the pesticide to make a significant response or due to delayed neurotoxicity. In erythrocytes and other tissues, the enzyme glutathione peroxidase, containing selenium as a prosthetic group, catalyzes the destruction of H_2O_2 and lipid hydroperoxides by reduced glutathione, protecting membrane lipids and haemoglobin against oxidation by peroxides (Robert, 1990). The decreased enzyme activity in all tissues can be interpreted by the inhibition action on the enzyme by the organophosphorus insecticide. This finding can be correlated with the report that organophosphates also

inhibit enzymes-esterase, protease, peroxidase- and slightly increase the activity of catalase.

Superoxide dismutase enzyme had a significant increase in gill and liver tissues on short-term exposure to the pesticide. Brain tissues also showed a slight increase which was not significant. Increase in enzyme activities indicates a defensive mechanism to overcome the insecticide-induced stress. Superoxide dismutase has been recognized to play an important role in body defense mechanisms against the deleterious effects of oxygen free radicals in biological systems (Fridovich, 1975; Kellogg and Fridovich, 1977). On long-term exposure to the pesticide the decrease in enzyme activity was found elevated in gill and liver tissues. But brain tissue showed an insignificant decrease, might be showing a tendency to decline after an increase on short-term exposure.

Available reports indicate that the enzyme activities associated with antioxidant defense mechanism are altered by insecticides both *in vivo* and *in vitro* (Patel and Chakrabarti, 1982). The elevated antioxidant enzyme activities protects the cell by scavenging mechanism on reactive oxygen species that are produced on stress, induced by chlorpyrifos. Antioxidant enzyme activities were found lowered on a long-term exposure to the insecticide. But the change in certain cases like brain SOD in 8.2ppb group, GST and SOD in gill tissue of 8.2ppb group were not found significantly changed. This can be explained on a dose-dependent relationship to the response of the enzymes and their effectiveness of these antioxidant enzymes to provide a long-term defense towards stress. The decrease in all other cases might be due to failure in providing a defense mechanism on a continuous production of reactive oxygen species for a prolonged exposure to the pesticide. This might have led to the cellular and cell-membrane damage and the inhibition on the synthesis of these antioxidant enzymes that happened by the structural alteration in these synthetic apparatuses.

This is evident from the consequent increase in malondialdehyde (MDA) levels in all the three tissues on long-term exposure to the chlorpyrifos. The

increased rate of lipid peroxidation has led to increased production of malondialdehyde. MDA is the end-product of lipid peroxidation. Conjugated diene is an intermediate compound in lipid peroxidation process. The changes in MDA levels were also not significant in brain tissue of any of the groups on short-term exposure to chlorpyrifos. In gill tissue a significant change was observed only in 16.4ppb group. A significant increase in liver MDA level observed was only in the highest sublethal concentration group. The insignificant changes in MDA levels might be indicating the effectiveness of the antioxidant enzymes to withstand the oxidative stress on chlorpyrifos exposure. Thus the amount of MDA and conjugated diene were not found increased in various tissues in different groups on short-term exposure to the pesticide. Antioxidant enzymes and other defensive parameters have provided protection against lipid peroxidation keeping the extent of lipid peroxidation less and MDA and conjugated diene levels low. But the increase, both in MDA and CD was prominent in tissues after long-term exposure to the insecticide. This might be indicating the extent of lipid peroxidation that was induced on long-term toxic exposure by chlorpyrifos.

Lipid peroxidation is known to occur as a result of GSH depletion. Glutathione protects from lipid peroxidation and there exists a close correlation between low level of GSH and accumulation of the malondialdehydes. Bagchi et al (1995) have shown that different classes of pesticides induce production of ROS and oxidative tissue damage.

The detection of oxidative stress has relied largely on the quantification of compounds such as conjugated dienes, hydroperoxides as well as malondialdehyde which are formed by degradation of initial products of free radical attack (Janero, 1990). The reaction of MDA with thiobarbituric acid is one of the most widely used estimators of oxidative stress (Liu et al, 1997).

Increased reactive oxygen species (ROS) production can lead to increased synthesis of antioxidant enzymes. Failure to remove ROS by antioxidant defences can result in oxidative damage to key biological molecules, including DNA (Sahu,

1991; Kehrer, 1993). Organophosphorus compounds are poisons with a neuroparalytic and enzymatic action. The basis of their toxicity lays in the capacity of their selective effect on enzymes of nerve tissues-cholinesterase-which lead to excessive accumulation of acetylcholine in the organisms, giving rise to complex poisoning symptoms.

Gultekin et al. (2000) reported that chlorpyrifos-ethyl directly inhibited catalase without any effect on SOD. Therefore, more H_2O_2 will accumulate and less will be oxidized to H_2O. Thus the increased superoxide radicals will inhibit both catalase and glutathione-peroxidase so that the H_2O_2 will greatly accumulate in the medium leading to inhibition of SOD and increased superoxide radicals. Kono and Fridovich (1982) also has reported inhibition of catalase activity by superoxide radicals. SOD and CAT activity have shown to be inhibited by singlet oxygen and peroxyl radicals according to Escobar et al. (1996). According to Achuba (2002), the levels of lipid peroxidation and superoxide dismutase activity were higher in the liver compared to the kidney of the two fish species. This could be attributed to the fact that the liver is the principal site of metabolism of xenobiotics and generation of superoxide anion, and therefore becomes the more susceptible organ of free radical induced tissue damage

In vitro administration of chlorpyrifos-ethyl resulted in the induction of erythrocyte lipid peroxidation and changed the activity of antioxidant enzymes, in studies by Gultekin et al. (2000). This suggested that ROS and/or free radicals might have involved in the toxic effects of the pesticidal use of chlorpyrifos-ethyl. SOD activity increased initially and decreased thereafter in liver and kidney of 60 days phosalone exposed rats in study by Janardan Reddy (1996).

The results from the present investigation conclude that the organism made an attempt to survive in chlorpyrifos-introduced aquatic medium with the help of antioxidant enzymes. These enzymes might have tried to prevent the abrupt changes in homeostasis on chlorpyrifos exposure. Thus their activities were found elevated on short-term pesticide-exposure.This was continued until the organism was not

capable to overcome the insecticide-induced stress. The increased lipid peroxidation on continuous exposure to chlorpyrifos decreased the efficiency of antioxidant enzymes. This is thought to be due to either by inhibition as a result of increased rate of substrate production by lipid peroxidation or by the inability of the organ system, due to altered structure, to synthesize new enzymes for resuming the defense mechanism.

Chapter

5

Effects of chlorpyrifos on biological membranes in *Oreochromis mossambicus*

Contents

5.1 Introduction

Before a pollutant can be distributed in an organism it must pass across a membrane and enter into cellular spaces (Tinsley, 1979). Many toxic substances or their metabolites result in cell injury by reacting primarily with biological membranes.

Diffusion of a xenobiotic across the gill epithelium will depend largely on lipid solubility (Randall et al., 1996). Membranes play a vital role by controlling the movement of pollutants and other chemicals through concentration gradients on either side of membrane boundaries. These processes are necessary for the normal sequence of functions, particularly metabolism.

Xenobiotic-induced subcellular pathology reflects perturbations of function and structure at the molecular level. In many cases, the earliest detectable changes of primary events are associated with a particular type of subcellular organelles such as lysosomes, endoplasmic reticulum and mitochondria (Moore, 1985).

5.1.1 Branchial ATPases:-

Gills, the site of respiration and osmotic regulation, are the main targets of acid toxicity in fish (Wood, 1989). The respiratory system is the most important interface of fish with the surrounding environment and is very often the first target of a potential toxicant. The gills of fish are very vulnerable to pollutants and lethality is usually due to alterations in respiratory homeostasis. The gills are a multipurpose organ directly involved in a variety of functions including: respiratory gas exchange, osmoregulation, acid-base balance and nitrogenous waste excretion. Since respiratory gases must pass through the lamellar epithelium by diffusion, this surface is quite delicate compared to the rest of the surface of the fish. There is a large flow of water over the lamellae even in a resting fish (approximately 70mL/ minute in a 0.5kg trout). Gill epithelium has a permanent contact with the aquatic environment

and is provided with very large surface area of the secondary epithelium that is not as protected as that of the skin and mouth. Therefore, there is ample opportunity for dissolved or suspended materials to come into contact with the lamellae and cause injury to the respiratory tissues (Heath, 1987).

The gill epithelium is located between two liquid compartments of very different ionic composition. The gills are the site not only of entry for selected ions essential to life (Na^+, K^+, Cl^-) but also for extrusion of other ions such as HCO_3^-, NH_4^+ and H^+ which are the ionic forms of metabolic by-products (Renzis and Michel, 1984). In fish, the uptake of Ca^{2+}, Cl^- and Na^+ from the water has largely been attributed to mitochrondria-rich cells, the so-called chloride cells or ionocytes (Mc Cormick, 1995; Perry, 1997). Chloride cells are characterized by their high numbers of mitochondria, dense vesicular tubular network in the cytoplasm, ovoid nuclei and high levels of the transport protein, Na^+K^+-ATPase. Chloride cells' role in fresh water teleost gills in transepithelial Ca^{2+} and Cl^- uptake is well established, and its role in Na^+ uptake identified (Jurss and Bastrop, 2004).

Na^+K^+-dependent ATPase is an enzyme that actively transports sodium out of and potassium into animal cells through the plasma membrane. Na^+K^+ATPase, is the marker enzyme of chloride cells. The activity of the enzyme is strongly enhanced in the gills of marine fishes; the Na^+K^+-ATPase was implicated in the excretion of salt from marine fish. In stenohaline fish the Na^+K^+-ATPase activities are 4-10 times greater in marine species compared to those in fresh water (Kamiya and Utida, 1969; Jampol and Epstein, 1970). The only source of energy for ionic transfer across membranes is ATP; certain ions such as Na^+, K^+ and Ca^{2+} are transported by molecular carriers that hydrolyse ATP as a source of energy. The enzymes that effect the utilization of stored energy from ATP are ATPases (EC 3.6.1.4). Among those that play a role in ionic transport are Na^+K^+-ATPase and K^+-ATPase have been identified and studied (Hoar and Randall, 1984).

Although the fish's skin with its external secretions effectively protects the animal from its potentially hostile environment, the gills represent the weak spot in

this barrier (Eddy, 1981). The numerous gill lamellae comprising up to 90% of the total body surface and the ion-transporting cells (chloride cells) in the epithelial layer are vulnerable to aquatic toxicants. As a consequence, vital processes such as respiration, ion and water exchange, acid-base regulation and excretion of nitrogenous wastes are endangered (Heath, 1987; Evans, 1987).

5.1.2 Lysosomal hydrolases:-

Lysosomes are single-membrane-bound organelles that enclose a battery of hydrolytic enzymes like acid phosphatase, β-glucuronidase, cathepsin, arylphosphatase etc. Their function is to digest foreign and endogenous substances within the cell. Potentially these enzymes could destroy the cell, but in health they are retained within the lysosomes surrounded by a lipoprotein membrane. They have an acid environment, which is maintained by a membrane-bound Mg^{2+}-ATPase dependent proton pump (Ohkuma et al., 1982). These organelles are with numerous functions like sequestration of foreign compounds, immune response, protein and organelle turnover, embryonic development, apoptosis etc.

The level of lysosomal β-glucuronidase does not show immediate decline although it may decrease with a time lag of several days. β-glucuronidase seems unique among liver enzymes since no other enzymes so far examined show similar changes (Suzuki et al, 1975).

Lysosomal damage is well-established as a biomarker of stress in a wide range of vertebrates (Bayne, 1976; Moore, 1990; Tabata et al, 1990). Lysosomal membrane is often a target of injury by xenobiotics or their metabolites in addition to its role in sequestration (Moore et al, 1982). Bitensky (1963) developed a simple histochemical index for the degree of lysosomal activity, in which the permeability or fragility of the lysosomal membrane is measured by the time needed for substrate to enter the lysosome across the membrane. Many stressors have been shown to increase fragility.

Liver is the major site of metabolism of chlorpyrifos (ATSDR, 1997). If the lysosomal membrane is damaged or destabilized, then these marker enzymes are released. Hence the assay of these enzymes can be used as an index of lysosomal membrane damage. The release of lysosomal enzymes is related to necrosis or death of the cell or pathological or stressful conditions (Hawkins, 1980).

The present study was an attempt to investigate the activities of branchial ATPases and lysosomal hydrolases in the fishes exposed to different concentrations of the organophosphorus insecticide, chlorpyrifos.

5.2 Materials and Methods

Collection, acclimation of fish and method of dosing were the same as that described in section 3.2.B. Different groups of fishes were exposed to different sublethal concentrations of chlorpyrifos for three weeks and gill and liver tissues from control and pesticide treated groups were dissected out after 7 and 21 days of exposure to the pesticide.

5.2.1 Studies on branchial ATPase activity

a. Extraction of the enzyme:

Most commonly, membranes are isolated as microsomal fraction (Renzis and Michel, 1984). The gill tissues were washed and 5% gill homogenate was prepared using ice-cold 0.33M sucrose solution. Tissue homogenate was centrifuged at 3000g for 15 minutes and the supernatant obtained was again centrifuged in a cold refrigerated centrifuge at 12000g for 30minutes. The clear supernatant was again centrifuged at 35000g for 30 minutes. The pellet so obtained corresponds to the heavy microsomal fraction (Davis, 1970). The pellet was resuspended in cold 0.33M sucrose and used as the enzyme source.

b. Assay of Na^+-K^+-ATPase activity (Na^+K^+-dependent adenosine triphosphatase, EC 3.6.1.3)

Procedure:

Na^+-K^+-ATPase activity was determined by the method of Bonting (1970). Sample is added to the reaction mixture containing 0.2mL of 50mM $MgSO_4$, 0.2mL of 50 mM KCl, 0.2mL of 600mM NaCl, 0.1mL of 1mM EDTA, 0.1mL of 40mM ATP and 1mL of 184mM Tris-HCl buffer (pH-7.5). The mixture was incubated for 10 min. at 37^0C and then the reaction was stopped by adding 1ml of 10% ice-cold TCA. The supernatant obtained after centrifugation at 1300g for 10min. was used for the determination of inorganic phosphate liberated from ATP by the method of Fiske and Subbarow (1925). Specific activity of Na^+K^+-ATPase was defined as micromoles of inorganic phosphorus liberated/minute/mg tissue protein. Protein was estimated by the method of Lowry et al (1951) as given under section 3.2.B.4.

c. Assay of Ca^{2+}-ATPase activity (EC 3.6.1.9)

Procedure:

Ca^{2+}-ATPase activity was estimated by the method of (Hjerten and Pan, 1983). 0.1ml tissue homogenate was added to the reaction mixture containing 100µL of 0.05M $CaCl_2$, 100µL of 0.01M of ATP and 0.1mL 0.125M of Tris-HCl buffer (pH-8.0). The whole reaction system was incubated at 37^0C for 15min. and reaction was stopped by adding 10% TCA. The supernatant obtained after centrifugation at 1300g was estimated by Fiske and Subbarow (1925) method for the amount of inorganic phosphorus liberated/minute/mg protein.

d. Assay of Total ATPase activity (EC 3.6.1.4)

Procedure:

Total ATPase activity was estimated by the method of Evans (1969). 0.1ml of enzyme source was added to the reaction mixture containing 1.5mL of 0.1M Tris-HCl buffer (pH-7.0), 0.1mL of 0.01M ATP, 0.1mL of 0.01M NaCl, 0.1mL of 0.1M

$MgCl_2$, 0.1mL of 0.1 M KCl. Then the system was incubated at 37^0C for 15min and further reaction was stopped by adding 10% TCA. The contents were centrifuged and the supernatant solution was used for the estimation of inorganic phosphorus liberated, by Fiske and Subbarow (1925) method. Specific activity was expressed as micromoles of inorganic phosphorus liberated /minute/mg protein.

e. Estimation of inorganic phosphorus (Fiske and Subbarow, 1925)

Reagents:

1. Ammonium molybdate reagent: Add 25gm Ammonium molybdate to 200mL of distilled water. Added the molybdate solution to 300mL of 10N H_2SO_4 and diluted to 1L with water.
2. Amino naphthol sulphonic acid (ANSA) reagent: Grind 0.2gm ANSA with 1.2gm of Na_2SO_4 and 1.2gm sodium bisulphate. Keep the mixture in freezer. At the time of use, dissolve 0.25gm in 10mL distilled water.
3. Standard solution: Weighed 35.1gm AR potassium dihydrogen phosphate accurately, dissolved and made up to 100mL with distilled water; 1mL contains 80μg phosphorus

Procedure:

Added 1mL ammonium molybdate to 0.1mL of the sample taken in test tube and incubated at room temperature for 10minutes. Then added 0.1mL Amino naphthol sulfonic acid, read after 5 minutes, the blue colour at 660nm.

5.2.2 Studies on lysosomal hydrolases

Lysosomal enzyme activity was investigated in different sub cellular fractions from liver tissue. Liver tissue was homogenized in 0.33M ice-cold sucrose solution and the homogenate was centrifuged at 600g for 10minutes in a high speed refrigerated centrifuge. The sediment (referred as nuclear fraction) of nuclei, unbroken cells and plasma membrane was separated. The supernatant was again centrifuged at 15000g for 30 minutes. The pellet (lysosomal fraction) obtained was separated from the supernatant (soluble fraction). Both nuclear and lysosomal

fractions were resuspended in citrate buffer containing 0.2% Brij-35. Activity of acid phosphatase and β-glucuronidase were determined in all the three fractions (Plummer, 1988).

a. Assay of Acid phosphatase activity (ACP) (EC 3.1.3.2)

Procedure:

Acid phosphatase activity was assayed by the method of Anon (1963). 0.5ml of p-nitro phenyl phosphate (400mg %) was mixed with an equal volume of 0.1M-citrate buffer of pH 4.8. The enzyme source was added and incubated for 30minutes at room temperature. After incubation, the reaction was stopped by the addition of 4ml of 0.1N NaOH. The absorbance of the solution was measured at 410nm. The amount of p-nitrophenol liberated by acid phosphatase per hour per mg protein gives specific activity. Protein was estimated by the method of Lowry et al (1951).

b. Assay of β-glucuronidase activity (EC 3.2.1.31)

Procedure:

β-glucuronidase assay was carried out by the method of Kawai and Anno, 1971. p-nitophenyl β-D-glucuronide in 0.1M citrate buffer (pH 4.5) was used as the substrate. The reaction system containing substrate and enzyme source was incubated at room temperature for 30minutes and the reaction was stopped by adding 0.2N sodium carbonate solution. The absorbance was read at 400nm in a UV-visible spectrophotometer. The enzyme activity is expressed in terms of μg of p-nitrophenol liberated per hour per gram protein using p-nitrophenol as standard. Protein was estimated by the method of Lowry et al (1951).

c. Lysosomal stability index

Lysosomal stability index is defined as ratio of lysosomal activity to soluble activity in a tissue. Thus the index was calculated as the ratio of enzyme activity in lysosomal fraction to the enzyme activity in soluble fraction, in the tissue.

5.3 Statistical Analysis

Statistical Analysis of results was performed by Single-Factor ANOVA and subsequent comparisons by LSD (Zar, 1996).

5.4 Results

Responses of branchial ATPases and hepatic acid hydrolases from different subcellular fractions of *O.mossambicus* treated with sub cellular concentrations of chlorpyrifos are given:-

5.3.1 Studies on branchial ATPase activity

Table 5.1: Effect on branchial Na^+K^+-ATPase activity in *Oreochromis mossambicus* exposed to different concentrations of chlorpyrifos for 7days and 21 days (Fig. 5.1)

One-way ANOVA revealed that after 7 days of exposure, there was a significant difference ($P<0.001$) between group means. Using LSD analysis (LSD=0.71), it was found that chlorpyrifos-treated groups are having significantly lower values compared to control group and acetone control group. Significantly higher values were observed in control and acetone control but between them, there was no significant difference. Also 8.2ppb group showed significantly higher value compared to 27.3ppb group and 16.4ppb groups and significantly lower values compared to control and acetone control.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	11.28±0.68	11.39±0.86^{NS}	9.02±0.55^{c}	7.97±0.26^{c}	7.54±0.56^{c}
21 days	11.37±0.36	11.44±0.25^{NS}	6.47±0.76^{c}	5.42± 0.54^{c}	4.12±0.78^{c}

Values are expressed as mean±SD of a group of 6 fishes

Specific activity is expressed as micromoles of inorganic phosphorus liberated/minute/mg of protein

c-significantly different from control; $P< 0.001$; NS-not significant

Single-factor ANOVA and further LSD analysis (LSD= 0.65) revealed that, after 21 days of exposure, a significant difference (P<0.001) was observed between different group means. Group 27.33ppb showed significantly lowest value compared to the rest. All chlorpyrifos-treated groups showed significant difference between them. Control and acetone control were having significantly higher values but between them the difference was not significant.

Table 5.2: Effect on branchial Ca^2+-ATPase activity in *Oreochromis mossambicus* exposed to different concentrations of chlorpyrifos for 7days and 21 days (Fig. 5.2)

One-way ANOVA revealed that after 7 days of exposure, there was a significant difference (P<0.001) between group means. . LSD analysis (LSD =1.7) revealed that 27.33ppb group showed significantly the lowest value compared to all the rest. The groups 16.4ppb and 8.2ppb showed significantly lower values compared to that of control group and acetone control group; but there was no significant difference between them. Control group and acetone control group did not show significant difference between them though the values were significantly higher compared to the rest.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	14.57±0.74	13.22±1.48^{NS}	9.35±1.18^{c}	10.11±1.62^{c}	8.58±2.03^{c}
21 days	13.29±2.54	14.62±1.73^{NS}	7.6±1.26^{c}	5.6±0.88^{c}	6.57±1.21^{c}

Values are expressed as mean±SD of a group of 6 fishes

Specific activity is expressed as amount of inorganic phosphorus liberated/minute/mg of protein

c-significantly different from control, P< 0.001level; NS-not significant

After 21 days of exposure, there was a significant difference (P<0.001) between group means. One-way ANOVA and further LSD analysis (LSD=1.879) revealed that no significant difference was observed between control group and acetone control group. Significantly higher values were observed in 8.2ppb group followed by groups 16.4ppb group and 27.33ppb group, compared to control.

Significantly lower values were observed in 16.4ppb group but no significant difference was observed between 27.33ppb group and 16.4ppb group.

Table 5.3: Effect on branchial Total-ATPase activity in *O.mossambicus* exposed to different concentrations of chlorpyrifos for 7days and 21 days (Fig. 5.3)

After 7 days of exposure, there was a significant difference ($P<0.001$) between group means. One-way ANOVA and further LSD analysis (LSD=2.09) revealed that all chlorpyrifos-treated groups showed significantly lower values compared to control group and acetone control group. Significantly higher values were observed in control group and acetone control group but between them the difference was not significant.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	33.45±2.3	32.32±1.27^{NS}	27.41±1.22^{c}	21.07±1.85^{c}	16.02±2.17^{c}
21 days	32.79±2.03	33.49±1.61^{NS}	12.36±4.82^{c}	13.01±1.18^{c}	9.89±2.37^{c}

Values are expressed as mean±SD of a group of 6 fishes

Specific activity is expressed as micromoles of inorganic phosphorus liberated/minute/mg of protein

c-significantly different from control, P< 0.001level; NS-not significant

One-way ANOVA revealed that there was a significant difference ($P<0.001$) between group means, after 21 days of exposure to chlorpyrifos. Further LSD analysis (LSD=3.137) revealed that 27.3ppb group showed significantly the lowest value compared to all the rest. All chlorpyrifos-treated groups showed significantly lower values compared to control group. Acetone control group did not show significant difference from control group.

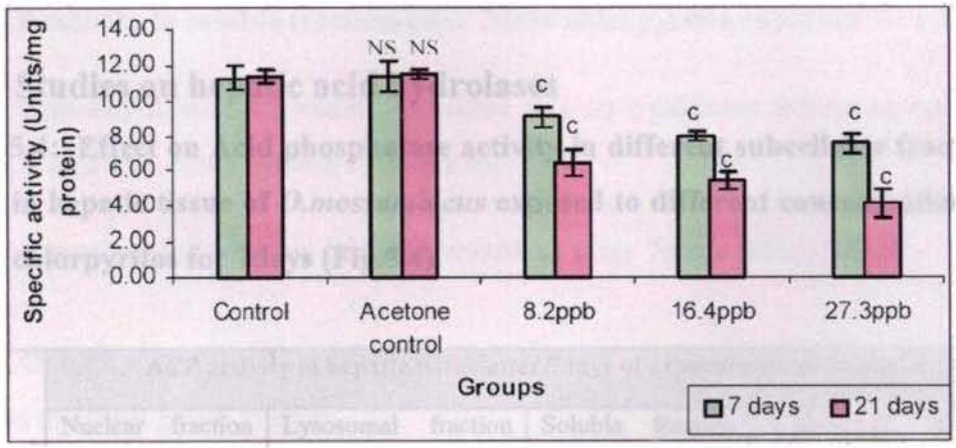


Fig.5.1: Branchial Na^+K^+-ATPase activity in *O.mossambicus* exposed to chlorpyrifos

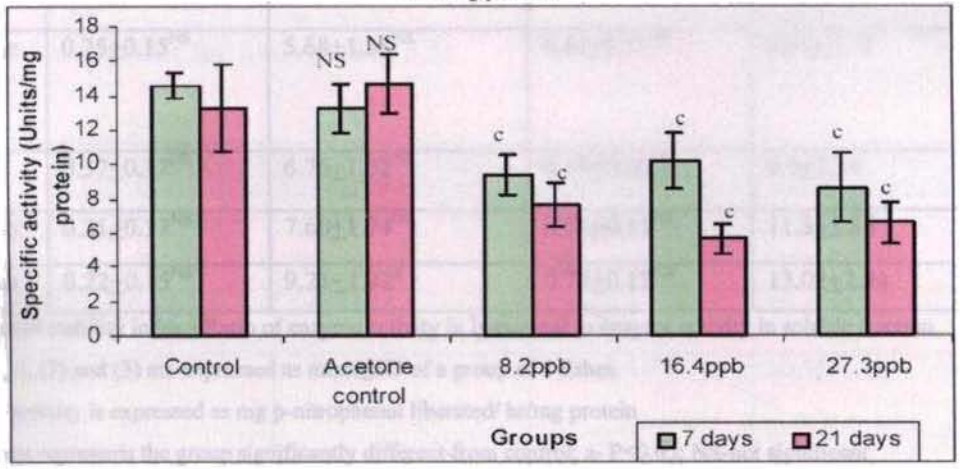


Fig.5.2: Branchial Ca^{2+}-ATPase activity in *O.mossambicus* exposed to Chlorpyrifos

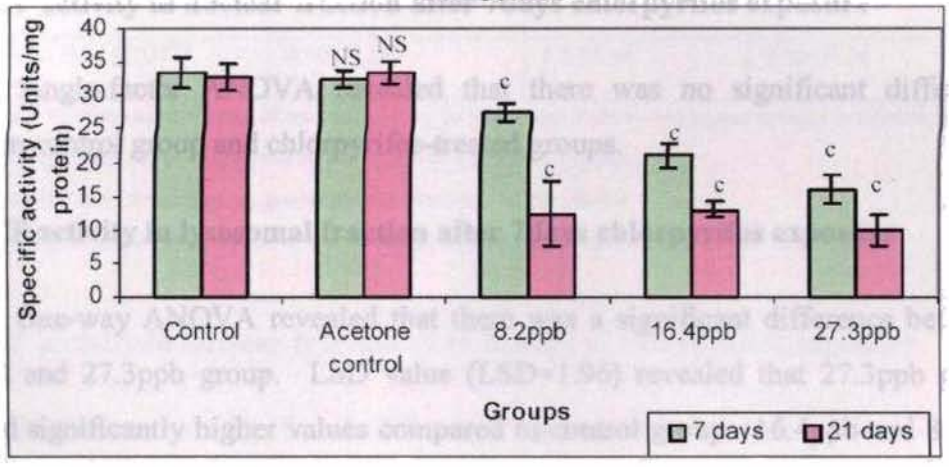


Fig.5.3: Branchial Total ATPase activity in *O.mossambicus* exposed to chlorpyrifos

Values are expressed as mean±SD of a group of 6 fishes
One unit is expressed as micromoles of inorganic phosphorus liberated/minute/mg protein
c-significantly different from control; P< 0.001level; NS-not significant

5.3.2 Studies on hepatic acid hydrolases

Table 5.4: Effect on Acid phosphatase activity in different subcellular fractions in hepatic tissue of *O.mossambicus* exposed to different concentrations of chlorpyrifos for 7days (Fig.5.4)

Groups	ACP activity in hepatic tissue after 7days of exposure to chlorpyrifos			
	Nuclear fraction (1)	Lysosomal fraction (2)	Soluble fraction (3)	Lysosomal stability index* (4)
Control	0.24±0.13	5.85±2.17	0.64±0.18	9.86±4.66
Acetone control	0.25±0.15^{NS}	5.68±1.38^{NS}	0.61±0.17^{NS}	9.96±3.71
8.2ppb	0.37±0.17^{NS}	6.76±1.02^{NS}	0.69±0.09^{NS}	9.9±1.56
16.4ppb	0.21±0.17^{NS}	7.60±1.74^{NS}	0.71±0.15^{NS}	11.3±3.86
27.3ppb	0.22±0.15^{NS}	9.21±1.92^{a}	0.72±0.11^{NS}	13.02±2.94

*Lysosomal stability index= Ratio of enzyme activity in lysosomal to enzyme activity in soluble fraction

Values (1), (2) and (3) are expressed as mean±SD of a group of 6 fishes

Specific activity is expressed as mg p-nitrophenol liberated/ hr/mg protein

superscript represents the group significantly different from control; a- $P<0.05$; NS-not significant

(1) ACP activity in nuclear fraction after 7days chlorpyrifos exposure

Single-factor ANOVA revealed that there was no significant difference between control group and chlorpyrifos-treated groups.

(2) ACP activity in lysosomal fraction after 7days chlorpyrifos exposure

One-way ANOVA revealed that there was a significant difference between control and 27.3ppb group. LSD value (LSD=1.96) revealed that 27.3ppb group showed significantly higher values compared to control group. 16.4ppb and 8.2ppb showed significant difference, though the values seemed to be higher. No significant difference was observed between control and acetone control group.

(3) ACP activity in soluble fraction after 7days chlorpyrifos exposure

One-way ANOVA revealed that there was no significant difference between control group and chlorpyrifos-treated groups.

(4) Lysosomal stability index in *O.mossambicus* after 7days chlorpyrifos exposure

One-way ANOVA revealed that there was no significant difference ($P>0.05$) between different group means.

Table 5.5: Effect on Acid phosphatase activity in different subcellular fractions in hepatic tissue of *O.mossambicus* exposed to different concentrations of chlorpyrifos for 21days (Fig.5.5)

Groups	ACP activity in hepatic tissue after 21days of exposure to chlorpyrifos			
	Nuclear fraction (1)	Lysosomal fraction (2)	Soluble fraction (3)	Lysosomal stability index* (4)
Control	0.25±0.12	5.68±1.54	0.63±0.2	10.42±5.72
Acetone control	0.31 ± 0.28^{NS}	6.01 ± 1.6^{NS}	0.59 ± 0.14^{NS}	10.55±3.48
8.2ppb	0.18 ± 0.42^{NS}	3.41 ± 0.97^{c}	1.19 ± 0.49^{c}	3.57±2.44
16.4ppb	0.14 ± 0.17^{NS}	2.14 ± 0.72^{c}	1.2 ± 0.35^{c}	1.9±0.76
27.3ppb	0.14 ± 0.07^{NS}	0.90 ± 0.28^{c}	1.5 ± 0.46^{c}	0.66±0.24

*Lysosomal stability index =Ratio of enzyme activity in lysosomal fraction to enzyme activity in soluble fraction

Values in (1), (2) and (3) are expressed as mean±SD of a group of 6 fishes

Enzyme activity is expressed as mg p-nitrophenol liberated/ hr/gm protein

c-significantly different from control, $P< 0.001$ level; NS-not significant

(1) ACP activity in nuclear fraction after 21days of chlorpyrifos exposure

There was no significant difference between control and pesticide-treated groups.

(2)ACP activity in lysosomal fraction after 21days of chlorpyrifos exposure

There was a significant difference ($P<0.001$) between control group and pesticide-treated groups. Single-factor ANOVA and LSD analysis (LSD=1.314) revealed that chlorpyrifos -treated groups showed significantly lower values compared to control.

(3) ACP activity in soluble fraction after 21days of chlorpyrifos exposure

There was a significant difference ($P<0.001$) between control and chlorpyrifos-treated groups. One-way ANOVA and LSD analysis (LSD=0.41) control group showed significantly lower values compared to chlorpyrifos-treated groups. There was no significant difference between control group and acetone control group.

(4) Lysosomal stability index in *O. mossambicus* after 21daysof chlorpyrifos exposure

Single-factor ANOVA revealed that chlorpyrifos-treated groups showed significantly lower values ($P<0.001$) compared to control. Further LSD analysis (LSD=0.239) revealed that acetone control group was not significantly different from control group. Among chlorpyrifos-treated groups, 8.2ppb group and 27.3ppb groups showed significant difference between them.

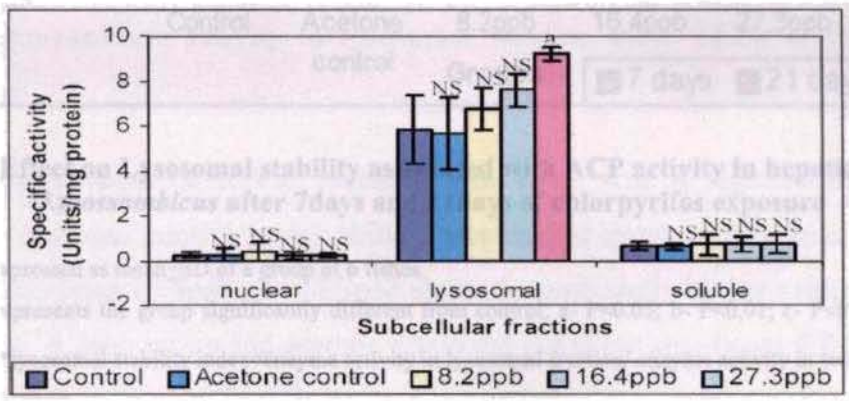


Fig.5.4: Effect on Acid phosphatase activity in different subcellular fractions in hepatic tissue of *O.mossambicus* exposed to different concentrations of chlorpyrifos for 7days

One Unit is expressed as mg p-nitrophenol liberated/hour/mg protein

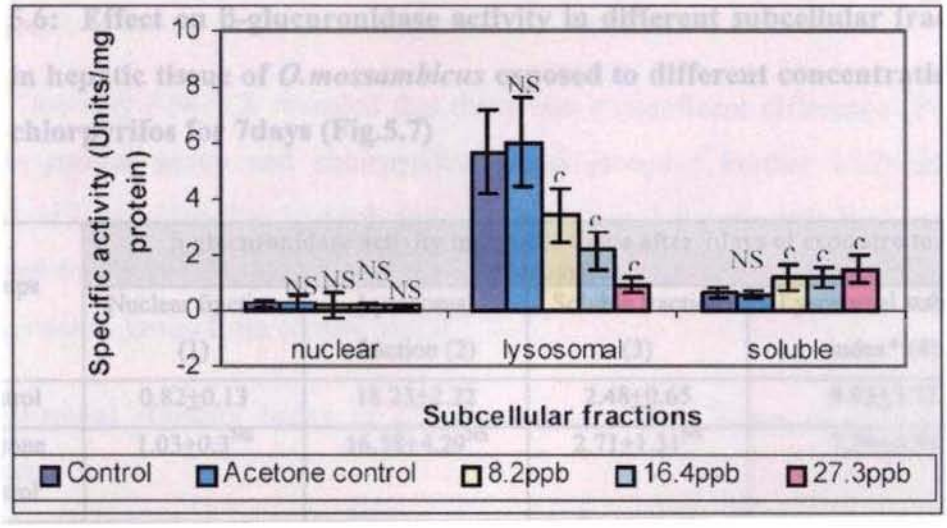


Fig.5.5: Effect on Acid phosphatase activity in different subcellular fractions in hepatic tissue of *O.mossambicus* exposed to different concentrations of chlorpyrifos for 21days

One Unit is expressed as mg p-nitrophenol liberated/hour/mg protein

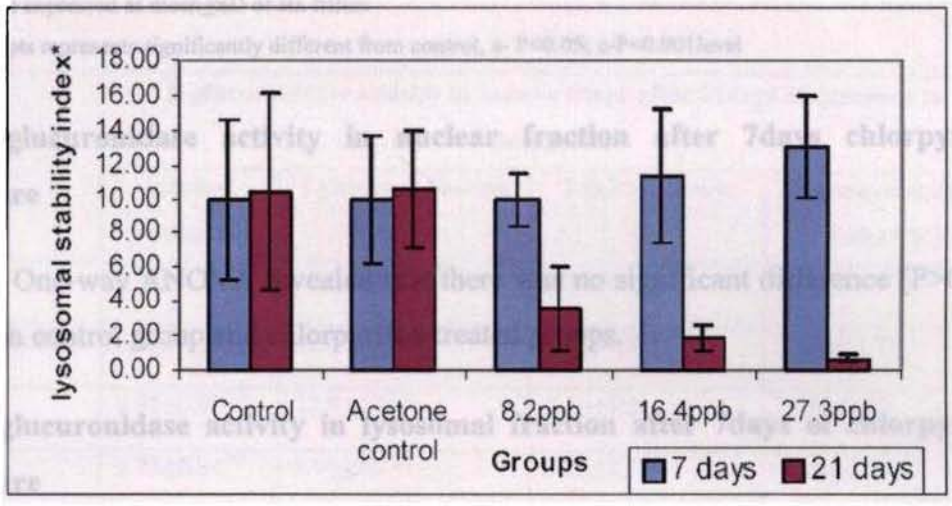


Fig.5.6: Effect on Lysosomal stability associated with ACP activity in hepatic tissue of *O.mossambicus* after 7days and 21days of chlorpyrifos exposure

Values are expressed as mean±SD of a group of 6 fishes

superscript represents the group significantly different from control; a- P<0.05; b- P<0.01; c- P<0.001, NS-not significant; *lysosomal stability index=enzyme activity in lysosomal fraction/ enzyme activity in soluble fraction

Table 5.6: Effect on β-glucuronidase activity in different subcellular fractions in hepatic tissue of *O.mossambicus* exposed to different concentrations of chlorpyrifos for 7days (Fig.5.7)

Groups	β-glucuronidase activity in hepatic tissue after 7days of exposure to chlorpyrifos			
	Nuclear fraction (1)	Lysosomal fraction (2)	Soluble fraction (3)	Lysosomal stability index* (4)
Control	0.82±0.13	18.23±2.22	2.48±0.65	9.93±3.22
Acetone control	1.03 ± 0.3^{NS}	16.58 ± 4.29^{NS}	2.71 ± 1.31^{NS}	7.79±4.54
8.2ppb	0.63 ± 0.3^{NS}	19.99 ± 4.73^{NS}	3.49 ± 1.53^{NS}	7.44±5.87
16.4ppb	0.88 ± 0.2^{NS}	23.77 ± 5.37^{c}	4.78 ± 1.49^{a}	5.61±2.94
27.3ppb	0.81 ± 0.12^{NS}	28.37 ± 2.91^{c}	4.40 ± 1.4^{a}	7.19±2.98

*Lysosomal stability index =Ratio of enzyme activity in lysosomal fraction to enzyme activity in soluble fraction

Values in (1), (2) and (3) are expressed as mg p-nitrophenol liberated/hr/gm protein

Values are expressed as mean±SD of six fishes

Superscripts represents significantly different from control, a- P<0.05; c-P<0.001level

(1) β-glucuronidase activity in nuclear fraction after 7days chlorpyrifos exposure

One-way ANOVA revealed that there was no significant difference (P>0.05) between control group and chlorpyrifos-treated groups.

(2) β-glucuronidase activity in lysosomal fraction after 7days of chlorpyrifos exposure

Single-factor ANOVA revealed that there was a significant difference (P<0.05) between control group chlorpyrifos-treated groups. LSD analysis (LSD= 4.7) revealed that 16.4ppb and 27.3ppb showed significantly higher values compared to control. 8.2ppb group and acetone group did not show significant difference from control group.

(3) β-glucuronidase activity in soluble fraction after 7days of chlorpyrifos exposure

One-way ANOVA revealed that there was a significant difference ($P<0.05$) between control group and chlorpyrifos-treated groups. Further LSD analysis (LSD=1.517) revealed that 16.4ppb and 27.3ppb showed significantly higher values compared to control group. No significant difference was observed in 8.2ppb and acetone control group from control group.

(4) Lysosomal stability index in *O. mossambicus* after 7days of chlorpyrifos exposure

There was no significant difference ($P>0.05$) between control group and pesticide-treated group.

Table 5.7: Effect on β-glucuronidase activity in hepatic tissue of *O.mossambicus* exposed to different concentrations of chlorpyrifos for 21days (Fig.5.8)

Groups	β-glucuronidase activity in hepatic tissue after 21days of exposure to chlorpyrifos			
	Nuclear fraction (1)	Lysosomal fraction (2)	Soluble fraction (3)	Lysosomal stability index* (4)
Control	0.82±0.13	17.62±2.48	2.54±0.79	7.56±2.59
Acetonecontrol	0.9 ± 0.11^{NS}	18.46 ± 2.07^{NS}	1.2 ± 0.65^{NS}	10.21±3.72
8.2ppb	0.98 ± 0.40^{NS}	12.8 ± 2.25^{c}	5.54 ± 1.3^{c}	2.39±0. 51
16.4ppb	0.73 ± 0.34^{NS}	9.95 ± 1.71^{c}	9.05 ± 2.39^{c}	1.15±0.32
27.3ppb	0.18 ± 0.11^{c}	4.88 ± 1.85^{c}	10.82 ± 3.98^{c}	0.6±0.54

*Lysosomal stability index =Ratio of enzyme activity in lysosomal fraction to enzyme activity soluble fraction

Values in (1), (2) and (3) are expressed as mg p-nitrophenol liberated/hr/gm protein

Values are expressed as mean±SD of six fishes superscript represents the group significantly different from control; c- $P<0.001$, NS-not significant

(1) β-glucuronidase activity in nuclear fraction after 21days of chlorpyrifos exposure

Single-factor ANOVA revealed that there was significant difference ($P<0.001$) between control and the pesticide treated, 27.3ppb group. No significant difference was observed between control group from 16.4ppb and 8.2ppb groups. Acetone control group was also not significantly different from control group.

(2) β-glucuronidase activity in lysosomal fraction after 21days of chlorpyrifos exposure

One-way ANOVA and further LSD analysis (LSD value=2.4) revealed that there was a significant difference ($P<0.001$) between control group and pesticide-treated groups. Chlorpyrifos-treated groups showed significantly lower values compared to control.

(3) β-glucuronidase activity in soluble fraction after 21days of chlorpyrifos exposure

One-way ANOVA releaved that there was a significant difference ($P<0.05$) between control and chlorpyrifos-treated groups. LSD value obtained was 2.55. Chlorpyrifos-treated groups showed significantly higher values compared to control group.

(4) Lysosomal stability index in *O.mossambicus* after 21days of chlorpyrifos exposure

One-way ANOVA and further LSD analysis (LSD=0.21) revealed that there was a significant difference ($P<0.001$) between control group and chlorpyrifos-treated group. No significant difference was observed between control group and acetone control group. 27.3ppb group was significant different from 16.4ppb group and 8.2ppb group.

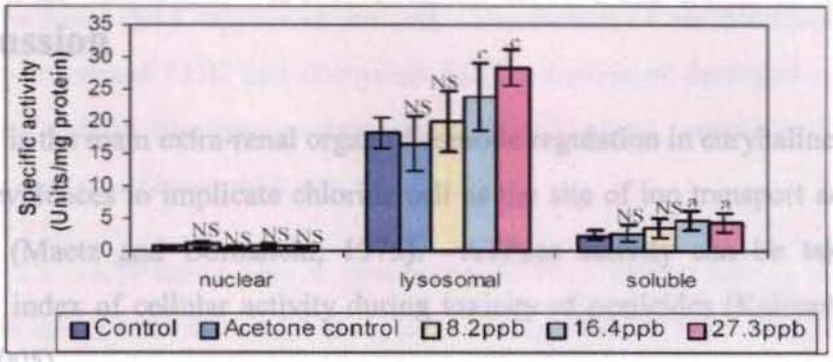


Fig.5.7: Effect on β-glucuronidase activity in different subcellular fractions in hepatic tissue of *O.mossambicus* exposed to different concentrations of chlorpyrifos for 7days

One Unit is expressed as mg p-nitrophenol liberated/hour/gm protein

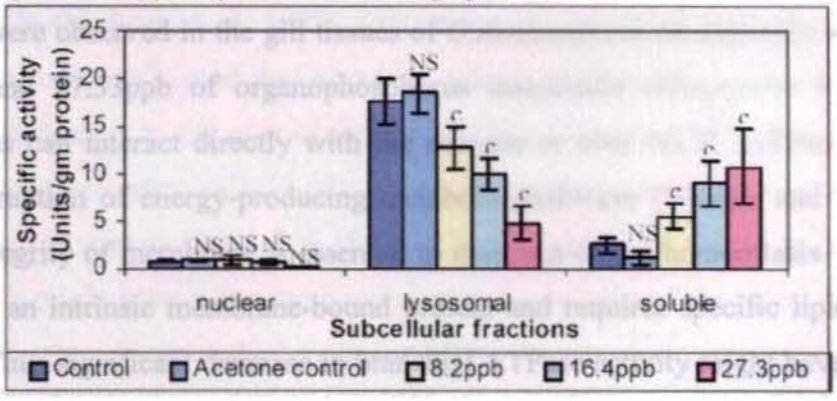


Fig.5.8: Effect on β-glucuronidase activity in different subcellular fractions in hepatic tissue of *Oreochromis mossambicus* exposed to different concentrations of chlorpyrifos for 21 days

One Unit is expressed as mg p-nitrophenol liberated/hour/gm protein

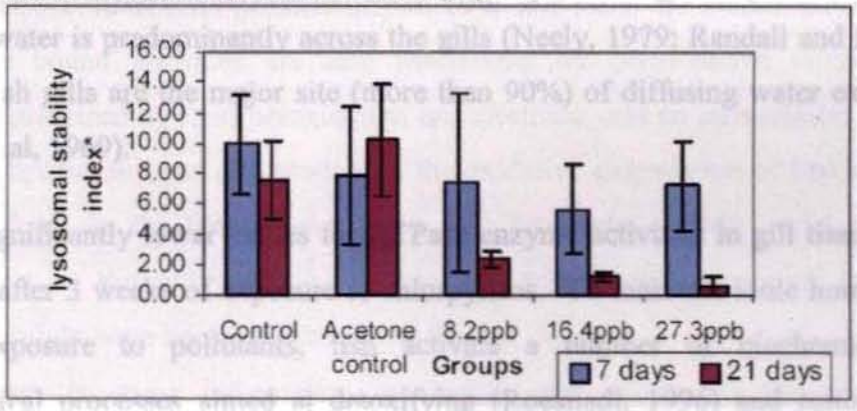


Fig.5.9: Effect on Lysosomal stability associated with β-glucuronidase activity in hepatic tissue of *O.mossambicus* after 7days and 21days of chlorpyrifos exposure

Values are expressed as mean±SD of a group of 6 fishes

superscript represents the group significantly different from control; a- P<0.05; b- P<0.01; c- P<0.001, NS-not significant; *lysosomal stability index= enzyme activity in lysosomal fraction/ enzyme activity in soluble fraction

5.5 Discussion

Gill is the main extra-renal organ of osmotic regulation in euryhaline teleosts. There are evidences to implicate chloride cell as the site of ion transport across gill epithelium (Maetz and Bornancin, 1975). ATPase activity can be taken as a meaningful index of cellular activity during toxicity of pesticides (Kalivaradan and Ramudu, 2005)

A significant inhibition on Na^+K^+-ATPase, Ca^{2+}-ATPase and Total ATPase activities were observed in the gill tissues of *O.mossambicus* on exposure to 8.2ppb, 16.4ppb, and 27.33ppb of organophosphorus insecticide chlorpyrifos for 7days. Xenobiotics can interact directly with the enzyme or alter Na^+K^+-ATPase activity due to disruption of energy-producing metabolic pathways (Watson and Beamish, 1980). Integrity of membrane is essential to maintain cation homeostasis. Na^+-K^+-ATPase is an intrinsic membrane-bound protein and requires specific lipids for its activity. Thus significant decrease in branchial ATPase activity might have resulted from chlorpyrifos-induced damage to the cell membrane lipids and proteins. The results revealed that inhibition of various ATPase activities by pesticide affect seriously the transport of ions across gill epithelial membrane. Xenobiotic uptake from the water is predominantly across the gills (Neely, 1979; Randall and Brauner, 1993). Fish gills are the major site (more than 90%) of diffusing water exchanges (Motais et al, 1969).

Significantly lower values for ATPase enzyme activities in gill tissues were observed after 3 weeks of exposure to chlorpyrifos. To maintain ionic homeostasis during exposure to pollutants, fish activate a number of biochemical and physiological processes aimed at detoxifying (Roesijadi, 1996) and restoring the branchial ion uptake machinery (McDonald and Wood, 1993). The inhibitory effect on Na^+K^+-ATPase is generally ascribed to structural and functional damage to the gills as a result of accumulation of toxicant on a long-term exposure (Pelgrom et al, 1995). The histopathological finding of complete loss of architecture of epithelial cells also supports the decreased activity of branchial ATPases. Sodium pump

maintains the ionic fluid balance of the cell. Impairment of aerobic respiration as observed by increased LDH and decreased SDH activities or deranged membrane integrity can disrupt the energy-dependent sodium pump, resulting in altered intracellular content of ions and water.

The Ca^{2+}-ATPase is also a membrane ATPase whose role in the cell is essential and evident but that could also play a role in transepithelial transport and be implicated in the regulation of plasma Ca^{2+} concentration. (Renzis and Michel, 1984). Ca^{2+} influx is facilitated by a Ca^{2+} channel in the apical membrane of the chloride cells (Verbost et al, 1989). Extrusion of Ca^{2+} from the cell across the basolateral membrane is mediated by a high-affinity Ca^{2+} ATPase and possibly by a Na^{+}, Ca^{2+} exchanger in the gills of fresh water fish (Zanatta et al, 2001). Chloride cells, the ion-transporting cells in gills, play an important role in the maintenance of ionic balance in teleosts (Perry S F, 1997). Thus the structural alteration induced by the pesticide might have led to the non-functional condition of the apical membrane of the gill. In the present study the enzyme inhibition was more prominent on a long-term chlorpyrifos exposure than on a short-term exposure.

One of the major causes of a loss of membrane fluidity is lipid peroxidation. It increases the membrane permeability to ions and loses the membrane integrity. Membrane bound enzymes are also inactivated by peroxidative injury. The aldehydes produced by lipid peroxidation are cytotoxic, and an intracellular pigment known as lipofuscin is an end-product of the oxidative degradation of lipids. These pigments form in the lysosomes (Stripp and Trombetta, 1994). So the lipid peroxidation in the gill epithelial tissues and subsequently occurred lysosomal disruption might have been contributed for the cell injury.

There is some information concerning the effects of the chlorinated hydrocarbon pesticicide DDT on sodium-potassium activated ATPase activity in fathead minnow gills. Desiah and coworkers (1975) measured the activity of gill $Na^{+}K^{+}$-ATPase using membrane fraction and reported an inhibitory effect of water-borne DDT after 225 days of exposure. Janicki and Kinter (1971a) studied the effect of DDT (5.0ppm or 1.4×10^{-5}M *in vitro*) on the $Na^{+}K^{+}$-ATPase of the eel (Anguilla

rostrata) and found that 43% of the total enzyme activity was inhibited. When the intestinal sac was filled with Ringer solution containing 50ppm of DDT, there was a 47% inhibition of water absorption. Since Na^+K^+-ATPase is generally meant to be the machinery in the teleost, they concluded that the inhibitory activities of DDT on the ATPase eventually result in impairment of fluid absorption in the intestinal sac.

Indeed, there are reports indicating that various fish ATPases are sensitive to organochlorine insecticides (Koch, 1969; Cutkomp et al, 1971; Janicki and Kinter, 1971b). In the present study, chlorpyrifos contains three chlorine atoms in one molecule. The observations in this study are in agreement with *in vivo* data on disrupted osmoregulation as judged by changes in Anguilla.

Gill is the first internal organ that comes in direct contact with the insecticide, chlorpyrifos. The enzyme, Cytochrome P-450, involved in biotransformation process is present in the highest amount in the membranes of the ER, especially in the microsomal fraction. Thus the binding of hydrophobic organophosphate compound with microsomal lipids and proteins might have occurred and adversely affected the enzyme activity. Lipid-free Na^+K^+-ATPase is completely inactive (Hoar, 1984). Subsequent interaction of chlorpyrifos with lipid molecules might have reduced the synthesis and activity of ATPase enzymes in gill tissue.

Some chemicals require activation, in the endoplasmic reticulum by monoxygenases, to become carcinogenic (Robert, 1990). Hence the monooxygenases and other xenobiotic-metabolizing enzymes present in the ER might have favoured the production of chlorpyrifos-oxon. According to Huff et al (1994) chlorpyrifos-oxon is 300-400 times more toxic than chlorpyrifos. The metabolite formed might have induced more damage than the parent compound.

Changes in the fluctuation of ions, or the direct action of pesticide might have caused intracellular damage by poisoning mitochondria. This is expected to lead to alteration in energy metabolism and a decreased synthesis of ATP. Loss of cellular energy then might have affected energy requiring activities such as ion pumps, membrane maintenance and protein synthesis which led to further losses in

membrane integrity. As the cytological effects are irreversible, the cell might have entered a state of necrosis as observed in histological findings.

Acid and alkaline phosphatases are known as inducible enzymes. Their activity goes up when there is toxic impact and the enzymes begin to counteract the toxic effect. In 27.3ppb group a significant increase was observed in acid phosphatase activity, only in lysosomal fraction after exposure for 7days to the insecticide. But in the other chlorpyrifos-treated groups like 16.4ppb group and 8.2ppb group an increase was observed both in lysosomal fraction and soluble fraction. The present observation is in consonance with the findings of Reddy et al (1986). He studied the changes in the activity of acid phosphatase in the hepatopancreas of the rice field crab (*Oziotelphusa senex*) after exposure of the pesticide sumithion. He reported that the increase in the acid phosphatase activity after the pesticide exposure could be attributed to lysosomal activity.

β-glucuronidase was found to be slightly elevated in all fractions on a short-term exposure to chlorpyrifos. This might be due to the increased detoxication activity by the cells involved in destruction of xenobiotics on a short-term exposure. This observation can be supported by a report by Connell (1984) that some pesticides are capable of inducing increased activity of hepatic enzyme systems. More damage to liver at higher sublethal exposure than at lower sublethal levels, was reported by Kalivaradan and Ramudu (2005) in *O. mossambicus* exposed to chlorpyrifos for 7days.

But the ACP and β-glucuronidase enzyme activities were found to be decreased significantly after 21days of exposure to chlorpyrifos. ACP activity in all the studied subcellular fractions was found to be decreased. This is expected to be due to cell damage induced by the insecticide. The organophosphorus insecticide might have inhibited the phosphatase enzyme hence the enzyme acid phosphatase showed a marked decline.

Simon (1953) after conducting different toxicant tests noted that the uncoupling of oxidative phosphorylation was the possible cause for the inhibition of

acid phosphatases. These findings were similar in *Ophiocephalus punctutas* exposed to copper (Srivastava and Pandey, 1982) and in *Notopterus notopterus* exposed to phenolic compounds (Dalela et al., 1982).

Lability to the lysosomal membrane might have resulted in the release of lysosomal enzymes to cytoplasm. Thus an increased enzyme activity was observed in soluble fraction. Lysosomal hydrolases are thought to contribute to the degradation of damaged cells and hence facilitate their replacement by normal tissue (Gopalakrishnan, 1990). This observation can be correlated with the morphological finding of hepatobiliary hyperplasia at certain regions of liver tissue.

Cytochrome P-450 monoxygenase is the main enzyme involved in the metabolism, namely, desulfuration of the insecticide chlorpyrifos. Thus the hepatic tissue exposed to chlorpyrifos is more prone to lipid peroxidation and cell injury. This might have resulted in cell death.

Binding of lysosomal membrane with chlorpyrifos and in turn its disruption might have resulted in the release of acid hydrolase enzymes that present inside the organelle. Thus a marked decrease was observed in the marker enzymes like acid phosphatase and β-glucuronidase in lysosomes. This might have led to the elevated levels of the enzymes in cytoplasmic fraction. The ratio of enzyme activity in lysosomal fraction to that in soluble fraction gives the lysosomal stability index. This ratio was found to decrease on prolonged exposure to chlorpyrifos. Thus a marked decrease was observed in the stability of lysosomal membrane. Binding of chlorpyrifos and chlorpyrifos-oxon to the membrane might be responsible for a decreased lysosomal membrane stability. The stability was found to decrease as the insecticide concentration increased. Due to the pollutant effluent, toxicants cause changes in size, quality, membrane lability and lysosomal stability (Leiand, 1983). Lysosomes are one possible indicator of well being of animals. Many types of stress can alter the membrane structure, which then becomes more permeable and permits substrates to enter lysosomes, and hydrolases to move out into the cytoplasm (Moriarty, 1983).

Damage to cell membrane can lead to the entry of these enzymes to the associated capillaries. Thus enzymes released from the damaged cells might have entered the body fluids and involved in the destruction of other cellular organelles.

The damage to the lysosomal membrane may be conceptualized as an increase/activation or decrease/inhibition of the lysosomal hydrolases or labilising/stabilizing effects due to changes in membrane permeability effected by the contaminants (Hawkins, 1980). However, the membrane stability or fragility of the lysosomes can be altered under certain physiological and pathological conditions (Bitensky etal, 1973).

Lipid peroxidation is a highly destructive process and alters the structure and function of cellular membrane (Kale and Sitaswad, 1990). Disrupted tissues are known to undergo lipid peroxidation at a faster rate than normal ones (Anjali and Kale, 2001). Observations from the present investigation revealed the toxic nature of the insecticide chlorpyrifos. The branchial ATPase inhibition was found to be leading to impaired ion-transport and imbalance in homeostatic mechanism. Binding of lipophilic chlorpyrifos is expected to be responsible for the rupturing of the lysosomal membrane. This might have led to the release of hydrolase enzymes to the surrounding cellular environment. Hence the entry of hydrolases itself might have led to partial-digestion of the cells.

Chapter

6

Effects of Chlorpyrifos on Haematological parameters and Electrophoretic pattern of serum proteins in *Oreochromis mossambicus*

Contents

6.1 Introduction

Fishes are resources of great economic importance. Presence of chemicals such as pesticides in marine ecosystem affects fish- immensely directly or indirectly in various ways. Usually, alteration in pH of water, decrease in oxygen content, presence of toxic ingredients, cumulative effects of pesticides and various other compounds are some of the major causes of homeostatic disturbances to fish. Haematological tests are important diagnostic tools for toxicological investigations. Recent speculations have suggested that they may be equally valuable as indicators of disease or stress due to pollutants and environmental fluctuations in fishes. The blood plays an important and inevitable part in all immune systems. Blood being the medium of intercellular transport, comes in direct contact with various organs and tissues of the body, therefore, the physiological state of an animal at a particular time is reflected in its blood. Pesticides rapidly bind to the blood proteins and induce haematological changes. These changes are of some value in assessing the impact of exposure under natural conditions and may also serve as tools for biological monitoring (Thomas et al, 2007).

In addition to enzymatic alterations, changes in other proteins also occur in fish exposed to various types of environmental stresses including pesticides. It has been suggested that the environmental perturbations including stresses caused by the pesticides may suppress the expression of some genes and activate the others to produce specific mRNAs which may subsequently be translated into specific proteins, the stress proteins (Adams and Rinne, 1982; Pelham, 1985). Varieties of pollutants have proved to alter the protein metabolism in fish (Palanichamy et al, 1989).

6.2 Materials and methods

6.2.1 Studies on haematological parameters

Fishes were collected and maintained as described in section 3.2.B. The fishes were exposed to chlorpyrifos at different sublethal concentrations of LC_{50} value and also to acetone for a short-term period of 7days and a long-term period of 21days.

a. Collection and processing of blood samples:

Blood was collected directly from cardinal vein lying under the gills of the fish using 1mL syringe and transferred into a bottle containing 0.2% EDTA as anticoagulant.

b. Determination of haematological parameters

The blood was analysed using Cellcounter Automated Analyzer (Celltak marketed by Pan Company in India) for the haematological parameters RBC (red blood cell) count, WBC (white blood cell) count, Hb (haemoglobin) and PCV (packed cell volume).

6.2.2 Studies on Electrophoretic pattern of serum protein

a. Collection and processing of blood samples

Blood was collected without anticoagulant and kept at room temperature for half an hour. Hemolysis-free serum was collected by centrifugation for 10minutes at 3000rpm and stored in polypropylene bottles at -20^0C until used for electrophoretic analysis.

b. SDS-PAGE analysis:

The basic methodology adopted was as described by Laemmli et al (1970) with some modifications. Standardisation of the technique was performed as the percentage of separating gel is a critical parameter in all electrophoretic separations of different proteins in the sample. Separating gels of 12.5%, 11.5% and 11% were tried to choose an ideal percentage, which gives a better electrophoretic separation. Finally, a separating gel of 11.5% concentration, prepared from a 30% stock of acrylamide and bisacrylamide monomers was selected along with a stacking gel of 6%. The concentration of serum samples to be loaded on the gel was also standardized to get an ideal resolution.

Reagents for electrophoresis:

A. Stock acrylamide solution (30%)

Acrylamide	-29.1g
NN'-methylene bisacrylamide	-0.8g

The mixture was dissolved in minimum quantity of water and made up to 100ml using distilled water. The mixture was filtered using Whatman No.1 filter paper and stored in amber coloured bottles in a refrigerator.

B. Gel buffers:

a. Separating Gel Buffer

1.5M Tris	- 18.17g
SDS (Sodium dodecyl sulphate)	- 0.4g

The pH was adjusted to 8.8 using 2M HCl and the solution was made up to 100mL using distilled water.

b. Stacking gel buffer

0.5M Tris	- 6.05g
SDS	- 0.04g

The pH was adjusted to 6.8 using 2M HCl and the solution was made upto 100mL using distilled water

c. Electrode buffer

Tris - 3g

Glycine - 14.4g

SDS -1g

Adjusted Tris pH to 8.6 with HCl and added SDS. Made upto 1000mL using distilled water.

C. Polymerizing Agent

Ammonium persulphate (APS) - 10% (freshly prepared)

D. 10% SDS solution - 10g SDS in 100mL distilled water

E. Composition of 11.5% gel

a. Separating gel

Acrylamide and NN' methylene bisacrylamide -11.5mL

Separating gel buffer - 6mL

Water - 12.7mL

10%SDS - 300µL

TEMED - 30µL

APS - 100µL

b. Stacking gel

Acrylamide and NN'Bisacrylamide - 2mL

Stacking gel buffer - 2.5mL

Water - 5.4mL

10%SDS - 100µL

TEMED (N,N,N',N'-Tetramethyl-ethylene diamine)- 10µL

APS - 40µL

The separating gel components were mixed gently and poured into the prepared cassette. Few drops of butanol were over layered to prevent meniscus formation and the gel was left undisturbed to set for 30minutes. After polymerization of the separating gel, the overlaying butanol was removed and the cassette was washed with double distilled water and dried. The prepared stacking gel mixture was then poured over the separating gel. The comb was placed in the stacking gel and allowed to set for 30minutes.

After the gel got solidified the comb was removed without distorting the shape of the well. The gel was carefully set on the electrophoretic apparatus after removing the clips, bottom spacers etc. with the plate having the "U" shape cut facing the upper tank using clamps and screws provided. The electrode buffer was added to the tanks and care was taken to prevent entrapment of air bubbles at the bottom of the gel. The electrodes were then connected to the power pack.

F. Sample buffer

Stacks of sample buffer with SDS were prepared as follows:

Glycerol : 2mL

β-mercaptoethanol : 1mL

Stacking gel buffer : 1.8mL

Bromophenol blue : 0.6mL (0.5%)

10%SDS : 1mL

G. Staining solution (500mL)

Coomassie Brilliant Blue R 250 - 0.75gm

Methanol - 230mL

Acetic acid - 40mL

Distilled water - 230mL

H. Destaining solution (500mL, freshly prepared)

Methanol - 25mL

Acetic acid - 35mL

Distilled water - 440mL

I. Marker: Protein molecular weight marker was purchased from GENEI, Bangalore.

Sample application and electrophoresis

Serum stored at -20^0C was brought to room temperature. 10µL of the sample was mixed with 90µL of distilled water. 50µL of this mixture was then mixed with 50µL of sample buffer with SDS and boiled for 1minute.

10µL of protein molecular weight marker was mixed with 60µL of sample with 2%SDS and boiled for 1minute.

The prepared samples were applied into the wells of the stacking gel and layered with running buffer in order to avoid disturbance to the sample. A constant voltage of 60volts was applied until the dye front crossed the stacking gel and it was increased to 140volts and electrophoresis was continued until the dye front reached the bottom of the gel.

Staining the gels:

Immediately after the completion of electrophoresis, the gels were carefully separated from the trays into plasic trays and washed in tap water to remove excess SDS. After staining the gels for two hours in Coomassie Brilliant Blue R 250, the excess stains were washed off and the gels were immersed in destaining solution.

Determination of Molecular Weight:

Molecular weight of standards used as molecular markers for SDS PAGE were 205000, 97400, 66000, 43000, 29000, 20100, 14300, 6500, 3000D, daltons. Rf values of the standard markers were calculated by using the following formula

$$\text{Rf, Relative front} = \frac{\text{Distance moved by solute}}{\text{Distance moved by dye}}$$

Using these Rf values calculated for standard markers, a graph was drawn with Rf and $\log_{10}$ of the molecular weights of the standard proteins on a semi-log graph. The Rf values of unknown samples were calculated and extrapolated on a standard graph to determine the molecular weight.

6.3 Statistical analysis

Statistical analysis was carried out by Single-factor ANOVA and followed by further LSD (Least significant difference) analysis (Zar, 1996). Significant difference in pesticide treated group from control group is represented along with Mean±SD.

6.4 Results

Responses to various haematological parameters are recorded. The electrophoretic pattern of serum proteins obtained after SDS-PAGE analysis is also presented under results.

Table 6.1: Haematocrit values from *O.mossambicus* exposed to chlorpyrifos for 7days and 21days (Fig.6.1)

Exposure period	Control	Acetone Control	8.2ppb	16.4ppb	27.33ppb
7 days	7.8±1.6	7.07 ± 3.4^{NS}	7 ± 0.68^{NS}	7.4 ± 0.64^{NS}	7 ± 1.97^{NS}
21 days	7.8±1.59	8.33 ± 1.32^{NS}	7.07 ± 1.25^{NS}	11.65 ± 3.77^{a}	9.59 ± 3.83^{NS}

Values are expressed as mean±SD of a group of 6fishes
Haematocrit is expressed as %
a- significantly different from control; P<0.05; NS-not significant

No significant difference (P>0.05) was observed between control and acetone control and between control and pesticide treated groups when exposed for 7days. After exposure of 21days significantly (P<0.05) higher values were observed in

16.4ppb group. No significant difference was observed between control and other chlorpyrifos-treated groups.

Table 6.2: Haemoglobin values from *O.mossambicus* exposed to chlorpyrifos for 7days and 21days (Fig.6.2)

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	2.63±0.26	2.97 ± 0.44^{NS}	2.66 ± 0.35^{NS}	2.7 ± 0.38^{NS}	2.83 ± 1.59^{NS}
21 days	2.93±0.27	3 ± 0.28^{NS}	3.02 ± 1.02^{NS}	3.19 ± 0.76^{NS}	2.87 ± 0.72^{NS}

Values are expressed as mean±SD of a group of 6fishes
Haemoglobin is expressed as gm%
NS-not significant

No significant difference (P>0.05) was observed between control and acetone control and between control and pesticide treated groups when exposed for 7days and 21days.

Table 6.3: RBC count -values from *O.mossambicus* exposed to chlorpyrifos for 7days and 21days (Fig.6.3)

After 7 days of chlorpyrifos exposure no significant difference (P>0.05) was observed either between control group and acetone control or between control and pesticide treated groups after 7days of exposure to chlorpyrifos.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.33ppb
7 days	1.8±0.41	1.85 ± 0.50^{NS}	1.75 ± 0.83^{NS}	1.79 ± 0.77^{NS}	2.10 ± 0.46^{NS}
21 days	1.69±0.37	1.75 ± 0.23^{NS}	2.23 ± 0.54^{a}	2.29 ± 0.53^{a}	2.38 ± 0.36^{a}

Values are expressed as mean±SD of a group of 6fishes
Number of RBCs in millions/cumm
a- significantly different from control; P< 0.05; NS-not significant

One-way ANOVA revealed that there is a significant difference (P<0.05) between control group and pesticide treated groups after 21days of exposure to chlorpyrifos. LSD value obtained was 0.493.

Table 6.4: WBC count- values from *O.mossambicus* exposed to chlorpyrifos for 7days and 21 days

A significant difference (P<0.05) between group means of control and pesticide treated groups was observed. LSD value obtained was 584. The 27.3 group was highly significant followed by 16.4ppb when compared to control, after 7 days of exposure to the pesticide. But no significant difference was observed between control and acetone control and between control and 8.2ppb group.

Exposure period	Control	Acetone control	8.2ppb	16.4ppb	27.3ppb
7 days	1150±488.8	1116.6±402^{NS}	1650±750^{NS}	1916.6±462^{a}	2016.7±318.8^{a}
21 days	1300±352	1133.3±320^{NS}	1616.6±116.9^{NS}	1083±608^{NS}	1016±354.5^{NS}

Values are expressed as mean±SD of a group of 6fishes
Number of WBCs in number/cumm
a-significantly different from control; P<0.05; NS-not significant

No significant difference (P>0.05) was observed between different groups after the exposure to chlorpyrifos.

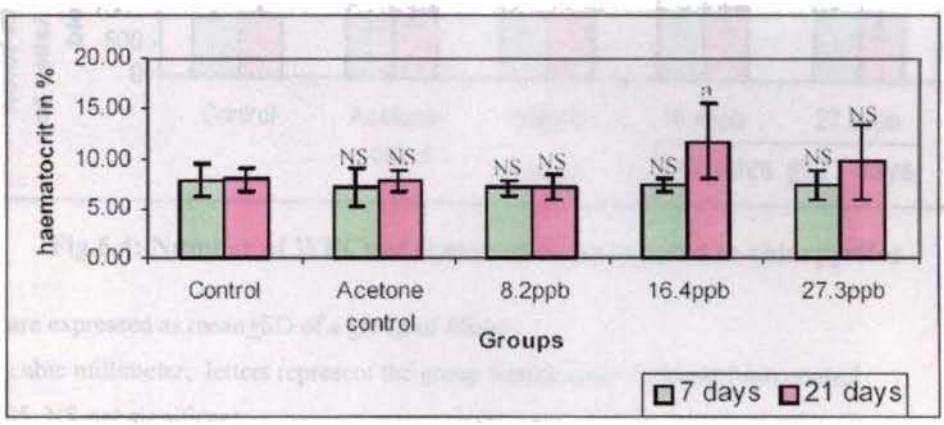


Fig. 6.1: Haematocrit values of *O.mossmbicus* exposed to chlorpyrifos

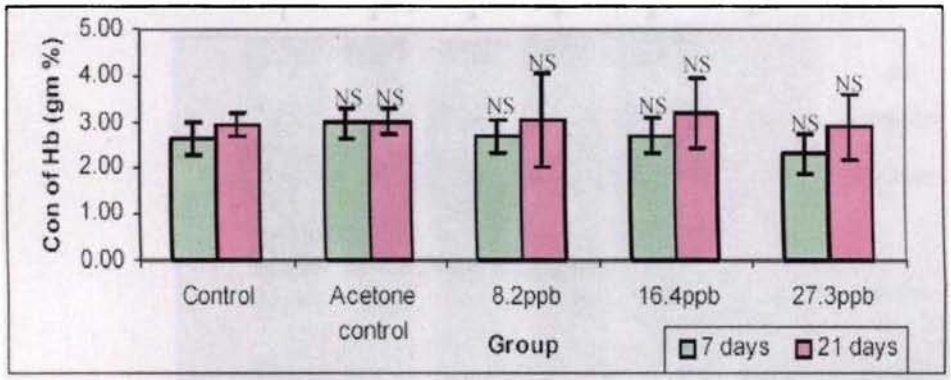


Fig.6.2: Haemoglobin values of O. mossambicus exposed to chlorpyrifos

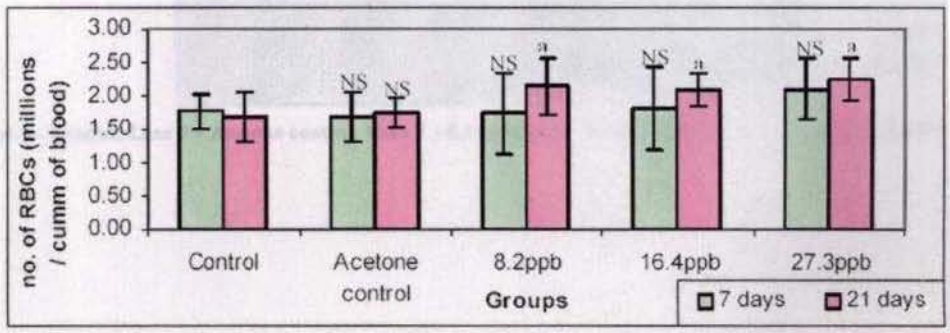


Fig.6.3: Number of RBCs of *O.mossambicus* exposed to chlorpyrifos

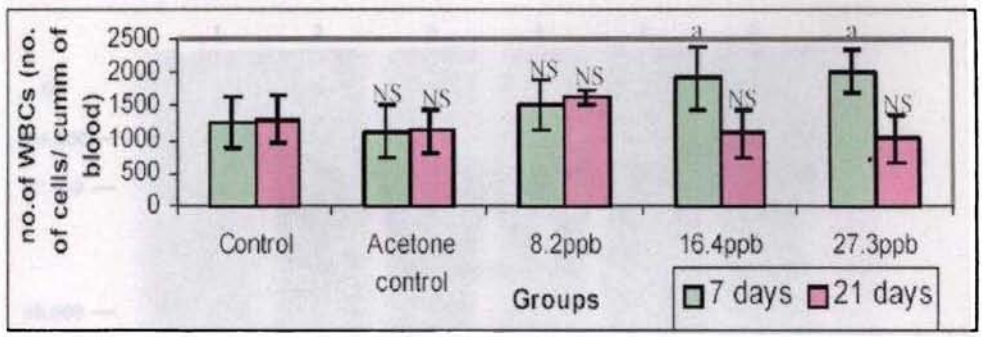


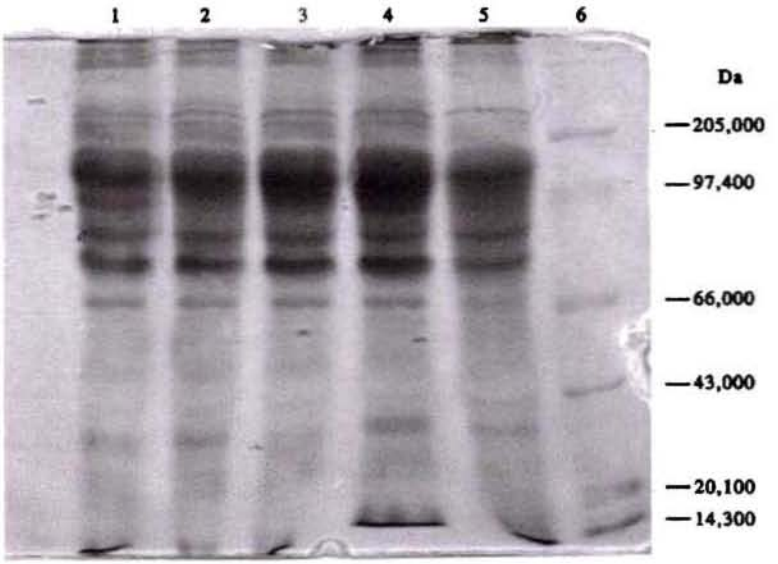
Fig.6.4: Number of WBCs of *O.mossambicus* exposed to chlorpyrifos

Values are expressed as mean±SD of a group of 6fishes

cumm- cubic millimeter; letters represent the group significantly different from control;

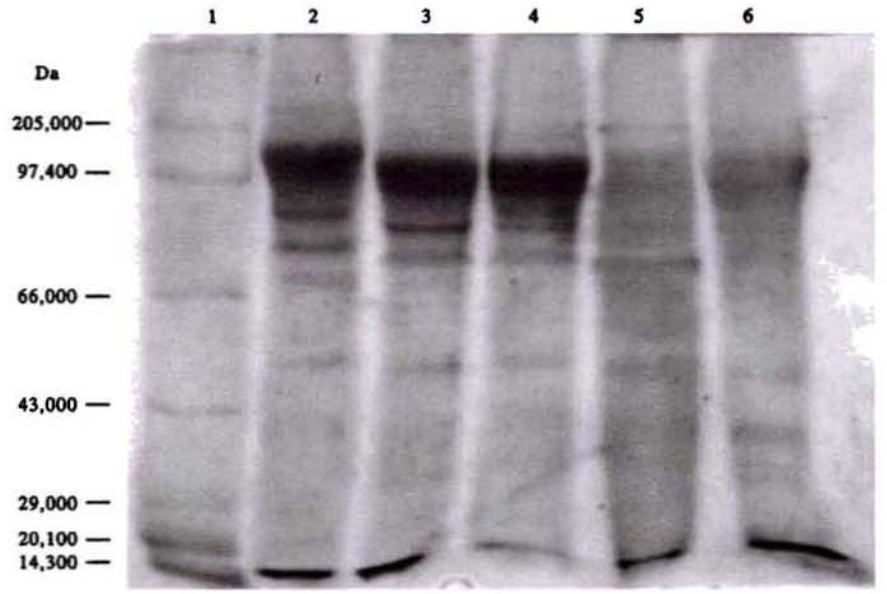
a- P<0.05; NS-not significant

Plate -1



Lane 1 - Control; Lane 2 - Acetone control; Lane 3 - 8.2 ppbl; Lane 4 - 16.4 ppb; Lane 5 - 27.33 ppb; Lane 6 - Marker

Plate -2



Lane 1 - Marker; Lane 2 - control; Lane 3 - Acetone control; Lane 4 - 8.2 ppb; Lane 5 - 16.4 ppb; Lane 6 - 27.33ppb

SDS PAGE Analysis of serum proteins of *Oreochromis mossambicus* exposed to technical grade chlorpyrifos for 7 days (Plate -1) and for 21 days (Plate -2)

6.5 Discussion

The responses to various haematological parameters like haemoglobin (Hb), packed cell volume (PCV), red blood cell (RBC) count, white blood cell (WBC) count are recorded in tables 6.1 to 6.4 and graphs 6.1 to 6.4. After 7days of exposure of the fish to chlorpyrifos, no remarkable changes were observed in haematological parameters like Hb, RBC count and PCV. This is expected to be due to the effectiveness of the defense mechanism provided by the antioxidant enzymes. Thus xenobiotic stress might not have adversely affected the haemopoietic system. But a significant increase was observed in PCV in 16.4ppb group, after 21 days of chlorpyrifos exposure. The increase in PCV was insignificant in 27.3ppb group.

A significant increase in RBC count was observed after 21 days of chlorpyrifos exposure. This might be due to the re-entry of the blood cells into the circulation. It is well known that during hypoxia the number of RBCs and haemoglobin content increases as reported by Valicre and Stickney (1972). But the haemoglobin content did not show significant variation, expected to be due to impaired haemoglobin synthesis.

Morphological changes happened in gill tissue after 21days of exposure can be correlated with the increased RBC count. The cardinal vein lying under the gill was the site of blood collection for hematological examination. Gill is the first internal organ that comes in direct contact with the pesticide exposure. Hyperemia with excess blood in the vessels supplying gill filament was observed with increased number of RBCs in the morphological studies of gill tissue. Fish might be trying to consume maximum amount of oxygen to meet the metabolic needs and compensate the stress condition.

The observation of increased RBC count in the present study is in agreement with the report of Svobodova et al (1994). According to him, the toxic substances can significantly damage the haemopoietic system of fish and in some cases they can

even cause an increased disintegration of erythrocytes. He reported that some haematological changes might be the result of a disorder in erythrocyte cell membrane permeability and/or the result of the activation of protective mechanisms. These mechanisms may include the release of erythrocytes from blood deposits and/or from haemopoietic tissues into the blood stream.

Lal et al (1986) also observed a significant increase in total erythrocyte count and haemoglobin content alongwith significant decrease in spleno-somatic index in *Heteropneustes fossilis* exposed to sublethal concentration of malathion for 8days. Study by Narendra Singh and Srivastava (1994) reported that treatment of *H.fossilis* with the insecticide formothion for 10days evoked significant rise in total erythrocyte count and haemoglobin content. He reported that a probable cause of haematological alterations might be due to the reason that fish are compensating for impaired oxygen uptake by release of erythrocytes from the spleen. Ramalingam et al (1992) have reported depressed tissue respiration in *Sarotheredon mossambicus* exposed to organophosphate insecticide. A state of stress might be associated with an increased secretion of catecholamines (Nakano and Tomlinson, 1967), which have been shown to cause polycythemia by contracting and partly emptying the spleen (Larsson, 1973).

An increase in WBC count was observed after 7days of exposure to the insecticide. This is expected to be to provide a defense mechanism against the stress response. Highly increased number of WBCs in pesticide treated groups compared to control might be due to increased rate of phagocytosis after a 7days of pesticide exposure. Chlorpyrifos, since it is hydrophobic, can bind with the membranes of WBCs, in the same way as it binds with every cell membrane. This binding might have led the WBCs to perform its function. Hence WBCs might have involved in engulfing the foreign substance. Thus the phagocytes might have involved in removing cell detritus. But failure to prevail this action brings an insignificant slight decrease in WBC count was observed after 21 days of exposure to chlorpyrifos.

The reactive species of a xenobiotic may bind to a protein, modifying it and altering its antigenicity. The xenobiotic is said to act as a hapten, ie, a small molecule that by itself does not stimulate antibody synthesis but will combine with antibody once formed. The resulting antibodies can then damage the cell by several immunologic mechanisms that grossly perturb normal cellular biochemical processes (Robert, 1990). According to Svobodova et al (1994), a decrese in leucocyte count and changes in differential leucocyte count are very characteristic findings in fish after toxic exposure to different pollutants. These changes lead to a decrease in the non-specific immunity of affected fish.

But a reduction in WBC count after 3 weeks exposure to chlorpyrifos in higher sublethal concentrations might have damaged the WBCs and the leukopoietic tissues. This might have reduced the number of WBCs and the organism might have suffered from impaired immunological response. This observation is in consonance with the investigation of acute toxicity of chlorpyrifos-methyl on Nile tilapia larvae by Chindah (2004). He observed a progressive reduction in the number of leukocyte and erythrocyte of the fish. The reduction in leukocyte and erythrocyte was significant indicating that the fish became anaemic.

In a study by Holladay (1996), Nile tilapia exposed to the organophosphate insecticide chlorpyrifos, at a concentration of 1μg/L displayed significantly lower total pronephros cell counts than did untreated controls. In addition, macrophages isolated from the pronephros of the treated fish had depressed phagocytic function relative to control fish.

This leukopenia may arise owing to increased activity of the inter-renal stress axis. Reduction in the number of circulating leukocytes is a response of fish to increased level of circulating ACTH and corticoid stress hormone (Srivastava and Agarwal, 1977). Significant reduction in the number of leukocytes has been reported in golden shiners (*Notemigonus crysoleucas*) and *Clarias batrachus* exposed to parathion and malathion respecticvely (Butler et al, 1969; Mukhopadhyay and

Dehadrai, 1980). Leukocytes combat against any toxicant introduced in the blood stream, both erythrocytopenia and leukopenia have developed due to exposure of formothion to catfish in a study by (Narendra Singh and Srivastava, 1994). On the other hand, Tovassoli (1975) reported that insecticides affect nervous system and since neural elements are associated with blood vessels and stroma haemopoiesis may also be affected.

Sublethal concentrations of malathion, an organophosphate insecticide, caused hematological and histopathological damage to channel catfish (*Ictalurus punctatus*). According to Areechon and Plumb (1990), deformities were found in the vertebrae. Blood samples showed increase in numbers of erythrocytes and decrease in numbers of leukocytes.

Gromysz-Kathowaska et al. (1985) reported a decline in haemoglobin, haematocrit (PCV) and RBC count by an organophosphorus insecticide in Japanese quail. This reduction can be related to the decreased RBC number, that indicates haemolysis, haemorrhage and reduced erythropoiesis in fishes on exposure to insecticide.

From the studies on *O.niloticus*, Girben-Perez (2006) reported that chlorpyrifos does not have any effect on number of red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration. However, the phagocytic index and the percentage of phagocytic active cells were significantly affected, which could indicate that phagocytic parameters from Nile tilapia are more sensitive than hematological ones to assess the effect of acute intoxication with chlorpyrifos.

Qualitative assessment of electrophoretic pattern of serum proteins revealed the reduced intensity of some protein bands after prolonged exposure of 21days to the insecticide chlorpyrifos. The insecticide exposure for a short-term period of 7days did not make much difference in the electrophoretic protein pattern from

8.2ppb, and 16.4ppb whereas the 66,000Da protein band was found to be with reduced bandwidth. The intensity in thickness of the bands between 2,05,000Da and 66,000Da appeared to be decreased in 16.4ppb and 27.33ppb pesticide treated groups when compared to the protein bands from control after a long-term exposure to the insecticide. This might be due to the inhibitory effect of the insecticide on the protein anabolic actions. The insecticide might have altered the functional conformations of the structural proteins in the cells and tissues. This is expected to result in the denaturation of these high molecular weight proteins. This might have impaired the normal metabolic processes. Similar but a few studies have been carried out to reveal the effect of pesticide on piscine serum electrophoretic pattern.

Similar trend was reported in the serum proteins of *Channa punctatus* under chronic exposure to organophosphorus and organochlorine insecticides in study by Sahai (1990) and by Ravinder et al. (1989) in the catfish *Clarius batrachus* exposed to Desis 2.8 E C.

The effect of carbaryl and BHC on blood serum protein of *Channa punctatus* and *Channa striatus* was repotrted by Thakur and Sahai (1989). Studies on blood serum proteins of *Channa punctatus* and *Channa striatus* assessed by electrophoretic techniques were reviewed by Sahai (1990). According to his report, on treatment with pesticides the number of protein fractions decrease, some completely disappear and sometimes new fractions appear. A comparative electrophoretic study on the tissue proteins of some catfishes was carried out by Hussain and Siddiqui (1974). El-Gendy et al. (1998) analyzed the blood serum of *Tilapia nilotica* electrophoretically for protein components. The total protein content of serum treated with two pesticides (edifenphos and glyphosate) was found to be decreased on different time periods, compared with control.

Tripathi and Shukla (1990a) demonstrated electrophoretic pattern by performing SDS-polyacrylamide gel electrophoresis of the cytoplasmic protein fractions of the liver and the skeletal muscle of *Clarias batrachus* exposed to

endosulfan and methyl parathion for 28days. The appearance of new protein bands at different time intervals after the exposure of the pesticide demonstrated clearly the alterations in the cytoplasmic protein pattern. These changes in the protein pattern in response to exposure to pesticides were expected to be attributed to the changes in the turnover (synthesis /degradation) of various proteins.

In a study by Arockia et al (2007), the fishes (*O. mossambicus*) were exposed to various concentrations of the carbamate pesticide methomyl for different durations revealed a definite pattern of variation in protein fractions. The fishes were exposed to lethal and sublethal concentrations of 96hr LC_{50} of methomyl for 15, 30 and 45 days, and tissues like brain, kidney, liver and muscle were dissected out and subjected to SDS-PAGE electrophoresis. The protein profile from every tissue showed wide variations from respective controls and the number of bands at particular regions were found as reduced in number and the reduction in number of bands increased as the concentration of pesticide increased. The least number of protein bands obtained on exposure to lethal concentration of pesticide for 96 hr.

Studies on *Clarius batrachus* under sublethal Malathion exposure revealed variations in serum proteins, this may be due to the alterations of protein mobility by Malathion binding (Mukhopadhyay and Dehadrai, 1980). The polyacrylamide gel electrophoretic study of protein fractions in the muscle of Tilapia irradiated and treated with malathion, endrin, sodium arsenate and mercuric chloride revealed striking differences in number, mobility and density of bands in muscle proteins compared to the control indicating that there are considerable inactivation of genetic loci for protein synthesis (Manna and Mukherjee, 1986).

During initial exposure to a xenobiotic, the first component, the concentration gradient between the environment and the fish, will be equal to the aqueously dissolved concentration of the xenobiotic in the environment. This will persist for some time because, immediately after entering the blood, lipiphilic compounds will dissolve in lipids and bind to proteins (Schmieder and Henry, 1988). Thus in the

present study, the reason for the loss of protein bands are expected to be due to the damage caused by the binding of chlorpyrifos with the proteins and the protein synthetic machinery, affecting adversely the anabolic reactions.

Earlier studies by Ramalingam and Ramalingam (1982) reported that the protein is likely to be converted to soluble proteins, which is an induction of change in protein metabolism. The breakdown of protein was confirmed qualitatively by micro-electrophoresis. All the regions at 24hr showed reduction in both high and low molecular weight proteins whereas at 48, 72 and 96hr an increase in protein fractions was observed suggesting the dissociation of high molecular weight proteins.

Electrophoretic pattern of serum proteins showed eight fractions in *C.punctatus* and seven fractions in *C.striatus* (Dhar and Chatterjee, 1984). According to them, on treatment with pesticides the number of protein fractions decreased and some completely disappeared and sometimes-new fractions also appeared. In the present investigation the reduction in the intensity and number of protein bands in serum subjected to different sublethal concentrations of chlorpyrifos was observed. This might be due to proteolysis of the serum proteins and the damage to the protein synthetic machinery.

The effect on BHC, an organochlorine pesticide on the blood serum protein profile and other haematological parameters like RBC count, WBC count and Hb content in an air breathing fish, *Channa punctatus* were investigated by Thakur and Sahai (1987). They suggested that biochemical studies like electrophoretic estimation of serum constituents like protein are very important in assessing the pesticide-induced stress in fish. The marked reduction in the serum protein fractions, RBC, WBC and Hb concentration is a good indicator, which might help, in the early detection of pollution by pesticides.

Chapter

7

Effects of chlorpyrifos on morphology of tissues in *Oreochromis mossambicus*

Contents

7.1 Introduction

The study of structural damage of organs or tissues is an integral part of pollution toxicology. Apart from haematological and biochemical effects pathoanatomical and histopathological changes in fish tissues and pollutant accumulation in fish are also investigated as part of the toxicity test procedure. Histopathology is an effective tool to visualize the stress-induced structural changes in cells and tissues.

Organisms have tremendous capacity to overcome the environmental stress conditions and thus to maintain the homeostasis. Cells, which have reached their limit of adaptability, begin to show structural changes, which indicate their failure to withstand the changed environment. If adverse conditions persist or if the initial pathological stimulus is severe, then these processes continue and progress into a sequence of events leading to cell death (Varanasi et al, 1989; Jehosheba, 2004). The extent of severity of tissue damage of a particular compound as toxicant depends on its toxic potentiality in the tissues of organisms (Murthy, 1986). Various chemicals with their varied mode of action in different tissues bring about certain architectural changes ultimately culminating in either death of the organism or making the organism less viable for survival.

The methods of established value in the evaluation of environmental impact at the organ and cellular levels are histology and cytology (Braunbeck et al, 1990). Histopathological techniques are rapid, sensitive, reliable and comparatively inexpensive tools for the assessment of stress-response to pollutants. Hence an attempt has been made to observe the possible structural changes in gill, liver and brain tissues of the teleost fish.

Gill is the major route of entry of any pollutant by virtue of its immediate contact with the medium. Uptake of xenobiotics from water by fish is determined by numerous factors, the most important of which are the transfer capacity of the gills and the physico-chemical properties of the compound. According to Murthy (1986)

respiratory distress is one of the early symptoms of pesticide poisoning and gills take part in metabolism and elimination of xenobiotics.

Liver, the first organ to face any foreign molecule through portal circulation is subjected to more damage (Jayantha Rao, 1982). The parenchymatous hepatic tissue has many important physiological functions and also detoxication of endogenous waste products as well as externally derived toxins, drugs, heavy metals and pesticides (Roberts, 2001a). Fish liver is particularly susceptible to chemical damage.

In the teleost nervous system, pathological changes related to neurons and their processes are only occasionally observed, and it is in the meninges and blood vessels, and the microglia, that the principal changes associated with neural dysfunction are normally observed. Brain plays a regulatory role in fish physiology and it is the most important organ in fish toxicology especially when pesticides are involved in their mode of action in the nervous system (Ware, 1983).

Hence the sublethal effects of chlorpyrifos on morphology of gill, liver and brain tissues from *O.mossambicus* were studied

7.2 Materials and methods

Collection, maintenance of fish and feeding were done as described before in the section 3.2.B.

A group of 6 fishes without sex determination, in duplicate were exposed to 27.3ppb (1/3 sublethal concentration of LC_{50} value) for observing the histopathological changes. The test solution and the water in which fishes were maintained, were renewed daily. Fishes were randomly selected from control groups and treated groups, for histopathological observations by sampling after 21 days of pesticide exposure with the sampling after 7 days also.

The tissues were collected by dissecting the fishes and transferred to 10% neutral formol saline immediately and kept for fixation for 24hours. These tissues

were dehydrated in ascending graded series of alcohol and were cleared in xylene until they became transluscent. Then the tissues were transferred to molten paraffin wax for 1 hour to remove xylene completely and impregnated with wax. Then the blocks were cut in a microtome to prepare sections of thickness 3 to 5 microns. Wax was removed in xylene and then cleared in descending graded series of alcohol and then brought to water. The sections were stained with haematoxylin and eosin and mounted in DPX (Roberts, 2001b) and observed under light microscope.

7.3 Results

The results of histological studies of gill, liver and brain tissues of *O.mossambicus* of control and actone control, and those exposed to sublethal concentration of chlorpyrifos are given in plates 3-23. The structural alterations were observed under light microscope in the sections of gill, liver and brain tissues of fish from treated group. The tissues of fishes from chlorpyrifos-treated groups appeared in a structure different from those of control group fishes.

The gill tissue of the control was observed as shown in plate 3. Generally, the gills of *O.mossambicus* comprised two sets of four holobranchs, forming the sides of the pharynx. Each holobranch consisted of two hemibranchs projecting from the posterior edge of the branchial arch or gill arch in such a way that the free edges diverged and touched those of the adjacent holobranchs. Close examination of the hemibranchs of a fresh gill shows that they consist of a row of long thin filaments, the primary lamellae, which project from the arch like the teeth of a comb. The surface area of each primary lamellae is increased further by the formation of regular semi lunar folds across its dorsal and ventral surface-secondary lamellae. The dorsal and ventral rows of secondary lamellae on each primary lamellae are staggered so that they complement the spaces in the rows of lamellae of adjacent filaments.

Gill tissue of fish exposed to acetone control is given in plate 21. It appeared in its intact architecture, similar to that of control. The changes observed in the gill tissue on exposure to chorpyrifos for 7 days (Plate 4&5) included hyperplasia of

Plate -3

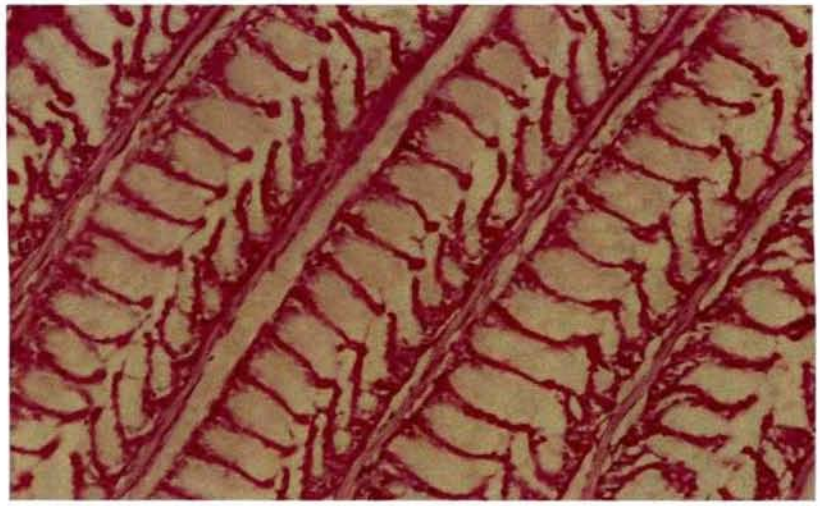


Plate -4

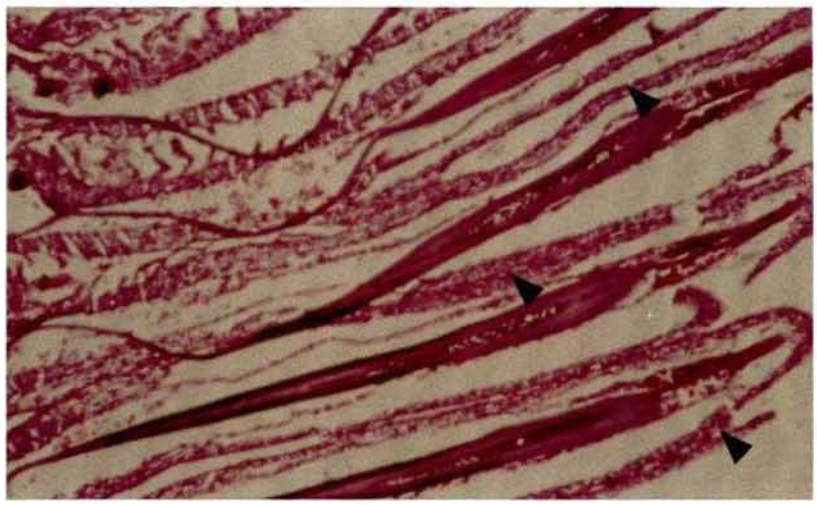
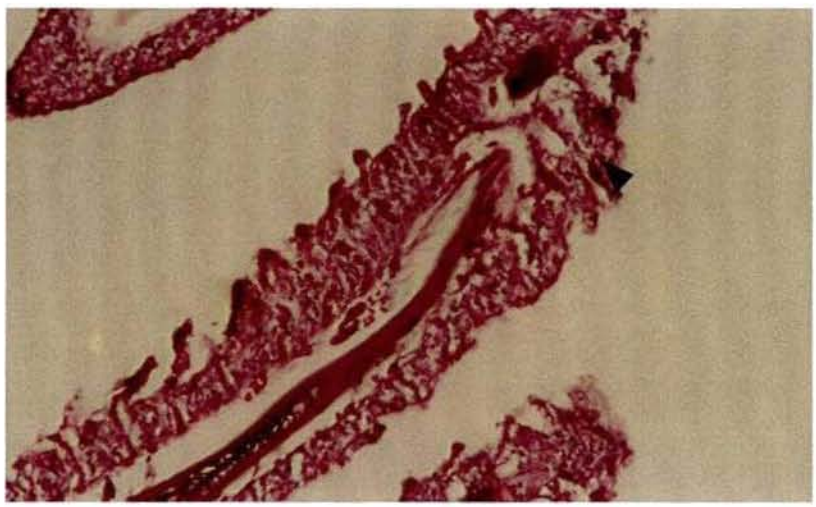


Plate -5



Light photo micrograph of cross-section of gill tissue of *Oreochromis mossambicus* showing histopathological effects of technical grade chlorpyrifos
Plate 3:Control (20x)
Plate 4: exposed for 7days showing desquamated and necrosed areas(arrowed)(20x)
Plate 5:exposed for 7days showing secondary lamellae clubbed (arrowed)(20x)

Plate -6

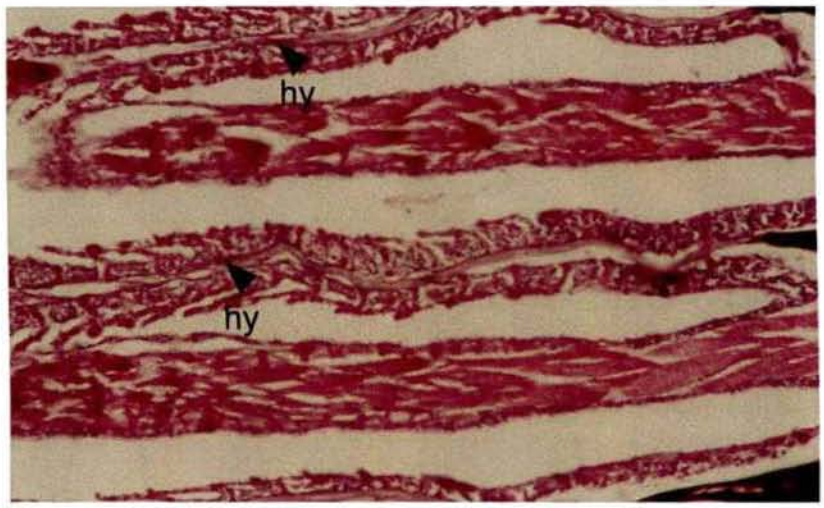


Plate -7

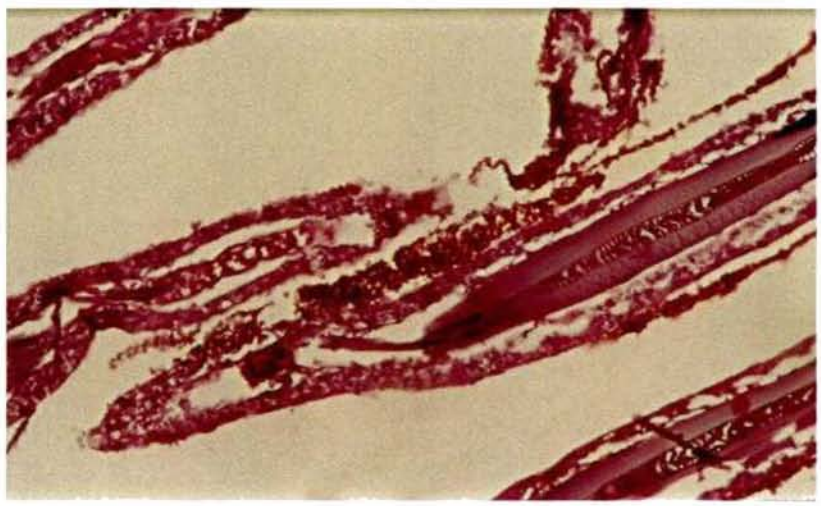
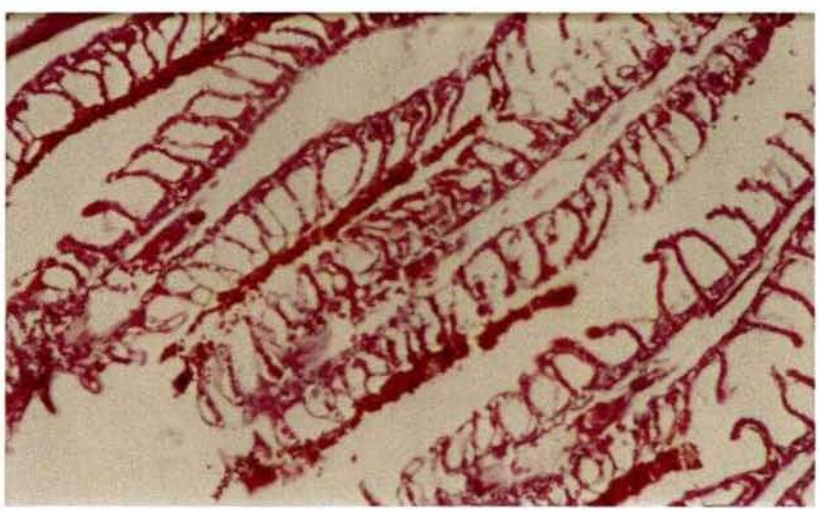


Plate -8



Light photo micrograph of cross-section of gill tissue of *Oreochromis mossambicus* showing histopathological effects of technical grade chlorpyrifos exposed for 21days
Plate 6: hy-hyperemia(20x)
Plate 7: loss of architecture (20x)
Plate 8: completely necrosed and desquamated gill filaments(20x)

Plate -9

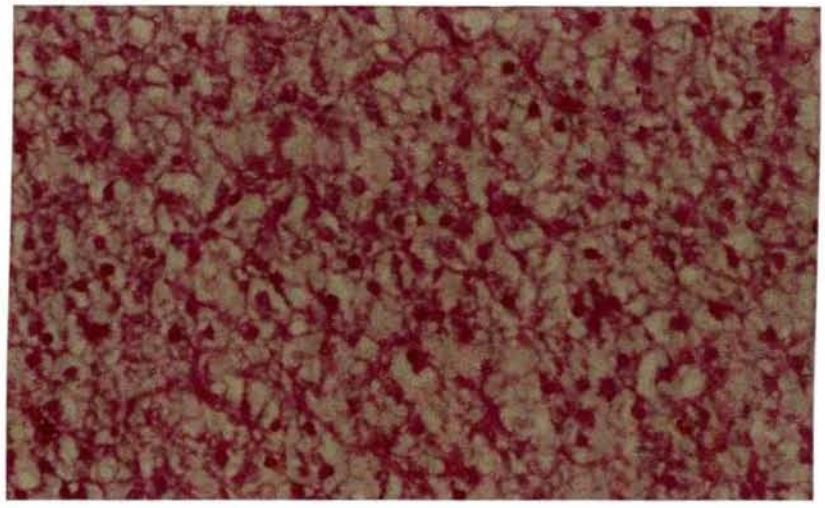


Plate -10

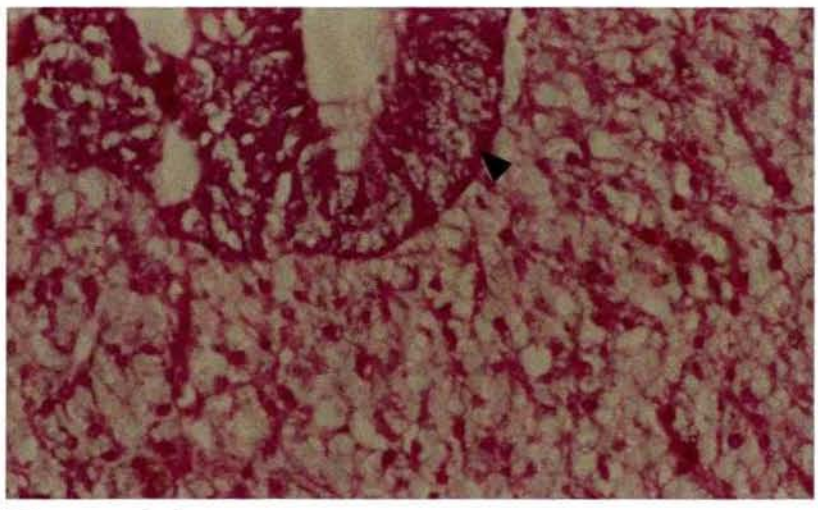
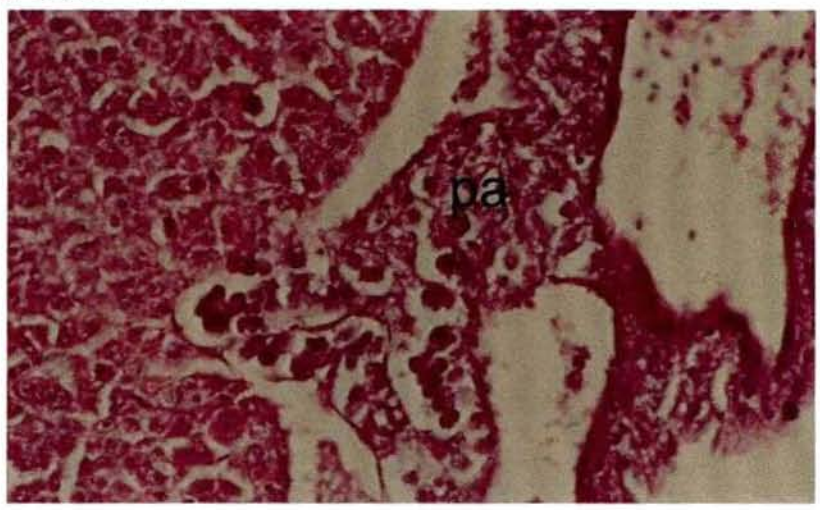


Plate -11



Light photo micrograph of cross-section of liver tissue of *Oreochromis mossambicus* showing histopathological effects of technical grade chlorpyrifos
Plate 9: Control (40x)
Plate 10: Control showing hepatopancreas (arrowed)(40x)
Plate 11: exposed for 7days showing swelling, rounding off and detachment of cells; pa-abnormally structured acinar cells showing necrosis of pancreas (40x)

Plate -12

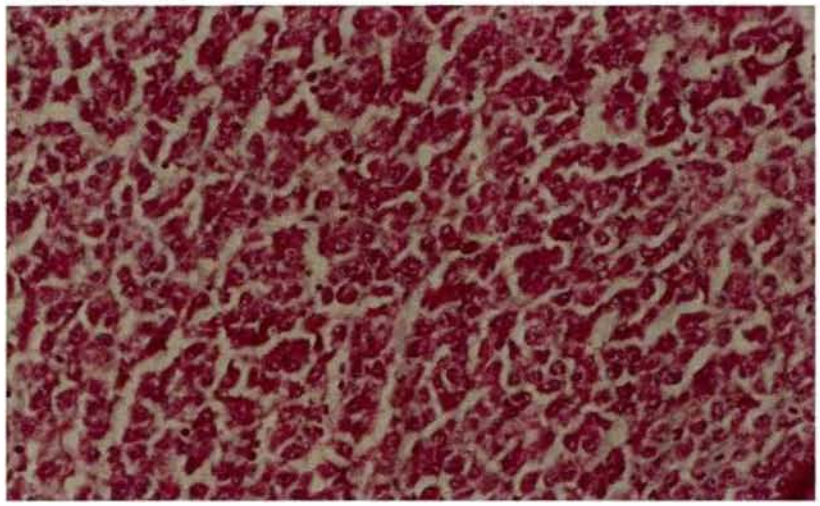


Plate -13

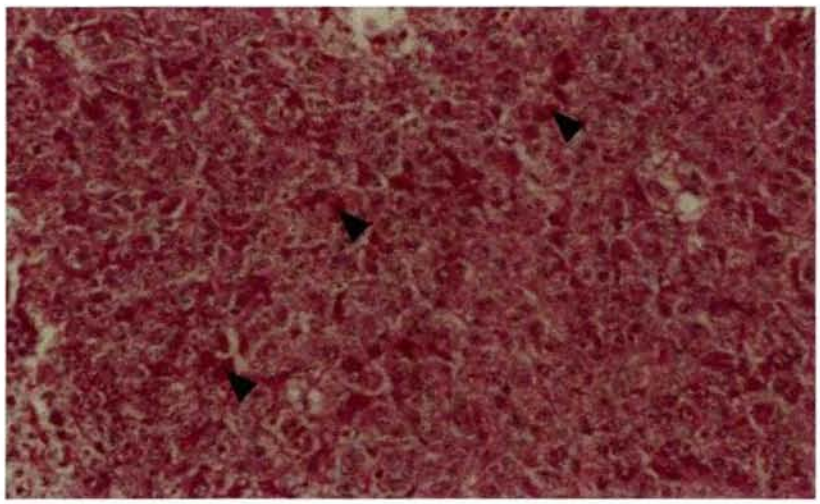
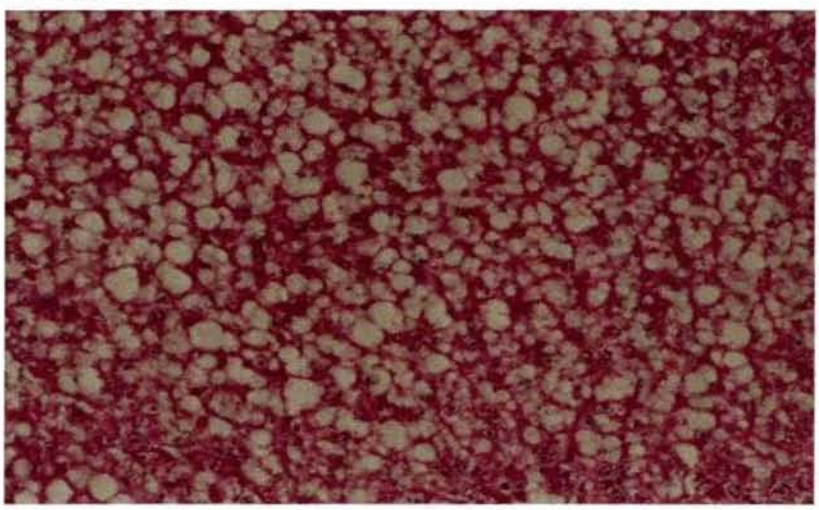


Plate -14



Light photo micrograph of cross-section of liver tissue of *Oreochromis mossambicus* showing histopathological effects of technical grade chlorpyrifos
Plate 12: exposed for 7 days showing rounding off and detachment of cells (40x)
Plate 13: exposed for 21days showing regenerating cells (arrowed)(40x)
Plate 14: exposed for 21days showing vacuolated areas with fat deposition(40x)

Plate -15

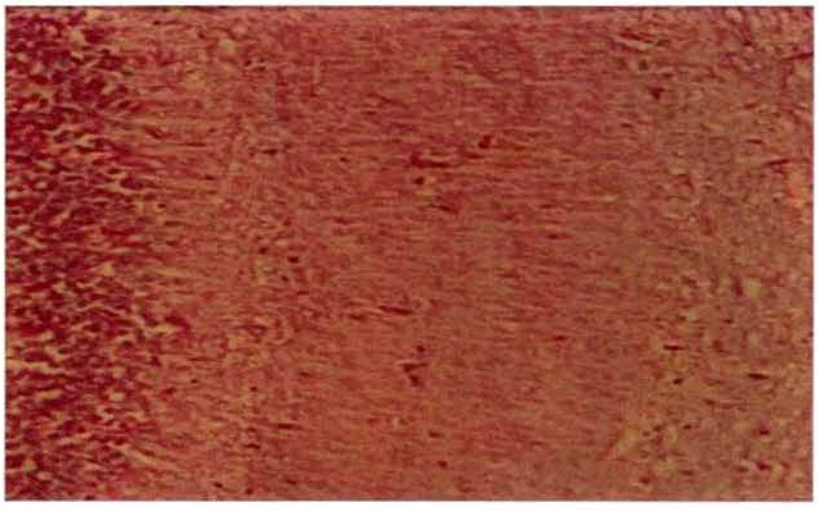


Plate -16

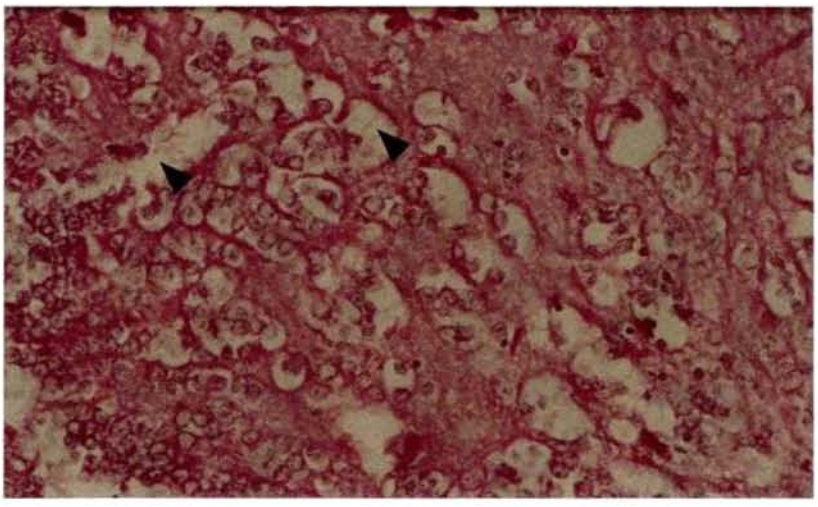
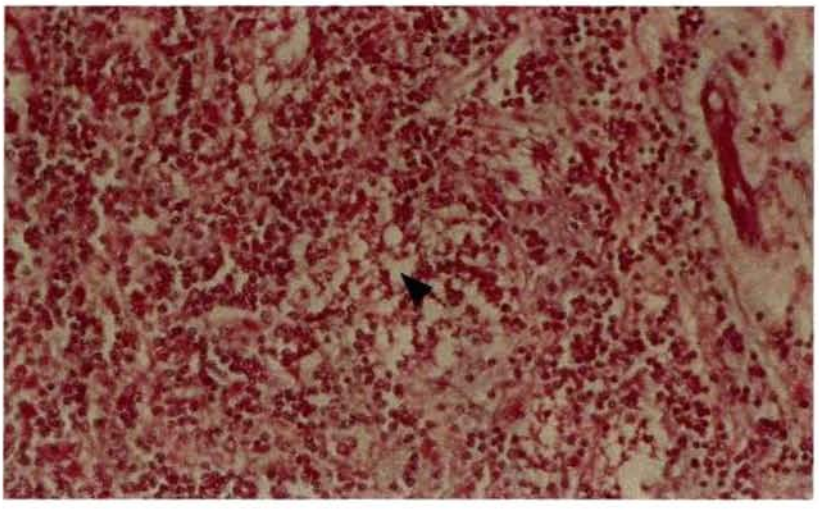


Plate -17



Light photo micrograph of cross-section of brain tissue of *Oreochromis mossambicus* showing histopathological effects of technical grade chlorpyrifos
Plate 15: control (20x)
Plate 16: exposed for 7 days showing vacant areas of degenerating neurons (arrowed)(40x)
Plate 17: exposed for 7 days showing areas of degenerating neurons (arrowed)(40x)

Plate -18

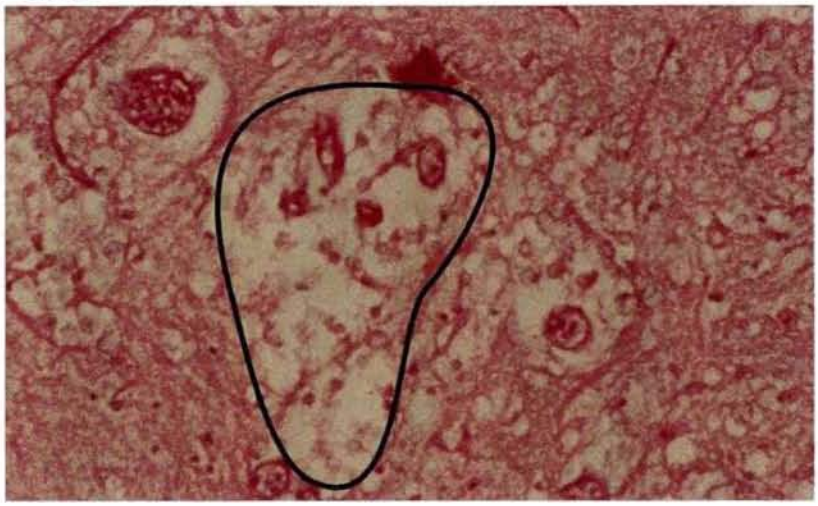


Plate -19

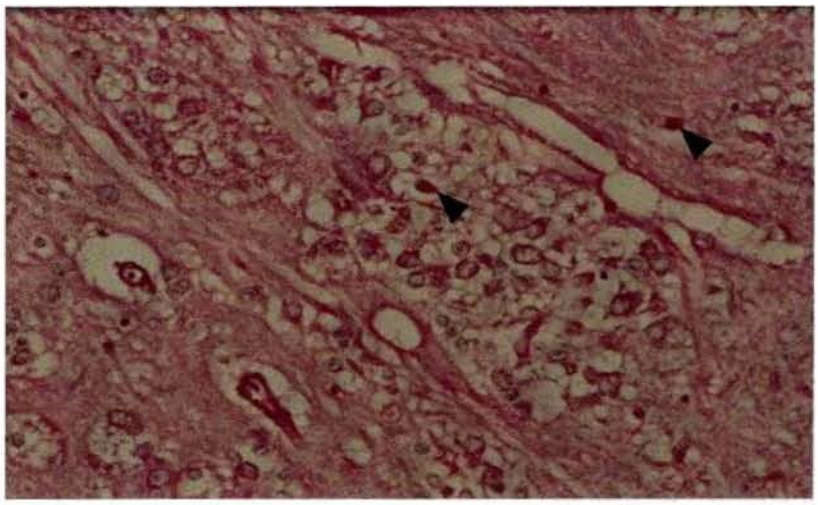
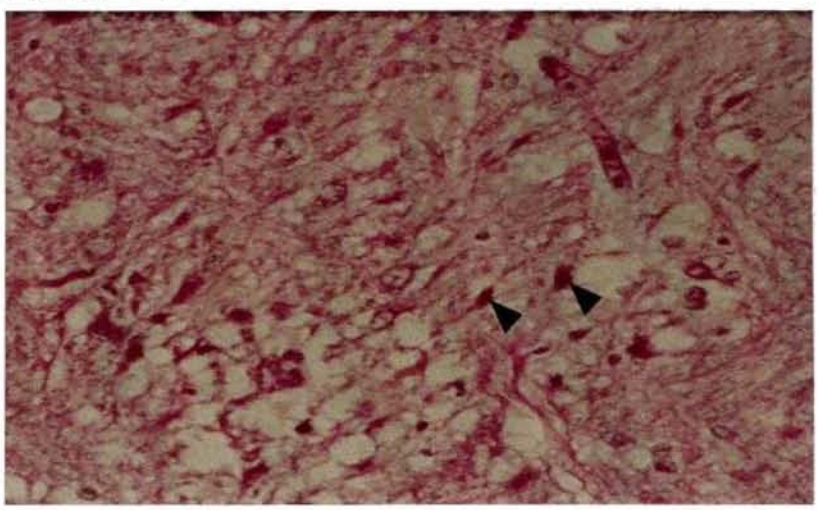


Plate -20



Light photo micrograph of cross-section of brain tissue of *Oreochromis mossambicus* showing histopathological effects of technical grade chlorpyrifos
Plate 18: exposed for 21 days showing encephalomalacia (marked)(40x)
Plate 19: exposed for 21 days showing demyelinated areas and pycnotic nuclei (arrowed)(40x)
Plate 20: exposed for 21 days showing demyelinated areas and pycnotic nuclei (arrowed)(40x)

Plate -21

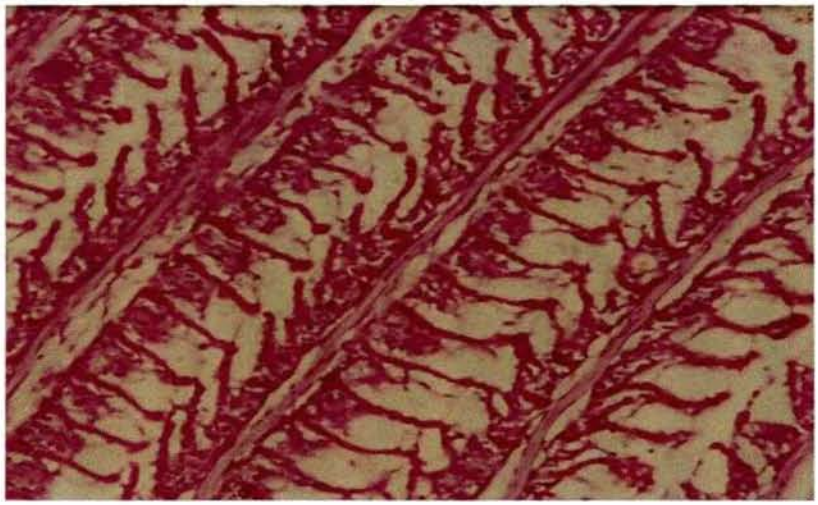


Plate -22

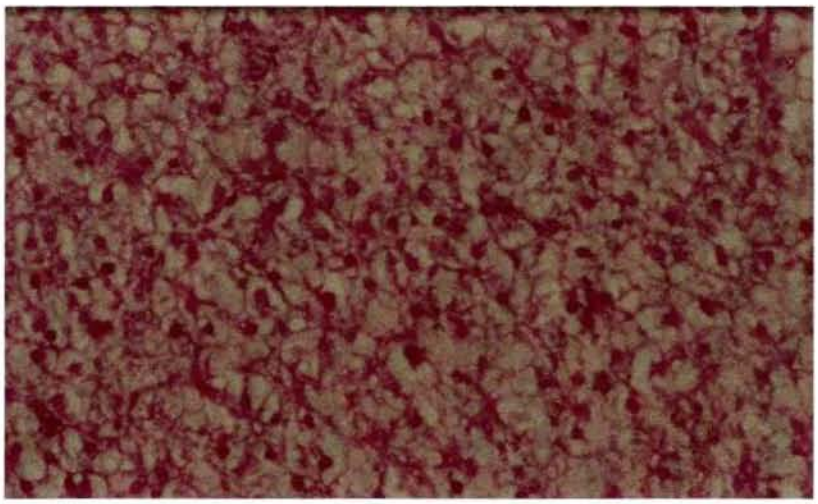
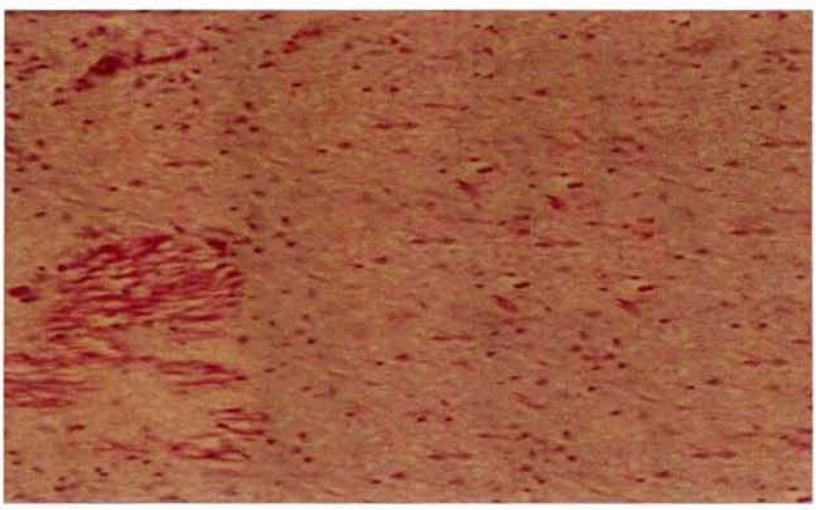


Plate -23



Light photo micrograph of cross-section of tissues of *Oreochromis mossambicus* showing histopathological observation
Plate 21: gill tissue exposed to acetone for 21 days (20x)
Plate 22: liver tissue exposed for 21 days (40x)
Plate 23: brain tissue exposed for 21 days (40x)

epithelial lining of secondary lamellae leading to their fusion and clubbing. Many epithelial cells appeared necrosed and desquamated from the mucosa.

The changes observed in the gill tissue on exposure to chorpyrifos for 21 days (Plate 6,7&8) included hyperemia indicated by engorged capillaries. Complete loss of architecture of gill filaments was observed. Completely necrosed and desquamated gill epithelial cells and increased mucus production were also seen on prolonged exposure to chlorpyrifos.

The liver tissues of pesticide treated fishes showed structural alteration unlike those from control group (Plate 9 &10). Generally, the liver in teleost fish is a compound organ in the form of hepatopancreas. Sinusoids, which are irregularly distributed between the polygonal hepatocytes, are fewer in number and are lined by endothelial cells with very prominent nuclei. Hepatocytes are polygonal and have a distinctive central nucleus with densely staining chromatin margins and a prominent nucleolus.

The liver tissue of fish exposed to acetone (Plate 22) appeared in regular network pattern of functional cells. The changes observed in the liver tissue on exposure to chlorpyrifos for 7days are shown in Plates 11&12. The changes included swelling and rounding off of hepatocytes, detachment of cells from each other. Pancreatic acini appeared to have lost its architecture. Cytoplasm of hepatocytes became more basophilic.

The changes observed in the gill tissue on exposure to chorpyrifos for 21 days are shown in Plate 13&14. The cells had distinct vacuoles in cytoplasm indicating fatty change. Necrotic areas appeared prominent. Hyperplasia or regenerating hepatic cells at certain regions were observed.

Brain tissue from tilapia is generally as shown in Plate 15. In the brain of fishes, five major regions are distinguished. They are telencephalon, diencephalon, mesencephalon, metencephalon and mylencephalon. In fishes, the roof of the telencephalon is covered with membranous tissue and lateral ventricle does not exist.

The diencephalon is the region that contains the third ventricle and is composed of the distinct components- epithalamus, thalamus and hypothalamus. The epithalamus consists of pineal body and the habencular nuclei, which connects with thalamus. The hypothalamus is more readily defined and usually relatively large in fishes. It appears to comprise mainly nuclei responsible for coordination of forebrain stimuli and lateral line impulses. The mesencephalon is relatively large and anatomically subdivides into the optic tectum, which provides the roof of the third ventricle, and the tegmentum, which is its floor. It contains the center of the visual sense, as well as the integration center between this sense and the other senses of locomotion.

The cerebellum or metencephalon occupies the interior portion of the dorsal wall of the fourth ventricle and is composed of a cortex and medulla. The metencephalon is the integration center between the auditory sense and the sense of the lateral line.

The brain tissue of fish exposed to acetone (Plate 23) did not show any deterioration in its structure and appeared in a structure similar to that of control. Exposure On exposure to chlorpyrifos for 7days, the major change observed in brain tissue was degenerating neurons, vacant areas (Plate 16&17). Prolonged exposure to chlorpyrifos (Plate 18, 19&20) resulted in encephalomalacia. Presence of pycnotic nuclei with demyelinating neurons was also observed.

7.4 Discussion

Cellular responses to pollutant-induced sublethal injury provided highly sensitive indicators of environmental impact (Hose et al., 1996).

The gills are among the most delicate structures of the teleost body. Gill is a multifunctional organ involved in gaseous exchange, acid base balance, transport of Na^+, Ca^{2+}, Cl^- and nitrogenous secretion (Perry, 1997). Morphological studies have provided further support for the idea that opecular membranes and skin are excellent models for branchial salt secretion (Foskett et al, 1982). Their external location and intimate contact with the water make them liable to damage by any irritant materials,

whether dissolved or suspended, in the water. The most frequently observed changes when the gill filaments come in contact with chlorpyrifos-an irritant-is swelling of lamellar epithelial cells or edema of the subepithelial space.

Lamellar edema is most frequent following the exposure to chemical pollutants such as heavy metals, red tides and certain pesticides (Roberts, 2001a). Increased mucus secretion renders a defense mechanism to the stress by the pesticide but the same fails when it is exposed for a prolonged time.

Hyperplasia was generally more pronounced towards the distal tip of the lamellar filament resulted in clubbing. As the time of exposure to chlorpyrifos increased, the different lamellar filaments became desquamated and completely lost their architecture indicating its failure to overcome the stress. Hyperemia with excess blood in the vessels supplying gill filament was observed with increased number of RBCs. This observation can be correlated with the increased RBC count in haematological examination, after 21days of exposure of fish to chlorpyrifos.

Gill is the first organ that comes in direct contact with the insecticide. Binding of hydrophobic organophosphate with various lipid and protein groups of gill epithelial cells might be the reason for altered stucture of gill filaments. In the present study, on exposure to chlorpyrifos, morphological observation can be correlated with the inhibition of ATPase enzymes involved in ion transport mechanism in microsomal preparation of the gill epithelial cells. Loss of structural integrity of the gills may easily lead to a drop in the concentration of blood electrocytes, such as sodium, chloride and calcium. Mallatt and Stinson (1990) have described that electrolyte loss occurs after exposure of freshwater fish to pesticides.

These findings are in good agreement with the reports of Rao et al (2003) and Jauch (1979). Rao et al. (2003) observed the bulging of secondary lamellae at the terminal ends, lesions and erosions at the base of lamellae on 12^{th} day of exposure of *O.mossambicus* to chlorpyrifos. A thick coat of mucus on the gill filaments was found to be persisting on 18^{th} day of exposure. According to Jauch (1979), fenthion

upon 96-hr exposure induced gill lesions, including hyperplasia and desquamation of the epithelium and thrombosis in the secondary gill lamellae.

A study by Gopalakrishnan (1990) elucidates the effect of organophosphorus pesticide Dimecron on the gill and liver of the fish *Etroplus*. Dimecron induced branchial congestion in the gill filaments. Edematous fluid lifted the respiratory epithelium in a few secondary lamellae, which were found thickened. The cells between the secondary lamellae were thickened to such an extent that the inter-lamellar spaces occluded, which gave the filament a compact appearance.

Epithelial hyperplasia with lamellar fusion, epithelial hypertrophy, telangiectasia, edema with epithelial separation from basement membranes, general necrosis and/or epithelial desquamation have reported following exposure to DDT and malathion (Walsh and Ribelin, 1975) and exposure to paraquat dichloride (Hendricks, 1979).

Liver tissue after 7days of exposure showed swelling and rounding off of hepatocytes and the starting of detachment of cells from each other. The pancreatic acinar cells appeared to loss the normal architecture. They found to be detached from the surrounding hepatic cells. The cytoplasm of the cells became more basophilic indicating the protein precipitation leading to the non-functional condition of hepatic tissue. On prolonged exposure to chlorpyrifos, vacuolated areas seen might be indicating fatty changes. This may subsequently lead to fibrotic changes. Hyperplasia observed at certain regions might be the feature of regenerating hepatic tissue. This might be the reason for slightly elevated ALT and ALP levels in hepatic tissue after 21 days of exposure to chlorpyrifos. These damages can result from a wide range of stimuli, from long-standing biliary obstruction, heavy metal or pesticide poisoning. In aquatic system, fish liver ultrastructure has proved to be particularly susceptible to low levels of environmental contaminants (Braunbeck et al, 1990).

Sudha Singh et al. and Tilak et al have also reported these changes. A Study by Sudha Singh et al. (1998) reported clubbing, vacuolation and also necrosis of

pancreatic tissue by the exposure of endosulfan and carbaryl on *Nandus nandus.* Intercellular spaces and spaces around pancreatic mass were also seen. They observed that after a long-term exposure of both endosulfan and carbaryl, the liver tissues were converted into spongy mass. The pancreatic tissues were seen shrunken and scattered due to heavy necrosis of hepatic mass.

Tilak etal. (2005) observed the similar changes in liver of *Catla catla.* The pathological changes included degeneration of cytoplasm in hepatocytes, atrophy, formation of vacuoles, and rupture in blood vessels, necrosis and disappearance of hepatocyte cell membrane disposition. Hepatic cords are found to be decreased in size and nucleus became pyknotic.

Radhaiah and Jayantha Rao (1992) reported moderate cytoplasmic degeneration in hepatocytes, formation of vacuoles, rupture in blood vessels and appearance of blood vessels among hepatocytes and pyknotic nuclei in the hepatic tissue of *T.mossambica* exposed to fenvalerate.

In the present study, brain tissue, after short-term exposure to chlorpyrifos, the vacant areas appeared due to the degenerating neuron as shown in Plate 16 &17. Encephalomalacia observed on prolonged exposure to chlorpyrifos, might be because of these degenerating and demyelinating neurons in the damaged brain tissue under toxic stress induced by chlorpyrifos. The present observations are in consonance with those from the studies by Tilak et al (2005). Since chlorpyrifos is an organophosphorus compound and organophosphates are neurotoxins, the chlorpyrifos intoxication caused chromatolysis, ie. Dissolution of the nissel bodies.

The orgnaophosphorus compounds are hydrophobic in nature; they have more affinity towards membrane lipids. The reactive species of xenobiotics produced by metabolism, bind to cell macromolecules including DNA, RNA and protein (Robert, 1990). In the present study, affinity of chlorpyrifos to the membrane lipid might have favoured it to pass across the blood-brain barrier. Further interaction of the insecticide with the neuronal cell membrane and cellular organelles might have led to their degeneration. Chlorpyrifos might have bound with various

lipid and protein groups making their metabolism impaired. Myelin sheath was seen as damaged. The degenerated neuronal cells might have been replaced by vacant areas resulting in encephalomalacia in brain tissue. Recent investigations have provided further evidence that organophosphorus insecticides, besides their typical action as inhibitors on AChE, interfere with the allosteric behaviour of the enzyme through interaction with the membrane lipids (Domenech et al., 1977).

Tilak et al (2001a) observed similar changes in *Labeo rohita* under chlorpyrifos toxicity. (Yacobu, 1999) reported swelling of axon, atrophy, necrosis and pyknotic nuclei in the brain tissue of the fish *Ctenopharyngodon idellus* under fenvalerate toxicity and severity of damage is more in lethal exposures than in sublethal exposure. Chlorpyrifos technical grade caused more degenerative changes in brain than in 20%EC exposure.

The present study revealed a visual evidence of sterical derangement in gill, liver and brain tissues of *O.mossambicus* on sublethal exposure to chlorpyrifos. The impairment of the cellular antioxidant defense or increases in the production of highly reactive free radical species may be the primary cause of cellular injury.

Chapter

8

Laboratory investigations on Pesticide residues in *Oreochromis mossambicus* exposed to chlorpyrifos

Contents

8.1. Introduction

Modern agriculture is mainly responsible for polluting soil through the non-judicious use of chemical fertilizers, herbicides, insecticides and fumigants. Pesticides particularly aromatic organic compounds are not degraded and they have long persistance time and exert cumulative effect (Trivedy, 2001).

Pesticide application was often being done without adequate emphasis on the dosage and method of application. Use of optimum quantities of pesticides will increase the bioefficacy of the pesticides. The adverse effects of pesticides observed in the field were caused by the defects in the dosage selected and in the application techniques followed than by the toxicity of the compounds. Farmers were using a wide range of pesticides indiscriminately. This has created undesirable side effects like pest resistance to pesticides, secondary pest outbreaks, resurgence, environmental pollution and residue hazards (Viswanathan, 1987). A survey done by Forensic Science Laboratories in India and recorded by Bami, in 1972, shows that nearly 50% of the human poisoning cases in India are due to pesticide poisoning especially organophosphorus group (Arun, 1984).

The interactions between environmental processes and physico-chemical properties of pollutants determine their distribution, fate and effect on living organisms (Connell and Gregory, 1984). The environmental and toxicological impacts of pesticides are highly dependent not only on the parent compound, but also on their metabolites (Kulkarni and Mitra, 1990).

Heavy and indiscriminate use of pesticides resulted in pesticide poisoning, development of insecticide resistance in pests, resurgence of pests, ill effects on the non-target organisms and contamination of food and the environment including soil, water and air (Singh and Dhaliwal, 1993). Due to various socio-economic factors, these compounds are still widely used in public health in most of the developing countries, though restricted for use in agriculture, due to cheapness and indigenous manufacture of technical grades of the pesticides (David et al., 1993).

The tendency of an insecticide to associate with organic phases in the environment depends on its partition coefficient value. Octanol/water partition coefficients (K_{ow}) express the relative affinity of a compound for organic phase compared to aqueous phase and are considered to be the key parameters in the estimation of environmental partitioning.

To assess the level of pesticide contamination in food commodities, intensive monitoring programmes have been established in many developed countries. In India, the importance of the toxic hazards due to pesticides was realized as early as in 1956 when a scheme on 'Exploration of toxicity hazards in the use of modern pesticides against cattle and human beings' was started at the Indian Agricultural Research Institute (IARI), New Delhi. The actual effective work was strengthened in the fourth five-year plan (1969-74). The investigations were confined to the estimation of pesticide residues in crops and their products. The residue problems along with their hazards associated with manufacturing and handling of pesticides were highlighted by several workers (Majumdar, 1959; Krishnamurthy, 1965; Pradham, 1965) and appropriate measures were suggested to encounter the problems. Four pesticide analytical units at IARI, New Delhi; Central Food Technological Research Institute (CFTRI), Mysore; Central Food Laboratory (CFL), Culcutta and Plant Quarantine Station, Bombay were started during the fourth five-year plan (Lal and Pradham, 1977).

In 1984, All India Co-ordinated Research Project (AICRP,1984) on pesticide residues involving 15 agricultural universities and ICAR institutions was launched by ICAR with the following objectives.

a. To organize and promote balanced use of pesticides in Indian agriculture
b. To monitor pesticide residues in agricultural commodities and in environment to prevent possible misuse/excessive use of pesticides
c. To study the fate of pesticides and their metabolism in tropical environment including stored grains

d. To ensure the supply of quality pesticides to the farmers by organizing and strengthening a program on quality control of pesticides.

Under All India Co-ordinated Research Project (AICRP) on pesticide residues, supervised trials are conducted and the monitoring of pesticide residues is being undertaken in many states. The Indian Council of Medical Research (ICMR) is also undertaking a taskforce study on monitoring and surveillance of food contaminant hazards in India in which the food commodities are monitored for the pesticide residues, heavy metals, aflatoxins etc. (ICMR, 1993).

Organophosphorus and carbamate insecticides are much less toxic to fish populations although significant mortalities may occur with some compounds such as parathion, methyl parathion, diazinon, malathion and chlorpyrifos at larvicidal rates below 1lb $acre^{-1}$ (Connell, 1984).

Chlorpyrifos that has been applied to the soil generally stays in the area where it has been applied because it sticks tightly to soil particles. Because of this, there is low chance that chlorpyrifos will be washed off the soil and enter local water systems. Also, since it does not mix well with water, if it does get into the natural waters, it will be in small amounts and will remain on or near the surface and will evaporate. Once in the environment (soil, air or water) chlorpyrifos is broken down by sunlight, bacteria or other chemical processes (ATSDR, 1997). But the use of chlorpyrifos as mosquito larvicide involved application of formulated product directly to freshwater bodies (Racke, 1993).

Use of chlorpyrifos is recognized as an economic approach to control pests' quality and quantity, but at the same time it can be harmful for many species after consumption of its residues in agricultural products (Rastelli et al., 2002 and Colosio et al, 2005).

Chlorpyrifos, the active ingredient in Dursban and Lorsban, is the most widely used insecticide in the world. In United States, chlorpyrifos is detected more frequently in surface waters than any other organophosphorus insecticide (US, 2000).

The major recipients of surface run-off are streams, lakes and coastal waters. The incidences of toxicity of pesticide residues to fish have been numerous. According to the report of Indian Council of Agricultural Research in 1967, the evidence for pesticidal contamination of water was originally obtained mostly from physiological responses of aquatic organisms like fish. The high susceptibility of fish to many of the insecticides gives an easy clue to the significant contamination of streams (Bindra, 1972).

As an organophosphorus insecticide containing both nitrogen and sulfur, chlorpyrifos lends itself to detection by gas chromatography using either a flame photometric detector (FPD) or a nitrogen phosphorus detector (NPD) (Rogers et al., 2006). Furthermore, with three chlorine atoms, an electron capture detector (ECD) is also an ideal candidate. GC-NPD seems to be the most common means of analyzing OP insecticides, and along with GC-FPD, is the detector of choice by United States Environmental Protection Agency (USEPA) (1986). However, ECD is frequently employed (Barra and Carmi, 1995; Macalady et al, 1985; Pablos et al., 1999) since it is allowed in USEPA Method 8141B for OP compounds, and it is much more common detector.

In connection with chronic toxicity tests, histopathological changes in fish tissue and residue levels of test substances in fish are very important parameters. Thus the level of residues of insecticide chlorpyrifos was investigated.

8.2 Materials and methods

In the present investigation, the residue levels of chlorpyrifos in the edible tissue of fish exposed to the chemical for various periods were studied. The insecticide was extracted from the edible fish tissue, then subjected for clean-up to obtain the insecticide with maximum recovery. This was then concentrated and analysed without delay by gas chromatography. The analysis was carried out at Sophisticated Test and Instrumentation Centre (STIC) associated with Cochin University of Science and Technology.

Chlorpyrifos

Details of reference chemical used in the study is given in **Table No.8.1**

Chemical	Physical appearance	Assay	Source
Chlorpyrifos	White, crystalline	Technical grade, 95%	Hindustan Insecticide Ltd, Kalamassery
Chlorpyrifos	Analytical Standard solution	PEST ANAL® 100ng/µL in acetonitrile	Accu Standard Inc., New Haven, CT, USA

8.2.1 Reagents:-

Since micro-level determinations were carried out, special precaution was taken to prevent contamination.

1. Acetonitrile- AR grade material
2. Hexane- HPLC grade material with melting point
3. Sodium sulphate- AR grade material
4. Florisil- AR grade material
5. Double distilled water
6. Glassware- All items of glassware were thoroughly washed with detergent solution using brush. They were rinsed sequentially with tap water, distilled water and acetone and were oven dried before use.

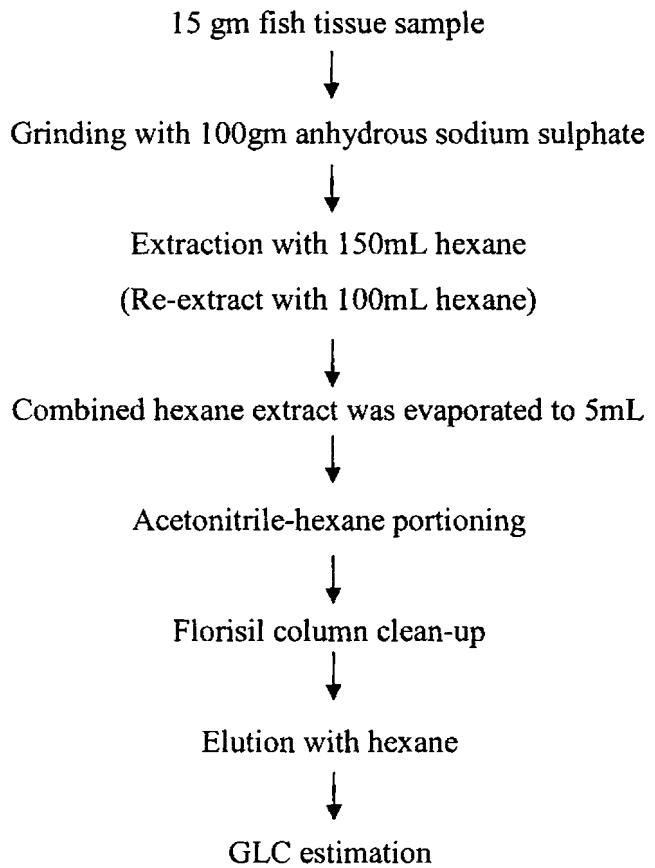
8.2.2 Gas chromatograph (GC)- Computer integrated GC system of Schimadzu Corporation, Koyoto, Japan was used for the study. The specifications of the equipment are given below.

Name : Schimadzu
Model : 2010A
Column Name : DB-1
Type of column : capillary column
Length : 30m

Inner diameter	: 0.25mm
Film thickness	: 0.25microns
Detector	: Electron capture detector (ECD)
Data analysis software	: GC solution

8.2.3 Experiment I- Standardisation of Gas chromatographic method for the determination of chlorpyrifos residues

A method was standardized for the extraction and estimation of residues of chlorpyrifos in edible fish tissue portions using GC. It involved standardization of GC conditions and standardization of extraction and clean-up method. Standard chlorpyrifos solution purchased from AccuStandard Inc. was used for the study.



Flow chart for residue analysis of fish tissue sample

a. Preparation of the sample for Extraction

For the study, collection and maintenance of fishes were performed as described in section 3.2.B. Healthy Fishes were divided into four groups of six fishes in each group. The I group was taken as control without pesticide. II, III and IV groups were exposed to sublethal concentrations of 8.2ppb, 16.4ppb and 27.33ppb respectively for 21 days. Water was renewed and the insecticide in known fixed concentration was added daily. Sampling was done after 7 and 21days of exposure to chlorpyrifos. The edible fish tissue portions from each group of fishes were collected separately and cumulative residue level was analyzed using gas-chromatographic technique.

The tissue samples drawn were extracted as far as possible, immediately after being collected. When the processing could not be done immediately, the samples were stored under refrigerated conditions at 4^0C, however the storing was not prolonged beyond 3days.

b. Extraction

Extraction of chlorpyrifos from fish tissue was performed by the method of Mills et al. (1963) with some modifications. Edible fish tissue portions after removing and discarding scales, fins, viscera, bones and skin were collected from each group and homogenized. Weighed out 15gm tissue was mixed with 100gm anhydrous sodium sulphate using mortar and pestle. This mixture was transferred to a cellulose thimble (33mm X 80mm, Whatman) and extracted with a Soxhlet extractor for five hours with 150mL hexane at a turnover rate of 8min. The extract was evaporated in rotary evaporator at 52^0C and transferred to a 150mL separatory funnel. Lipid extracts were partitioned four times with 30mL acetonitrile saturated with hexane and shaken vigorously for 2minutes each time. The separated acetonitrile layers were transferred into a 1L separatory funnel containing 650mL deionized water, 40mL saturated NaCl solution and 100mL hexane and shaken well to separate the organic layer from inorganic layer. The aqueous layer was transferred

to a second 1L separatory funnel and again extracted with 100mL hexane. The hexane extracts were combined and washed with two 100mL portions of water containing 5mL saturated NaCl. After washing, solvent was evaporated to near dryness in rotary evaporator and was diluted to 10mL and subjected to clean-up procedure.

c. Clean-up

Clean-up procedure was performed as per the procedure by AOAC (1995). Then activated the florisil and sodium sulphate respectively by keeping the former in a furnace at 675^{0}C for one hour and the latter in an oven at 160^{0}C for one hour. Added 5gm of activated anhydrous sodium sulphate, 25gm of activated florisil and 10gm of activated anhydrous sodium sulphate respectively to a chromatographic column with sintered disc (2.5cmX45cm). Sufficient amount of hexane was added to wet the column. Then added the extract to the column and eluted the column with 100mL of n-hexane controlling the solvent flow to around 2mL/minute. Collected the eluent in a conical flask, evaporated the eluent to 1mL in vacuum flash evaporator and repeated the evaporation using n-hexane and concentrated the eluent to a final volume of 5mL in a standard flask. Fish tissues from control group were also processed in the same manner and run simultaneously with the experimented samples.

8.2.4 Estimation

The sample solution after cleanup was subjected to analysis with the aid of Gas chromatograph with the following conditions.

Gas chromatograph:-

Temperature: Injector	: 250^{0}C
Column	:300^{0}C (temperature programmed)
Detector	:300^{0}C
Injection volume	: 1μL
Carrier gas	: Nitrogen

Total run time : 20minutes

Minimum detectable limit : 0.01ppm

8.2.5 Quantification

The amount of residue was measured by comparing the sample response with the response of standard by using the formula

$$\text{Pesticide residue in ppm} = \frac{\text{Sample area X concentration of standard injected X volume of extract}}{\text{Standard area X injected volume X weight of the sample(Gomez and Gomez, 1984).}}$$

8.3 Results

The quantified values of pesticide residues in edible tissue portions of *Oreochromis mossmabicus* exposed to sublethal concentrations of chlorpyrifos, are given in Table No.8.2.

Table No.8.2. Concentration of residues of chlorpyrifos in tissue of fish exposed at sublethal concentrations for 7 and 21 days

Exposure level (ppb)	Concentration (µg/gm) of chlorpyrifos residues at different stages of analysis		
	7days of exposure	21days of exposure	10days of recovery period
8.2	90.88	273.1	Below detectable level
16.4	252.6	531.8	236.4
27.3	800.6	1053.9	417.9

Gas chromatogram of chlorpyrifos studied is shown in Fig. No.8.1.The chromatogram showed 4 peaks at 9.745minutes and just at the beginning and at the ending of running the sample, for 20minutes duration. The peak of highest area was contributed by chlorpyrifos at 9.745minutes. The peaks eluted in the chromatogram

immediately at the beginning and at the end of the running are expected to be contributed by solvents used for diluting the standard compound.

Gas chromatogram for the fish tissue sample of control group is shown in Fig. No.8.2 (given in appendix). No peak was obtained at the same retention time of standard chlorpyrifos. This indicated the absence of chlorpyrifos in the control sample.

The chromatogram of samples collected after 7days of exposure from 8.2ppb, 16.4ppb and 27.3ppb sublethal concentration groups are shown respectively in Fig. 8.3, 8.4 and 8.5 (in appendix). The peaks contributed by chlorpyrifos showed the retention time in these figures respectively at 9.748, 9.749 and 9.751minutes. Similarly, the peaks eluted in chromatogram, Fig. No. 8.6, 8.7 and 8.8 (in appendix) were eluted respectively at retention time 9.749, 9.752 and 9.749 minutes. The peaks eluted indicated the presence of chlorpyrifos in these fish tissue samples after 21days of exposure to chlorpyrifos in sublethal concentrations respectively at 8.2ppb, 16.4ppb and 27.3ppb.

Gas chromatogram of fish tissue samples obtained after a recovery period of 10 days on withdrawal of pesticide exposure at sublethal concentration of 27.3ppb, 16.4ppb and 8.2ppb is shown respectively in Fig. No. 8.9, 8.10 and 8.11 (in appendix). The peaks eluted in Fig. No. 8.9 and 8.10 were before the retention time 9.745minutes. Thus it can be reported that these peaks were not contributed by chlorpyrifos. These are expected to be contributed by solvent or co-extractives.

Hence all the chromatogram except the control sample, showed the peak of chlorpyrifos.

Table No.8.3: Bioconcentration factors*of chlorpyrifos in edible tissue portions of fish *Oreochromis mossambicus* exposed to sublethal concentrations for a period of 21days.

Exposure period	Concentration of exposure		
	8.2µg/L	16.4µg/L	27.3µg/L
7days	90.88 (11.08)	252.6 (0.94)	800.6 (29.3)
21days	273.1 (4.06)	531.8 (32.43)	1053.9 (38.6)

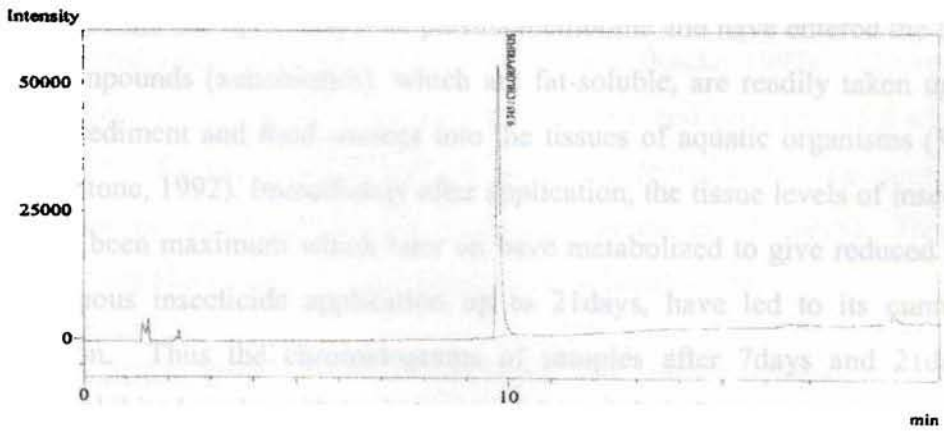
Figures in paranthesis denotes BCF

*Bio-concentration factor= accumulated concentration in tissue (µg/g)/ concentration in media(µg/L).

8.3 Discussion

GAS CHROMATOGRAM FOR STANDARD CHLORPYRIFOS

Analysis Date & Time : 12/5/2007 9:08:42 AM
User Name : Admin
Vial# : 1
Sample Name : CHLORPYRIFOS 0.9796PPM CRM-2
Sample ID : CHLORPYRIFOS 0.9796PPM CRM-2
Sample Type : Standard
Injection Volume : 1.00



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	9.745	372884	52784	0.000	ppm		1	CHLORPYRIFOS
Total		372884	52784					

The concentration of cumulative accumulation of the insecticide is recorded in table 8.2. The peaks eluted in samples after 7 and 21 days of exposure showed that as the concentration of pesticide applied increased, the residues present also was found to increase. Continuous exposure of fish to the insecticide resulted in bioaccumulation of the insecticide in the body of the organism and the accumulation was found to be more on exposure for 21days than for 7days. The samples from both duration, showed a tendency to increase (Fig.8.12)

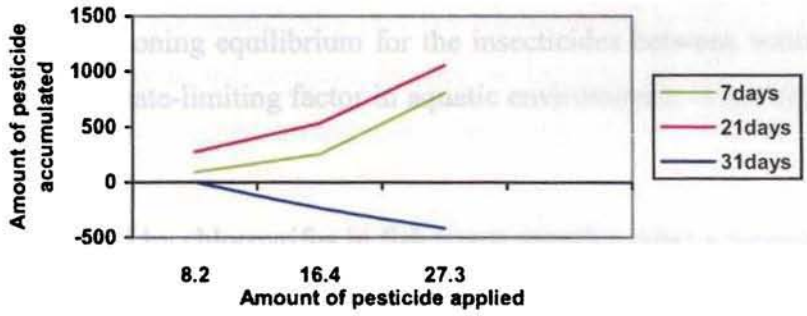


Fig.8.12: Graph showing relation between concentration of chlorpyrifos applied in sublethal dose and concentration of chlorpyrifos accumulated, in *O.mossambicus* after exposure for a period of 21days and after a recovery period of 10days (shown as 31days).

On exposure period, the hydrophobic chlorpyrifos might have been absorbed from water across the lipid bilayer of plasma membrane and have entered the tissues. Foreign compounds (xenobiotics), which are fat-soluble, are readily taken up from the water, sediment and food sources into the tissues of aquatic organisms (Walker and Livingstone, 1992). Immediately after application, the tissue levels of insecticide might have been maximum which later on have metabolized to give reduced levels. But continuous insecticide application up to 21days, have led to its cumulative accumulation. Thus the chromatograms of samples after 7days and 21days of exposure, exhibited peaks with higher area as the sub lethal concentration increased. Affinity of lipophilic chlorpyrifos to the tissue lipids might have resulted in the insecticide bioaccumulation in fish tissues.

Matsumura (1980) reported that the chemical characteristics like partition coefficient between water and organic solvent, water solubility and solvent solubility, in addition to chemical stability determine the distribution of chemical in water. Eg. Despite the low water solubility of DDT, bioaccumulation is high because of its high partition coefficient between lipid and water. Because lipophilicity is a factor of unquestioned importance in bioaccumulation of pesticidal compounds. Even in aquatic environments, where biological systems generally pick up pesticides against the gradient of concentration, the dose-accumulation relationship appears to hold for many lipophilic insecticides. This is probably because of the quick establishment of partitioning equilibrium for the insecticides between water and the organisms, so that the rate-limiting factor in aquatic environments is not the speed of transfer.

The peak eluted by chlorpyrifos in fish tissue samples, after a recovery period of 10days (given in table 8.2) has shown a tendency to decrease in chlorpyrifos concentration. The residue level of chlorpyrifos in these samples was found to be decreased in groups in the order 27.3ppb> 16.4ppb> 8.2ppb group. The level of chlorpyrifos was below detectable level in 8.2ppb group on a recovery period of 10days. In graph, the data reflected a tendency to decrease (Fig.8.12).

The decrease in the residue level after a recovery period might be due to enhanced rate of degradation of the insecticide. Half-life ($t_{1/2}$) of chlorpyrifos is short and the reported water photolysis period is 30 days (Racke 1993). Chlorpyrifos might have been converted to water-soluble non-toxic end product, 3,5,6-trichloro pyridinol (TCP). Shorter chemical half-life and quick degradation in environment is the advantage of organophosphorus pesticides (Ware et al, 1978). Thus the chlorpyrifos might have degraded and obtained its reduced levels.

Like many other chemical reactions, the initial rate of insecticide disappearance follows the law of "first order kinetics": rate of disappearance is related to the amount deposited (Matsumura, 1980). The hydrolysis rate of

chlorpyrifos in water followed simple first-order kinetics over the concentration range, $3x10^{-9}$ to $3x10^{-7}$ M (Richard et al, 1978).

According to Sumpter (1995) et al. the hydrophobic xenobiotics bioconcentrate and bioaccumulate in aquatic organisms. Different organisms will bioconcentrate to different extents. Even with a single organism, the bioconcentrated compound is unlikely to be equally spread through all tissues; it is much more likely to be concentrated preferentially in a few tissues, such as fat. He reports that what happens to these compounds once bioconcentrated within an organism is essentially unknown; they may be physiologically inactive whilst stored in adipose tissue, but when this fat is mobilized, the compounds may be freed to act elsewhere, or they may be metabolized into other compounds which may or may not be active as estrogens.

In the present study the observation of redsidue levels can be correlated with increased levels of Alanine amino transaminase and Alkaline phosphatase levels in liver, where the desulfuration of chlorpyrifos to chlorpyrifos-oxon takes place. Histopathological observation in the present study also supports the chromatographic finding of increased levels of chlorpyrifos residue in tissues. The morphological changes revealed that accumulation of chlorpyrifos resulted in structural alteration and degeneration of gill, liver and brain tissues making the organism unviable for survival.

A study by Rao et al. (2003) has reported the bioaccumulation of chlorpyrifos in *O.mossambicus* of size 3±1gm. The study reports that on exposure for a period of 18days, the accumulation of chlorpyrifos was detected more in viscera followed by body and head. The study reflected not only direct toxicity but also indirect effects by its accumulation in various tissues. Theses residues can add the pesticide residues to human food.

Venkateswara Rao et al. (2005) reported in his study that the accumulation of chlorpyrifos was found to be more in viscera followed by head and body. The

average bioconcentration factor (μg/gm) values were observed as 0.109, 0.009 and 0.004 for viscera, head and body respectively.

Smith et al (1966) reported investigations with C^{14}-dursban on fish, which are able to take in the substance from the water, for instance, after a mosquito control action. Fish ingested dursban only in slight amounts and rapidly metabolized it. The largest intake with 50ppm Dursban was during the first eight to ten hours of exposure in the water; the maximum was reached in 12 hours. It was established from the radioactivity that the largest concentration of residues were present in the viscera. After one day of exposure, they contained 0.08mmol/kg, the head 0.07mmol/kg, the skin 0.004mmol/kg and meat 0.003mmol/kg. The metabolic products were predominantly acetone-soluble after 30 hours exposure and consisted of Dursban plus hydrolysis products: 3,5,6-trichloro-2-pyridylphosphate,3,5,6-trichloro-2-pyridinol and ethyl O-(3,5,6-trichloro-2-pyridyl) thiophosphate (Kaemmerer and Buntenkotter, 1999).

Species sensitivity to chlorpyrifos varies considerably across kingdoms and phyla and even within species. In general, laboratory tests have shown that most species of aquatic plants, including algae and aquatic microorganisms are relatively resistant to chlorpyrifos exposure, with LC_{50}s (24-48hr) generally greater than 100μg/L.

There are reports on toxicity of chlorpyrifos to fish. Fish generally are less sensitive to chlorpyrifos in both acute and chronic exposures than invertebrates, although the sensitivity between species is considerable. Laboratory 96-hr LC_{50} for two of the most sensitive fish species ranged from 2μg/L for the freshwater bluegill to 0.4μg/L for the saltwater silverside. Chlorpyrifos was approximately 100 times more toxic (0.1-3μg/L) to fathead minnows in chronic exposures (32-200days) than in acute exposures, whereas concentrations causing chronic toxicity to silver sides were similar to those causing acute toxicity. Aquatic field studies with chlorpyrifos have shown effects on fish growth at 0.5μg/L or greater and decreased survival at levels greater than 1μg/L (Leeuwangh, 1994). Ambient water quality criteria for

acute toxicity are similar for freshwater and saltwater organisms (0.083 and 0.011µg/L, respectively) (USEPA, 1986).

Bioconcentration is the increase of pollutant concentration from water when passing directly into aquatic species. Many organic substances are much more soluble in lipids than in water. These compounds enter animals because they are lipid soluble and then accumulate in the body fat of animal (Randall et al., 1995). Reviews on uptake clearance of chlorpyrifos report that fish and other aquatic organisms absorb chlorpyrifos from water, with reported uptake clearances of 2-60mL/gm/hr. Elimination half-lives, range from 0.5 to 4days. Reported BCFs (Bioconcentration factor) range from approximately 50 to 5000, but bioconcentration is usually less than that predicted from equilibrium partitioning because of chlorpyrifos biotransformation. Biotransformation by fish appears to limit the accumulation of both waterborne and dietary chlorpyrifos (Barron et al., 1991, 1993). Extensive partitioning onto organic carbon may also limit chlorpyrifos bioconcentration. For example, the presence of organic materials (i.e., plants, sediment) in the exposure water limited bioaccumulation in goldfish due to adsorption of chlorpyrifos (Smith et al, 1966). USEPA (1992) reported that the average concentrations of chlorpyrifos in fish at industrial and background monitoring sites in the United States were 0.004 and 0.0004 mg/kg, respectively. The bioconcentration of degradates of chlorpyrifos is expected to be less than that of the parent compound because of greater polarity. For example, the BCF of TCP in mosquitofish was approximately 100 times less than that of chlorpyrifos (Neely et al., 1974).

Pharmacokinetic studies in fish have shown that chlorpyrifos is rapidly absorbed from exposed water and extensively metabolized. Chlorpyrifos uptake clearance and elimination half-life values in rainbow trout (*Oncorhynchus mykiss*) were 14.4mL/gm/hr and 66 hr, respectively (Murphy and Lutenske, 1986). Chlorpyrifos uptake clearance and elimination half-life in the three-spined stickleback (*Gasterosteus aculeatus*) were 57.4mL/gm/hr and 13.9hr, respectively (Deneer, 1994). Chlorpyrifos uptake clearance and elimination half-life in eels

(Anguilla anguilla), estimated from the data of Douglas and Bell (1990), were 2mL/gm/hr and 81.5 hr. Chlorpyrifos uptake clearance in eastern oysters (*Crassostrea virginica*) was 4.7mL/gm/day, and the elimination half-life was 1.6 and 2.2 days in whole oyster and oyster tissue, respectively (Thacker et al, 1992). The elimination half-life of chlorpyrifos in carp was 34.7 hr (Tsuda et al., 1992). Chlorpyrifos uptake clearance in guppies was 38mL/gm/hr, and the elimination half-life was 31-38 hr (Welling and de Vries, 1992). Uptake clearance estimated from the lipid-normalized data for guppies of Deneer (1993) was 26.5mL/gm/hr, and the elimination half-life was 41.5 hr.

Studies by Hedlund (1972, 1973) indicated that chlorpyrifos was rapidly metabolized and cleared by fish. The work with Hedlund (1972) with TCP indicated that it was cleared even more rapidly than chlorpyrifos by fish. Fish exposed to 1.1ppb of TCP for 6 days accumulated a whole-body concentration of 3.4ppb TCP, and after removal to freshwater nondetectable residues were reached within 3 days.

Study by Thomas and Mansingh (2002) reported that when the fish was transferred every 24 hr to freshwater and brackishwater aquaria containing insecticide at a level of 0.05mg/L, bioconcentration reached its peak within 8hr of exposure in freshwater and within 1hr in brackish water. And in spiking method with concentration of 0.005mg/L, these peaks were attained in 48hr in freshwater and 8-24hr in brackish water. Thereafter, the concentrations declined.

Field bioaccumulation factors are generally less than laboratory-measured BCFs, most likely because dissipation of chlorpyrifos in the water column does not allow sufficient time to establish equilibrium levels in the aquatic animal. van Wijngaarden (1993) concluded that recolonization following chlorpyrifos application was dependent on the timing of the application relative to insect emergence, availability of colonizing organisms, and the rate of chlorpyrifos dissipation. The disappearance of chlorpyrifos from water in micro- and mesocosm studies by Peter (1994) was reported as consistent despite the wide variation in system dimensions and in physical, chemical and biological properties. He inferred that the decrease in

chlorpyrifos concentration in water, from approximately day one to ten, was thought to be governed especially by partitioning processes. Neely and Blau (1977) used compartmental model to estimate the concentration of chlorpyrifos after a single application to earthern ponds. A decrease was observed in concentration of chlorpyrifos in water, and fish and he has plotted the data graphically.

The rapid dissipation of chlorpyrifos from aquatic ecosystems has important implications for aquatic risk assessment. Toxicity profiles observed during prolonged, constant concentration exposure in the laboratory may not accurately reflect toxicological responses to pulsed and rapidly declining concentrations in water under field conditions (Barron and Woodburn, 1995).

In general, aquatic and terrestrial microorganisms and plants are tolerant to chlorpyrifos exposure. Aquatic invertebrates, particularly crustaceans and insect larvae, are sensitive to exposure: LC_{50} values are less than 1µg/L and no-observed-effect concentrations may be below 0.1µg/L in laboratory studies. Fish appear to be less sensitive, with LC_{50} values generally between 1 and 100µg/L and no-observed effect concentrations of approximately 0.5µg/L. In general, saltwater and freshwater organisms exhibit similar sensitivity to chlorpyrifos, considering the extreme phylogenetic and species differences in toxicity (Barron and Woodburn, 1995).

In the Fig: 8.13, the lines represent the concentration of chlorpyrifos accumulated in fish tissues after 7 and 21days of continuous exposure to chlorpyrifos. If the lines are extrapolated, they reflect a tendency of increasing accumulation. The lines reach far higher values than the tolerable limit at LC_{50}. According to Agency for toxic substances and disease registry (ATSDR, 1997), the no-observable-adverse-effect level (NOAEL) for oral exposure to chlorpyrifos in man is 0.03mg/kg/day and the lowest-observable-adverse-effect level is 0.1mg/kg/day. In the present study, chlorpyrifos accumulation in edible tissue portion on exposure to *O.mossambicus* in the highest (1/3) sublethal concentration was found to be 1053ppb. Thus the continuous exposure to chlorpyrifos even at sublethal concentration has potential to contaminate the aquatic environment and

bioaccumulate in higher trophic levels. Toxic substances incorporated into the food chain at lower trophic levels may result in harmful effects to organisms at higher trophic levels, including man. Many estuaries and coastal waters are impacted to varying degrees by toxic substances released from land-based sources. Humans are at risk of developing serious health problems when they regularly consume fish and shellfish with high levels of toxic substances. Sublethal levels of pesticides may accumulate in fish from the water or food. By their nature, these diverse groups of toxic chemicals are often persistent in the environment and leach into aquatic ecosystems. Pesticides may act strongly on fish populations by indirectly affecting food chains. (Michael, 1997).

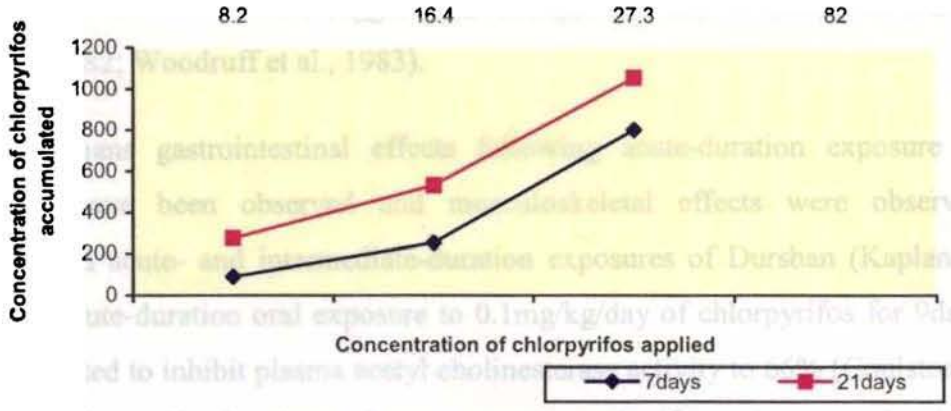


Fig.8.13 Graph showing relation between LC_{50} and accumulated concentration of chlorpyrifos

In a developing country like India, majority of the people coming below poverty line, select food items considering the price rather than the nutritive value. A high percentage of people in Kerala depend on fishing for their livelihood. Most of them include fish in their daily diet. Consumption of these fishes that are contaminated with pollutants like pesticide, open chances for these toxicants to enter human body and affect man, the non-target organism.

The higher levels of pesticides in fish than the minimum risk level can lead these toxicants to result in acute and chronic toxicity to humans.

Thus there is possibility for biomagnification- increase in concentration of pollutant in animal tissue in successive members of a food chain. The freshwater bodies surrounded by hectares of agricultural area, as in Kuttanad regions of Alapuzha district, are more prone to the frequent exposure of different kinds pesticides. All these pesticides may reach the water bodies by leaching and can be present in the body of aquatic organisms. Lethal concentration can kill the fish. When the fishes are suffering from sublethal effects as a result of cumulative accumulation of these pesticides, they may survive for longer times. In man, daily consumption of these fishes will cause the ill effects that are specific to the toxicant. This process will be damaging the organism silently, without causing any immediate abrupt changes. The changes may be at genetic level inducing genotoxicity. Results of studies conducted with rodent and insect cell lines suggest that chlorpyrifos may be genotoxic (Amer and Fahmy, 1982; Woodruff et al., 1983).

In humans gastrointestinal effects following acute-duration exposure to chlorpyrifos have been observed and musculoskeletal effects were observed associated with acute- and intermediate-duration exposures of Dursban (Kaplan et al., 1993). Acute-duration oral exposure to 0.1mg/kg/day of chlorpyrifos for 9days has been reported to inhibit plasma acetyl cholinesterase activity to 66% (Coulston et al, 1972). Chlorpyrifos has been shown to produce significant increases in sister chromatid exchanges (Sobti et al, 1982). It has also been reported that chlorpyrifos causes X chromosome loss (Woodruff et al., 1983). Thus the changes acquired by chlorpyrifos exposure might be transferred from generation to generation.

However, out of 45 fishes exposed to the highest sublethal concentration, for the study on pesticide residues, only four fishes survived. Increase in mortality with increase in exposure period could be affected by several factors, which may be acting separately and conjointly. Eg. Uptake of pesticides is time-dependent, which leads to a progressive increase of pesticides in body. Stability (life of pesticides) in environment and the rate of their detoxification in animal body also affect the mortality and exposure period (Matsumura, 1985). The binding of chlorpyrifos with acetylcholinesterase is irreversible. Thus the normal functioning of nervous system

might be requiring the synthesis of new molecules of enzyme. Further studies are needed to investigate the haematological, histopathological and biochemical changes during recovery period. Histopathological changes and residue levels of test substances in fish tissues are the most sensitive parameters for the evaluation of chronic toxicity effects and thus also for the derivation of maximum admissible concentrations.

Chapter

9

Summary and Conclusion

Contents

9.1 Summary and Conclusion

Pesticides are a diverse group of widely varying chemical structures ranging from simple inorganic substances to complex organic molecules. Unlike, most toxic agents, pesticides have been selected and synthesized for their biocidal properties and are applied to kill or control organisms. Thus they are all toxic to target and non-target organisms.

The ubiquitous use of pesticides in the environment and their potential exposure to aquatic organisms is increasing in parallel with modernization of agricultural areas.

The work presented here centers around the toxic action of chlorpyrifos on the euryhaline cichlid fish, *Oreochromis mossambicus*. The first chapter gives an introduction on environmental pollution by pesticides and its toxicity to aquatic organisms. In general, information on the toxic effects of organophosphorus pesticides on different aquatic organisms is detailed out in the review of literature.

The chapters from three to seven has presented the aspects like lethal toxicity, alteration in biochemical parameters, variations in antioxidant enzyme levels, changes in haematological parameters, and morphological alterations in brain, liver and gill tissues of the fish. An evaluation of chlorpyrifos residue in edible tissue portion of the fish by gas chromatographic technique has been documented in the eigth chapter.

The important observations can be summarized.

- LC_{50} value was found to be 82ppb. This indicated that chlorpyrifos, even in microgram quantities can induce toxicity to fish.
- Behavioural changes observed were erratic swimming, loss of equilibrium and hyperactivity before collapsing. Excess secretion of mucus was a prominent observation on exposure to the pesticide. During initial phase of

exposure, fish responded vigorously to mechanical stimulation but later failed to respond. The toxicity may be dose-dependent, in case of pesticide pollution and therefore, exposure to the highest sublethal concentration of pesticide, resulted in greater mortality rate and deviations from the normal behavioral pattern, than the lower sublethal concentrations.

- Depletion in protein content indicated the requirement of large amounts of protein under a toxic stress to compensate the energy demand and the metabolic requirements. Inhibition of acetylcholinesterase enzyme in all the tissues especially in brain revealed the potency of the organophosphorus pesticide chlorpyrifos to impair the synaptic transmission mechanism. The remarkable changes in activities of enzymes like LDH and SDH suggested the impaired oxidation of carbohydrates through TCA cycle. Variations in the liver-specific ALT and ALP enzyme activities indicated the role of the tissue in detoxification processes under chlorpyrifos toxicity. The toxicity resulted in impaired metabolism leading to disturbed homeostasis.
- Significant increase in RBC count with an insignificant change in Hb content and PCV, on a long-term exposure to chlorpyrifos indicated an attempt of organism to consume more amount of oxygen. The Increased RBC count showed a shift of blood cells from other tissues to vascular tissues to carry more oxygen under stress condition induced by chlorpyrifos. The RBCs were trying to compensate the pesticide-induced toxic stress.
- A significant increase in WBC count on short-term exposure followed by an insignificant decrease on long-term exposure to chlorpyrifos, was observed revealing the immunological responses to toxic stress. Increase in WBC count might be indicating aggressive phagocytic process, which is a sign of deviation from normal metabolism. Electrophoretic studies described the serum protein depletion in the organism under stress condition.
- Histopathological observations envisaged the deleterious anatomical and morphological alterations induced in gill, liver and brain tissues by sublethal toxicity of the insecticide chlorpyrifos. Each tissue showed specific sterical

changes and revealed the incapability of these tissues to withstand the toxic effects induced by chlorpyrifos.

- Various branchial ATP-ase enzymes were found to be inhibited on short-term followed by long-term exposure to chlorpyrifos.
- Lysosomal hydrolase activities were found to be elevated on short-term exposure but decreased on long-term exposure.
- Reduction in tissue levels of pesticide might be due to non-persistent character of organophosphorus insecticide. It might have metabolized to non-toxic water-soluble metabolite called 3,5,6-trichloropyridinol (TCP).
- Increased levels of antioxidants on a short-term exposure of 7days and thereafter their decrease on a long-term exposure showed the efficiency of antioxidant system to defend the insecticide-induced stress and the inability of the organism to overcome the toxic effect of chlorpyrifos on a long-term exposure to insecticide.

Organophosphorus pesticide is potent to cause acute damages, more significantly the chronic damages to aquatic organism. But the capacity to adsorb to the soil particles and its non-persistent nature has made the compound to be appreciated with a great market value. But its application to soil can kill the earthworms, we call farmer's friend, thus the use of chlorpyrifos causes damages in two ways- it joins with the soil particles and lessens the soil fertility by killing the earthworms. Birds like hen are also susceptible to chlorpyrifos toxicity.

The present study revealed that the organophosphorus insecticide chlorpyrifos is potent to cause toxic responses, even structural alterations, in aquatic organism like fish. Though it is degrading very readily, because of short half-life, the chances for acute toxicity are not avoidable. The high mortality rate at higher concentrations i.e. at 100ppb and the lower LC_{50} value proves the highly toxic nature of chlorpyrifos that it can cause damages to aquatic organisms.

The hazards of environmental contamination are usually associated with unexpected side effects due to pesticides or pesticide-derived compounds. Thus the

insecticide contamination of the major food and value-added export commodity-fish- may adversely affect the human health. A number of studies have elucidated the aquatic toxicity of different kinds of pesticides. These reports bring discussions on the deteriorating nature and the lethal effects of the pesticides on ecosystem. Pesticides, especially the non-degradable ones, even in minute levels, are capable to cause a stress to aquatic organisms. The toxic responses are reflected by the behavioral, biochemical and pathological changes. But concerted effort in reducing the use of pesticides and implementing natural remedies for pest-encroachment through organic farming can help resolving the problem of pesticide pollution.

Genotoxicity can be expected on exposure to chlorpyrifos. The review of related work have suspected the chances for significant increases in sister chromatid exchanges and X chromosome loss. But this perspective can be considered for future studies.

List of scientific papers consulted for the enrinchment of the present work are provided under bibliography. (REFERENCES)

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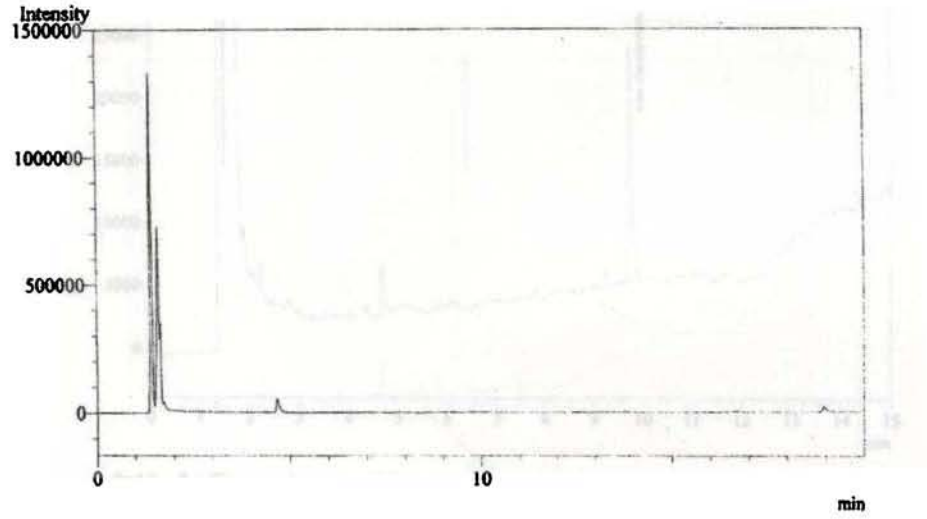
Appendices

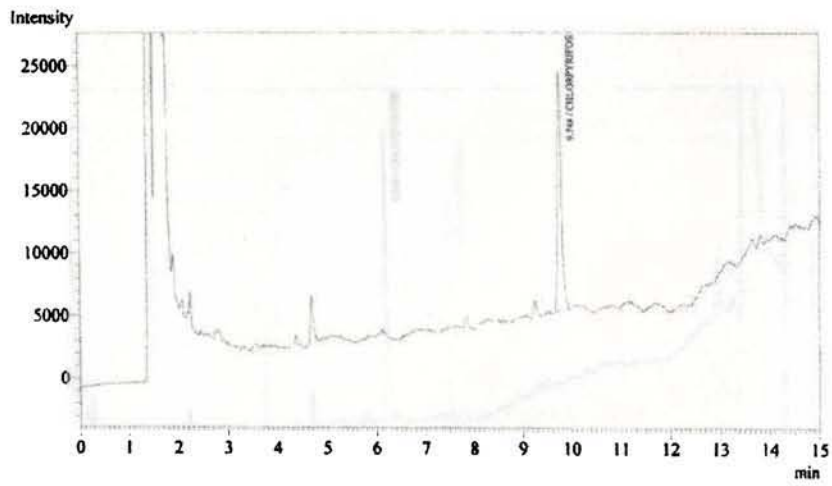
GAS CHROMATOGRAM FOR EDIBLE TISSUE PORTIONS FROM *OREOCHROMIS MOSSAMBICUS* OF CONTROL GROUP

Analysis Date & Time	: 12/5/2007 9:36:00 AM
User Name	: Admin
Vial#	: 2
Sample Name	: CONTROL FISH CUSAT
Sample ID	: CONTROL FISH CUSAT
Sample Type	: Unknown
Injection Volume	: 1.00



GAS CHROMATOGRAM FOR EDIBLE TISSUE PORTIONS FROM *OREOCHROMIS MOSSAMBICUS* EXPOSED TO CHLORPYRIFOS OF CONCENTRATION OF 8.2ppb FOR 7DAYS

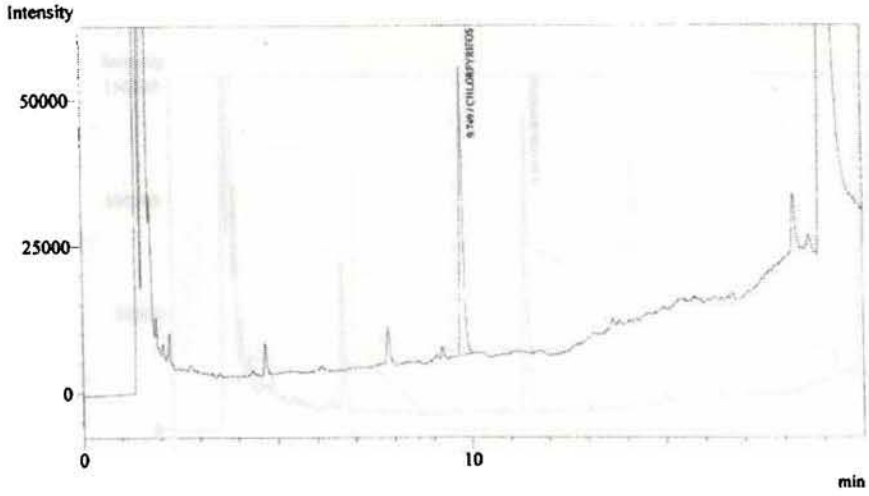
Analysis Date & Time : 12/5/2007 1:00:08 PM
User Name : Admin
Vial# : 10
Sample Name : FISH CUSAT
Sample ID : FISH CUSAT
Sample Type : Unknown
Injection Volume : 1.00



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	9.748	103782	18975	0.000	ppm		1	CHLORPYRIFOS
Total		103782	18975					

GAS CHROMATOGRAM FOR EDIBLE TISSUE PORTIONS FROM *OREOCHROMIS MOSSAMBICUS* EXPOSED TO CHLORPYRIFOS OF CONCENTRATION OF 16.4 ppb FOR 7DAYS

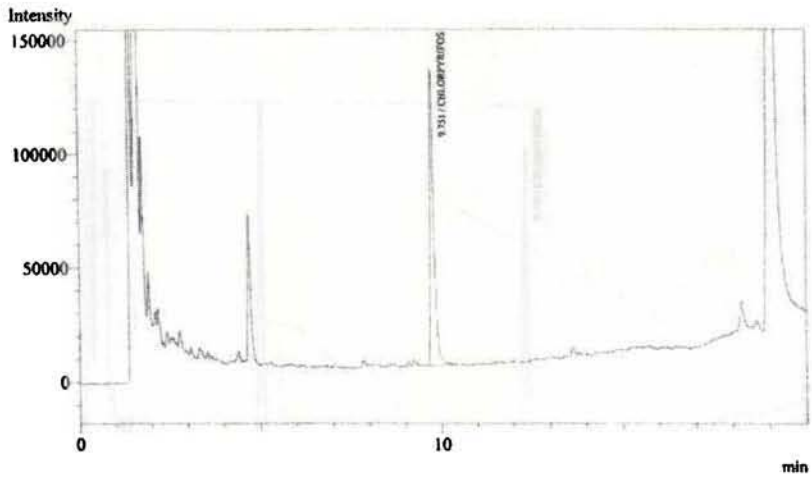
Analysis Date & Time : 12/5/2007 11:42:57 AM
User Name : Admin
Vial# : 7
Sample Name : FISH CUSAT
Sample ID : FISH CUSAT
Sample Type : Unknown
Injection Volume : 1.00



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	9.749	288438	48981	0.000	ppm		1	CHLORPYRIFOS
Total		288438	48981					

GAS CHROMATOGRAM FOR EDIBLE TISSUE PORTIONS FROM *OREOCHROMIS MOSSAMBICUS* EXPOSED TO CHLORPYRIFOS OF CONCENTRATION OF 27.33 ppb FOR 7DAYS

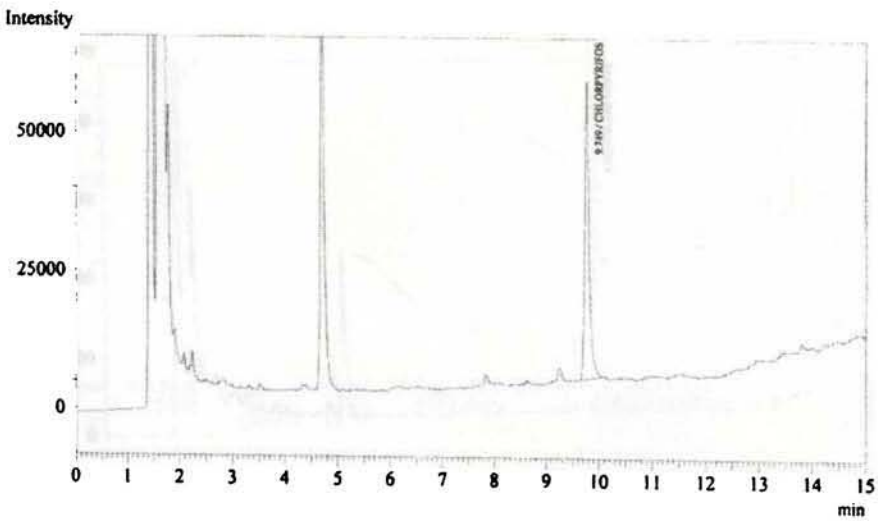
Analysis Date & Time : 12/5/2007 10:27:54 AM
User Name : Admin
Vial# : 4
Sample Name : FISH CUSAT
Sample ID : FISH CUSAT
Sample Type : Unknown
Injection Volume : 1.00



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	9.751	914274	129818	0.000	ppm		1	CHLORPYRIFOS
Total		914274	129818					

GAS CHROMATOGRAM FOR EDIBLE TISSUE PORTIONS FROM *OREOCHROMIS MOSSAMBICUS* EXPOSED TO CHLORPYRIFOS OF CONCENTRATION OF 8.2ppb FOR 21DAYS

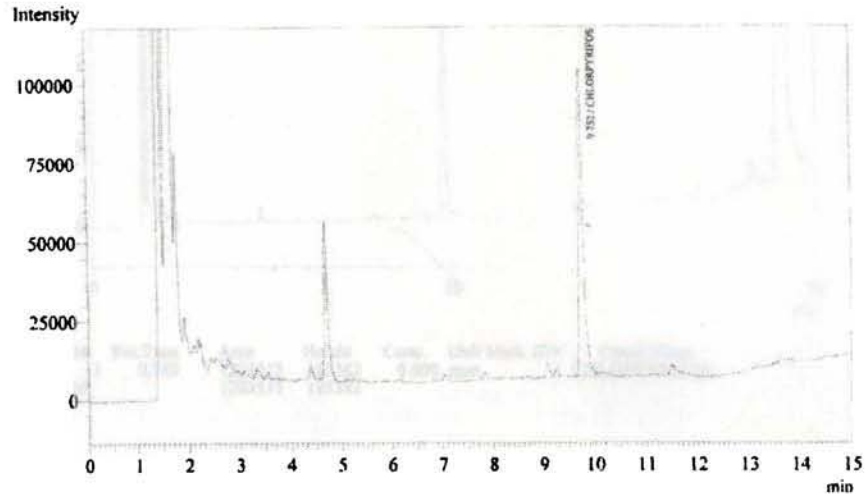
Analysis Date & Time : 12/5/2007 12:34:56 PM
User Name : Admin
Vial# : 9
Sample Name : FISH CUSAT
Sample ID : FISH CUSAT
Sample Type : Unknown
Injection Volume : 1.00



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	9.749	311882	53626	0.000	ppm		1	CHLORPYRIFOS
Total		311882	53626					

GAS CHROMATOGRAM FOR EDIBLE TISSUE PORTIONS FROM *OREOCHROMIS MOSSAMBICUS* EXPOSED TO CHLORPYRIFOS OF CONCENTRATION OF 16.4ppb FOR 21DAYS

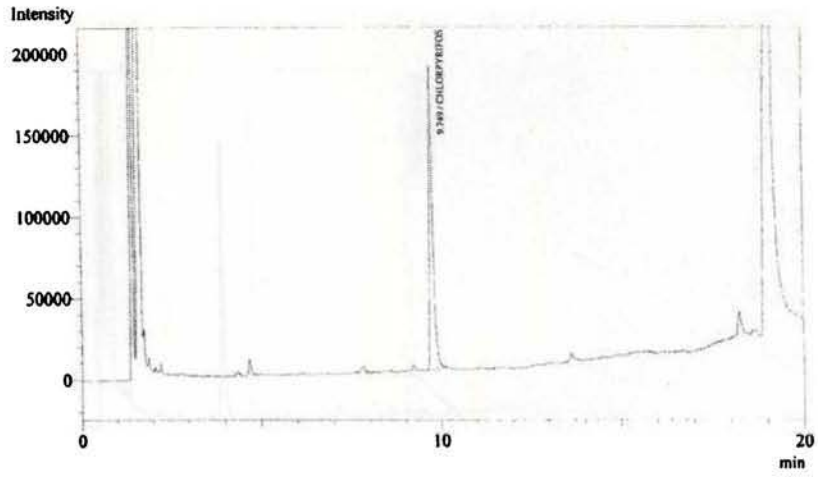
Analysis Date & Time	: 12/5/2007 12:07:26 PM
User Name	: Admin
Vial#	: 8
Sample Name	: FISH CUSAT 1/5 - 2- 21
Sample ID	: FISH CUSAT 1/5 -2- 21
Sample Type	: Unknown
Injection Volume	: 1.00



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	9.752	607263	97356	0.000	ppm		1	CHLORPYRIFOS
Total		607263	97356					

GAS CHROMATOGRAM FOR EDIBLE TISSUE PORTIONS FROM *OREOCHROMIS MOSSAMBICUS* EXPOSED TO CHLORPYRIFOS OF CONCENTRATION OF 27.33ppb FOR 21DAYS

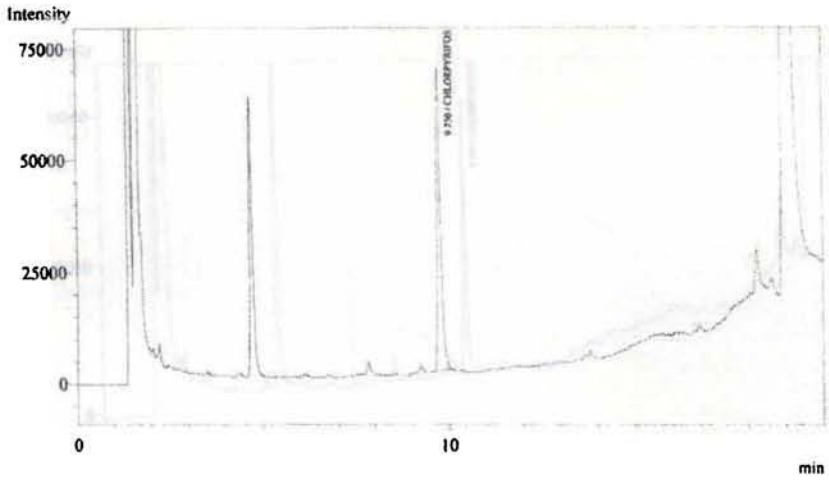
Analysis Date & Time : 12/5/2007 10:53:08 AM
User Name : Admin
Vial# : 5
Sample Name : FISH CUSAT 1/3-2-21
Sample ID : FISH CUSAT 1/3-2 21
Sample Type : Unknown
Injection Volume : 1.00



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	9.749	1203513	185382	0.000	ppm		1	CHLORPYRIFOS
Total		1203513	185382					

GAS CHROMATOGRAM FOR EDIBLE TISSUE PORTIONS FROM *OREOCHROMIS MOSSAMBICUS* ON A RECOVERY PERIOD OF 10 DAYS AFTER CHLORPYRIFOS EXPOSURE (27.33ppb)

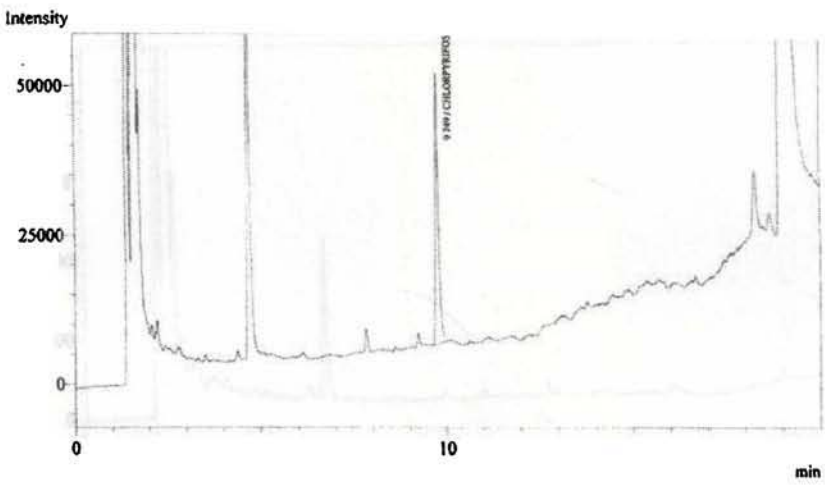
Analysis Date & Time : 12/5/2007 10:01:18 AM
User Name : Admin
Vial# : 3
Sample Name : FISH CUSAT
Sample ID : FISH CUSAT
Sample Type : Unknown
Injection Volume : 1.00



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	9.750	477309	67671	0.000	ppm		1	CHLORPYRIFOS
Total		477309	67671					

GAS CHROMATOGRAM FOR EDIBLE TISSUE PORTIONS FROM *OREOCHROMIS MOSSAMBICUS* ON A RECOVERY PERIOD OF 10 DAYS AFTER CHLORPYRIFOS EXPOSURE (16.4ppb)

Analysis Date & Time	: 12/5/2007 11:18:26 AM
User Name	: Admin
Vial#	: 6
Sample Name	: FISH CUSAT
Sample ID	: FISH CUSAT
Sample Type	: Unknown
Injection Volume	: 1.00



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	9.749	269898	45374	0.000	ppm		1	CHLORPYRIFOS
Total		269898	45374					

GAS CHROMATOGRAM FOR EDIBLE TISSUE PORTIONS FROM *OREOCHROMIS MOSSAMBICUS* ON A RECOVERY PERIOD OF 10 DAYS AFTER CHLORPYRIFOS EXPOSURE (8.2ppb)

Analysis Date & Time : 12/5/2007 2:35:26 PM
User Name : Admin
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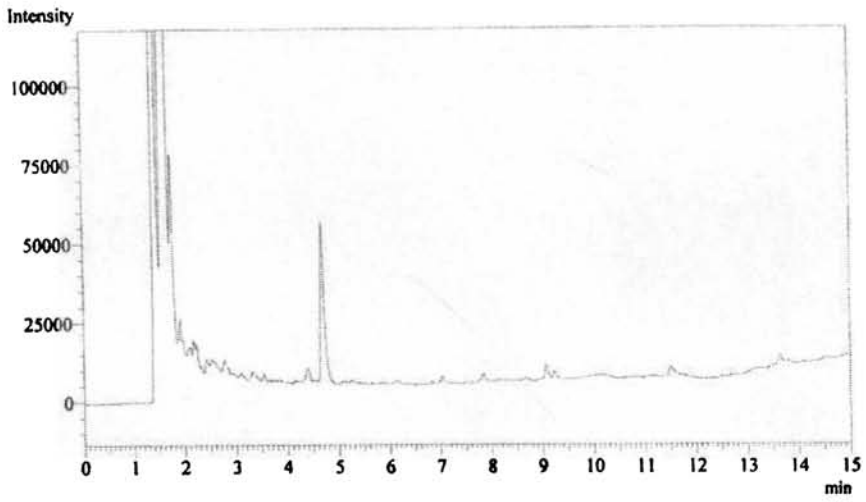
Sample Name : FISH CUSAT 1/5 - 2- 21
Sample ID : FISH CUSAT 1/5 -2- 21
Sample Type : Unknown
Injection Volume : 1.00



List of publication

Effect of chlorpyrifos on branchial ATPase enzymes of *Oreochromis mossambicus* (Peters), International Conference on Biodiversity Conservation and Management, Theme: Ecosystems and sustainable development, BIOCAM 2008, Book of Abstracts, EDB-08, 77 (Orally presented).

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